Using isothiocyanate plant allelochemicals as biocontrol against plant pathogenic *Ralstonia solanacearum* bacterium

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Abstract

Ralstonia solanacearum is a bacterial plant pathogen with a global status, able to infect many economically important crops including tomato and potato. Traditionally *R. solanacearum* has been controlled using agrochemicals. However, stricter legislations and environmental damage caused by agrochemicals have made these options unfeasible. Biofumigation is a biocontrol method that exploits *Brassica* plant allelochemicals called isothiocyanates (ITCs) and could be used to protect against crop disease. While recent studies have shown promising results, the potential for pathogen ITC tolerance evolution and the effects of ITCs on non-target microbes and crop hosts remain understudied. This thesis identifies that allyl-ITC is very effective at suppressing *R. solanacearum* pathogenic bacterium *in vitro* and *in vivo*. However, ITC exposure can also select for ITC tolerance evolution. Mechanistically, tolerance was associated with insertion sequence movement particularly in the megaplasmid. In multi-species communities, it was found that plant non-pathogenic *Pseudomonas* bacteria were less susceptible to ITC than *R. solanacearum* in liquid and soil microcosms and *in vivo* tomato mesocosms. Further, synergistic suppressive effects of ITC and *Pseudomonas* on *R. solanacearum* densities were observed. However, ITC effects were less specific in more complex rhizosphere communities, where ITC application reduced the diversity and affected the composition and potential connectivity of soil microbiota. Crucially, ITC application had negative effects on the plant flowering and biomass where *Pseudomonas* were present. The work presented here lay the groundwork for systematic understanding of ecological and evolutionary effects of biofumigation on *R. solanacearum* pathogen, non-target microbiota and crop host.

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I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1. General Introduction

1.1. Threats to food security

Food security is defined as an environment where every person has access to sufficient food and nutrition to sustain an active and healthy life (Myers *et al.*, 2017). The global population is projected to reach 10 billion by the mid-century (United Nations, 2019) and currently, over 800 million people are malnourished and underfed (Strange and Scott, 2005). Achieving global food security will require food production to increase by 50% by 2050 using the same amount of land (Chakraborty and Newton, 2011). Climate change acts as a barrier to feeding the growing population by driving unpredictable weather extremes, precipitation, humidity and increasing CO_2 levels (Anderson *et al.*, 2004). For example, El Niño, the periodic warming of the Pacific Ocean, has become increasingly intense and frequent in recent times leading to severe droughts, floods and hurricanes and directly affecting food production in countries like Papua New Guinea (Cobon *et al.*, 2016). As a result, the global yields of staple food crops including, wheat, rice, maize and soybean, are predicted to decline (Zhao *et al.*, 2017).

Globally, pests and diseases result in the loss of up to 30% of food production per year (Oerke and Dehne, 2004; Savary *et al.*, 2019) and developing nations, where food security is already strained, suffer the greatest crop losses (Savary *et al.*, 2019). The major plant pathogens are oomycetes, nematodes, parasitic plants, viruses, fungi and bacteria. Historically, incidences of crop disease outbreaks have resulted in devastating famines. For example, the Irish potato famine caused by the oomycete *Phytophthora infestans* resulted in the death of over a million people (Strange, 2003), and more recently, an outbreak of wheat blast disease in Bangladesh resulted in up to 100% crop losses (Callaway, 2016; Islam

et al., 2016; Mottaleb *et al.*, 2018). Climate change will likely reduce crop yields further by increasing the prevalence and severity of crop disease. For instance, warmer temperatures often promote soil-borne pathogen virulence and survival (Kaczmarek et al., 2014) and prolong the growing season, allowing more time for pathogen populations to establish and increase in densities (Jones *et al.*, 2014). In addition, increasing temperatures and atmospheric CO_2 has been reported to increase fecundity and growth of pathogens, including species of the fungal pathogen *Fusarium* (Melloy *et al.*, 2010; Webb, Brenner and Jacobsen, 2015). Species distribution models predict the warming climate could broaden pathogenic host range latitudinally and promote the emergence of new diseases. For example, *Pseudomonas syringae* pv. *actinidiae*, the causal agent of kiwifruit canker was once epidemic in Europe and New Zealand and has since spread to regions of China (McCann *et al.*, 2017), likely due to the warming climate. Ultimately, crop disease severely threatens food security in a warming world with a growing number of mouths to feed and a greater reliance on global trade, which facilitates the spread of known and emerging plant pathogens.

1.2. Agrochemical approaches to control crop disease

Agricultural systems have responded to the growing pressure to maximise food production by relying on more efficient continuous, monoculture cropping and intensive tillage, which also creates ideal conditions for pathogens to thrive (Strange and Scott, 2005; Nunes, Karlen and Moorman, 2020). Pathogen control has traditionally relied on agrochemicals like pesticides, herbicides, fungicides and insecticides. The uprising of agrochemicals during the 'Green Revolution' enabled food production to double in the last half-century (Savary *et al.*, 2012), and every year around 17 million kg of pesticides are applied in the UK (Fera

statistics, 2016). However, legislation against pesticides is becoming stricter due to unwanted side effects, complicating and limiting their use (Geiger *et al.*, 2010; Bolton *et al.*, 2012). For example, highly persistent and dangerous pollutant organochloride pesticides, such as aldrin and dieldrin, were banned in the UK for use in plant protection in the 1980's due to bioaccumulation, persistence in the environment due to their lipophilic nature (Jayaraj, Megha and Sreedev, 2016) and side-effects on non-target organisms, including seed-eating birds (Walker, 1983). These compounds were also linked to cancer in humans (Wolff *et al.*, 1993). Additionally, methyl bromide, a broad-spectrum fumigant once used to control against plant-parasitic nematodes, including *Meloidogyne* spp. and *Globodera* spp. and plant pathogenic microorganisms such as *Ralstonia solanacearum* and *Aspergillus parasiticus,* was discontinued in Europe under the Montreal Protocol in 2005 due to ozone depleting effects (Santos *et al.*, 2006). There are also concerns that pesticides could have wider environmental impacts through leaching into rivers and aquifers surrounding treated crop fields. A classic example of this is the case of neonicotinoid insecticides, such as imidacloprid and thiamethoxam, which are often applied as seed dressings and have been shown to accumulate in soil and contaminate groundwater and surface water via surface runoff and drainage (Morrissey *et al.*, 2015). The presence of low concentrations of neonicotinoids in aquatic systems are destructive to insects and crustaceans, causing widespread ecosystem harm. Moreover, inefficient pesticide use has facilitated the evolution of pesticide-resistant organisms. As an example of this, *Venturia inaequalis*, the causal agent of fungal apple scab disease, was often controlled in the late 1960's by application of the fungicide benomyl, yet resistance quickly developed by mutations in the gene encoding the target protein β-tubulin (Koenraadt et al., 1992). Due to the prevalence of resistance in the target pathogen, the production of the fungicide ceased. Reliance on

chemical pesticides will likely drive the evolution of further pesticide-resistant pathogens, which in some cases takes only a few years to develop (Hawkins *et al.*, 2019). As a result, fewer agrochemicals are available for growers, making it challenging to control pests and pathogens while simultaneously increasing crop production.

1.3. Alternatives to agrochemicals

Due to stricter legislations on pesticide use, effects on non-target organisms, environmental concerns and the growing problem of pesticide resistance, alternatives to agrochemicals are urgently required.

Resistant cultivars are a major method in controlling crop diseases and can be generated through traditional breeding, breeding using traits identified by mutagenesis or genetic engineering. These cultivars can often tolerate pathogen attack by producing antimicrobials, activating defence genes and triggering cell death (Levine *et al.*, 1994). For example, selective breeding for phenotypic traits, such as anthocyanin producing potato cultivars can reduce disease susceptibility (Wegener and Jansen, 2007). However, there are concerns the red colouration associated with anthocyanin production may deter consumers. Resistant cultivars of potato can be acquired by crossing wild-type *Solanum* species possessing disease resistance genes into the commercial potato genome (Jansky and Rouse, 2007). Additionally, naturally existing allelic variation in pepper has been exploited to enable resistance to bacterial spot (Jones *et al.*, 2007) through a six base pair deletion in the Bs5 gene, encoding a protein product that lacks two amino acids in a highly conserved domain (Iliescu et al., 2013).

Moreover, resistant crops can be generated using genetic engineering by modifying host plant DNA. The identification of key genes involved in the multi-layered plant immune

system has driven studies in overexpression of these genes to limit pathogen invasion. The plant's first line of defence against pathogen invasion relies on the detection of microbial/damage-associated molecular patterns (M/DAMPs) like flagellin, the bacterial elongation factor Tu and chitin (Zipfel *et al.*, 2006; Faulkner *et al.*, 2013), by host pattern-recognising receptors (PRRs) (Boller and Felix, 2009). For instance, the expression of the *Arabidopsis thaliana* PRR, elongation factor Tu receptor gene significantly reduced tomato susceptibility to *R. solanacearum,* the causal agent of bacterial wilt disease (BWD) (Kunwar *et al.*, 2018). Additionally, molecular geneticists have exploited plant host resistance (*R*) genes to enable broad-spectrum resistance in previously susceptible crops through genetic engineering (Hammond-Kosack and Parker, 2003). *R* genes detect and induce a local hypersensitive response at the site of infection and trigger systemic acquired resistance when infected by a pathogen carrying a specific avirulence (*avr*) gene (Pink, 2002). For example, in *Arabidopsis* the *R* gene, RRS1 confers resistance to *R. solanacearum*, potentially by binding DNA in several pathogen-induced promoters (Deslandes *et al.*, 2002). *R* genes can even be 'stacked', where numerous variants of *R* genes are genetically transformed into a susceptible plant, to enable longer-lasting and broader pathogen resistance (Orbegozo *et al.*, 2016). This has been demonstrated by the introgression of multiple *R* genes isolated from wild *Solanum* species into the cultivated potato (*Solanum tuberosum*) to confer long-lasting resistance against multiple strains of the oomycete that causes late blight, *Phytophthora infestans* (Roman *et al.*, 2017). Aside from PRR and *R* genes, overexpression of *Arabidopsis* NPR1, a gene induced by host salicylic acid secretion during pathogen attack, can increase resistance to an array of pathogens in a number of plant families (Chern *et al.*, 2001; Quilis *et al.*, 2008; Molla *et al.*, 2016). Despite these advances in transgenic disease resistant crops, the enhanced resistance is often associated with fitness costs, such as

reduced growth and altered development (Hammond-Kosack and Parker, 2003), and eventual pathogen evolution to overcome plant defences (McDonald and Linde, 2003).

Integrated pest management, a strategy that incorporates multiple agricultural practices to control crop disease acts to target pathogens from multiple directions. While its effects could be ecological, affecting pathogen densities, it could also reduce the potential for pathogen evolution by applying multiple, concurrently acting selective pressures which might impose evolutionary trade-offs that constrain pathogen adaptation. For instance, rotations with trap crops, like *Solanum sisymbriifolium*, have been shown to reduce parasitic potato cyst nematode populations and egg densities (Dandurand, Zasada and LaMondia, 2019). However, rotations are ineffective in the suppression of pathogens able to persist in soils for long periods such as the potato cyst nematode *G. pallida* and the powdery scab causal agent *Spongospora subterranea* (Fiers *et al.*, 2012; Hampson, 1985). Further, integrated pest management was used to successfully manage stem rust in wheat, a fungal epidemic disease in North America by integrating cultural practices (removal of the host required for pathogen reproduction), making use of advances in chemical control (development of quinone outside inhibitor fungicides) and a complex breeding programme (Pardey *et al.*, 2013). Thus, combining multiple crop disease control mechanisms may be a method of attaining long-term effective disease management.

1.4. Biocontrol

Biocontrol is a technique to manage crop disease using living organisms and has been an active area of scientific research particularly in the last 30 years (Droby *et al.*, 2016), due to the downfalls of agrochemicals. Many biocontrol reviews conclude that an effective biocontrol agent should be highly pathogen-specific with minimal effects on non-target

biota. Examples of biocontrol agents include nematode-destroying fungi (Mankau, 1980), probiotic pseudomonads and *Bacillus* spp. bacteria that induce plant-host systemic resistance (Weller, 2007), and viral myxomatosis disease to control rabbit pests in Australia (Di Giallonardo and Holmes, 2015). Biocontrol agents are generally expected to have neutral effects on the environment due to their natural origin, making them favourable over synthetic control mechanisms.

1.4.1. Plant growth-promoting bacteria as biocontrol agents

The most well-established form of soilborne plant pathogen biocontrol is the application or enrichment of plant growth promoting rhizobacteria (PGPR) which exist naturally in the soil. There are four principal mechanisms of PGPR biocontrol: (i) competition, (ii) antibiosis, (iii) predation and (iv) activation of plant systemic acquired resistance (Haas and Keel, 2003). Biocontrol using *Pseudomonas* PGPR could be a promising method to control crop disease. Notably, *Pseudomonas fluorescens* has been shown to prevent *R. solanacearum* invasion of tomato and potato roots by inducing host plant systemic resistance and releasing antibiotics such as 2,4-diacetylphloroglucinol (DAPG) (Duffy and Défago, 1999), orfamides (Loper and Gross, 2007), and producing siderophores like pseudobactin which limit the availability of Fe^{3+} for root-colonising pathogens (Buyer, Wright and Leong, 2002; Ran *et al.*, 2005). However, biocontrol using PGPR has not been widely adopted in agricultural systems due to insufficient colonisation, competition by existing soil microbiota, and often only partial and inconsistent disease control (Thomashow and Weller, 1988).

1.4.2. Bacteriophages as biocontrol agents

One example of a highly pathogen-specific biocontrol method is the introduction of bacteriophages. These are viruses that specifically infect bacteria and can exist either as lytic

(obligate parasites that reproduce and lyse the bacterial cell), or as lysogenic phages (incorporate their viral genome into the host cell genome). Phage present an attractive alternative to chemical pathogen control mechanisms as they are often pathogen-specific with neutral effects on commensal microorganisms (Skurnik, Pajunen and Kiljunen, 2007), they self-replicate at the target site during the course of an infection (Carlton, 1999), infect antibiotic-resistant bacteria (Kutter *et al.*, 2010), and can co-evolve alongside their bacterial hosts in an arms race, retaining their infectivity in the face of resistance evolution (Betts *et al.*, 2013; Mumford and Friman, 2017). Phage potential as biocontrol agents has been demonstrated against the bacterial plant pathogen *Ralstonia solanacearum* (Fujiwara *et al.*, 2011b; Wang *et al.*, 2017, 2019; Álvarez, López and Biosca, 2019). Particularly successful control of *R. solanacearum* has been reported when phages are applied as a 'cocktail' in combination with other phage types. For example, one study identified the lowest levels of BWD incidences in tomato when three different phages were applied in combination in greenhouse and field experiments (Wang et al., 2019). Similarly, the suppressive effects of phages on *R. solanacearum* densities can be increased when treated with both phage and probiotic *Bacillus amyloliquefaciens* bacterium in lab and greenhouse experiments (Wang *et al.*, 2017).

1.4.3. Allelochemicals

The root exudate of some plants includes biocidal allelochemicals. These are natural plant secondary metabolites, that can protect against disease by fungi and bacteria (Macías, Galindo and Galindo, 2007), herbivore attack and increase competitiveness with other plants (Bourgaud *et al.*, 2001; Bhadoria, 2011). Of these compounds, coumarins, a class of lactones, are particularly widespread and have antibacterial, antifungal and anticancer effects (Detsi, Kontogiorgis and Hadjipavlou-Litina, 2017), as well as suppressive effects

against weedy competitors (Pergo *et al.*, 2008; Haig *et al.*, 2009). They are believed to aid in iron uptake from low iron soils (Tsai and Schmidt, 2017) and induce plant systemic resistance (Stringlis *et al.*, 2018). Since their discovery, researchers have aimed to prime coumarin accumulation to enable improved crop disease tolerance by exogenous application of plant defence hormones like salicylic and jasmonic acid (Pastírová, Repčák and Eliašová, 2004; Barilli, Prats and Rubiales, 2010). Whilst these studies have successfully improved plant pathogen tolerance, the effects of coumarin release on non-target microbes could have broader ecosystem effects which also promote plant health (Stringlis *et al.*, 2018). Aside from direct suppression by root exudates, some plant allelochemicals can act as signal molecules to alert nearby plants of pathogen or herbivore attack (Dicke and Sabelis, 1987), which may simultaneously increase visibility to other potential attackers (Heil and Ton, 2008; Berens *et al.*, 2017). For example, upon pathogen invasion, some plants emit volatile signalling chemicals called terpenoids which induce defences in neighbouring plants and increase expression of defence-related genes in un-infected parts of the plant (Halitschke *et al.*, 2008). Improved knowledge of the plant immune system could allow these allelochemicals to be harnessed and used as crop protection mechanisms.

1.5. *Ralstonia solanacearum*

Ralstonia solanacearum is a Gram-negative β-proteobacteria, aerobic rod-shaped bacterium measuring 0.5-1.5 µm in length, with a single polar flagella tuft. It is a soilborne bacterial plant pathogen with a worldwide distribution and can infect over 200 different plant species spanning 50 families, including many agriculturally important crops, particularly those belonging to the Solanaceae family including aubergine, tomato and potato (Hayward, 1991). Other common non-solanaceous hosts include banana, peanut and ginger (Kelman,

1953; Elphinstone, 2005). *R. solanacearum* is the causal agent of bacterial wilt disease (BWD), also known as brown rot disease in potatoes, and notorious as one of the most devastating phytopathogenic bacteria in the world (Hayward, 1991; Mansfield *et al.*, 2012). The pathogen has been isolated from every continent except Antarctica, including virgin jungle soils, suggesting the origin of the species complex occurred before the fragmentation of Gondwana (Hayward, 1991).

R. solanacearum has undergone several reclassifications since its discovery in 1896 in line with technical advances. Originally classified under the genus '*Bacillus*' (Smith, 1896) based on its rod shape and association with solanaceous hosts, the identification of biochemical similarities to *Pseudomonas* species resulted in its reclassification as *Pseudomonas solanacearum* (Smith, 1914). The pathogen was then reclassified a third time as *Burkholderia solanacearum* due to its inability to produce fluorescent pigment (Yabuuchi *et al.*, 1992). Since then, advances in 16S rRNA sequence analysis, RNA-DNA hybridisation and fatty acid analyses have resulted in its most recent classification under the new *Ralstonia* genus (Yabuuchi *et al.*, 1995).

R. solanacearum strains are highly adept for genetic exchange *in planta* (Bertolla *et al.*, 2007) and share similar aetiology leading to disease (Patil, Gopal and Singh, 2012). However, they vary widely in their host range and virulence ((Cook, Barlow and Sequeira, 1991); Table 1.5.1). DNA-DNA homology studies indicate that the relatedness between isolates of this species often fall below the 70% threshold level commonly used to define a species (Fegan, 2005). As a result of this unusually broad phenotypic diversity, *R. solanacearum* is taxonomically considered a 'species complex', a cluster of closely related yet genetically distinct strains whose individual members may actually represent more than one species (Fegan, 2005; Peeters *et al.*, 2013). Prior to advances in DNA-based

classification, the *R. solanacearum* species complex was sub-divided into five races determined loosely by host range based on pathogenicity tests (Hayward, 1991), and six biovars determined by ability to metabolise disaccharides and hexose alcohols (Prior and Fegan, 2005). Whilst this classification system was once well-established, race designation was often inaccurate as it did not account for differences between host cultivar, growth conditions and inoculum densities (Gabriel *et al.*, 2006; Genin and Denny, 2011). As a result of the unreliability of race and biovar classifications, the *R. solanacearum* species complex is now categorised by phylotypes and sequevars (Hong *et al.*, 2012). Four phylotypes have been identified and represent a monophyletic cluster of strains determined by sequence data based on the internal transcribed spacer (ITS) region of the 16S-23S rRNA gene and hrpB gene sequences (Prior and Fegan, 2005). Phylotype II has been further divided into two subgroups IIA and IIB due to the extent of phylogenetic data (Castillo and Greenberg, 2007; Cellier *et al.*, 2012). Meanwhile, twenty-three sequevars have been identified and are distinguished by a highly conserved sequence based on the endoglucanase (egl) gene sequence (Prior and Fegan, 2005). The egl gene is essential to *R. solanacearum* pathogenicity and functions in host plant invasion and stem colonisation (Roberts, Denny and Schell, 1988a; Denny, 2007). Whilst these phylotypes are still commonly referenced in regard to the *R. solanacearum* species complex, there have since been further reclassifications based on sequencing advances using average nucleotide identity measures into three species; *R. solanacearum* (phylotype II), *R. pseudosolanacearum* (phylotype I and III) and *R. syzygii* (phyotype IV) (Remenant *et al.*, 2010; Safni *et al.*, 2014; Prior *et al.*, 2016). To date, the most well annotated *R. solanacearum* genome available is of strain GMI1000 belonging to phylotype I (Salanoubat *et al.*, 2002) which was isolated from tomato in French Guyana (Boudazin *et al.*, 1999). The total genome size of GMI1000 is 5.8Mb and comprises

two replicons: a 3.7Mb chromosome and a 2.1Mb megaplasmid with 67% G+C content. The mosaic structure of these replicons likely indicates the acquisition of genes through horizontal gene transfer (Salanoubat *et al.*, 2002).

Table 1.5.1. *Ralstonia solanacearum* species complex classification. Adapted from (García, Kerns and Thiessen, 2019).

Phylotype	Sequevar	Geographic origin	Biovar	Race	Species	Host plant(s)
I	12, 14, 16, 18	Asia	3, 4, 5	1, 4, 5	*R. pseudosolanacearum*	Solanaceae, ginger, mulberry
IIA	1, 2, 3, 4, 5, 6, 7, CIP10, CIP223, NCPPB3987	North America	2-T, 1, 2	1, 2, 3	*R. solanacearum*	Solanaceae, Musa spp.
IIB	1, 2, 3, 4, 5, 6, 7, CIP10, CIP223, NCPPB3987	North America	2-T, 1, 2	1, 2, 3	*R. solanacearum*	Moko disease, brown rot of potato, bacterial wilt of tomato and geranium
III	19, 20, 21, 22, 23	Africa	2-T, 1	1	*R. pseudosolanacearum*	Solanaceae
IV	8, 9, 10, 11	Oceania	2-T, 1, 2	1	*R. syzygii*	Solanaceae, blood disease of banana

1.5.1. Brown rot disease of potato

Whilst the *R. solanacearum* pathogen is most prevalent in tropical and subtropical regions (Hayward, 1991), temperate strains also persist in Europe and North America capable of infecting crops like potato and tobacco (Janse *et al.*, 2005). The first incidence of *R. solanacearum* to reach the UK was documented in 1992 in Oxfordshire (Stead, 1996). This

strain, belonging to phylotype II biovar 1 (previously known as race 3 biovar 2) is the causal agent of brown rot disease in European potatoes (Wenneker *et al.*, 1999). In extreme cases, infection by this phylotype can cause 75% reductions in potato yields (Cook, 1994; Elphinstone, Stanford and Stead, 1998; Elsas *et al.*, 2007), costing over approximately $950 million in potato crop losses globally every year (Champoiseau, Jones and Allen, 2018). This strain is unique to other *R. solanacearum* phylotypes in its ability to persist and infect plants in cold environments (Milling *et al.*, 2009; Cellier and Prior, 2010). Additionally, this phylotype has been shown to have much lower genetic diversity than *R. solanacearum* races originating from the rest of the world (Timms-Wilson, Bryant and Bailey, 2001). Phylotype II biovar I is believed to have originated from the Brazilian Amazon basin (Wicker *et al.*, 2011; Santiago *et al.*, 2017), and spread to Europe in contaminated potatoes, potentially by allied troops during World War II (Janse, 1996; Cellier and Prior, 2010; Wicker *et al.*, 2011), and proliferated excessively in the 1990s. It is now widespread in Europe although with a restricted distribution and few recorded outbreaks (van der Gaag *et al.*, 2019). Dispersal of this temperate phylotype most often occurs through planting of asymptomatic infected seed potatoes (Elphinstone *et al.*, 1996; Janse, 1996, 2012) or through field irrigation using contaminated river water. Water can become contaminated through run-off from potato processing plants or the handling of infected potatoes at water purification sites (Elphinstone, 1996; Janse, 1996; Hong *et al.*, 2012).

R. solanacearum is expected to become increasingly prevalent as the climate changes in response to global warming (Bebber, 2015; Castillo and Plata, 2016), in part due to greater reliance on irrigation during increasingly frequent drought conditions, but also due to the flooding of *R. solanacearum* contaminated rivers as extreme heavy rainfall events become more common (Wenneker *et al.*, 1999). The pathogen is especially difficult to

control due to its rapid adaptation rate, high invasion success (Milling *et al.*, 2009; Wicker *et al.*, 2011), and ability to persist in unfavourable environments for extended periods. For example, *R. solanacearum* has also been shown to persist in the soil for up to two years following removal of infected potato tubers (Elphinstone, 1996), and in surface water for several weeks depending on temperature (Janse *et al*., 1998; van Elsas *et al*., 2001). The pathogen has even been shown to survive for up to 5 hours on farm machinery (Fortnum and Gooden, 2008). Another factor impeding *R. solanacearum* control is its ability to asymptomatically infect weed hosts. Notably, *R. solanacearum* infects woody nightshade (*Solanum dulcamara*) and black nightshade (*Solanum nigrum*) in the UK. These weeds tend to live along river banks and their roots often float on the water, enabling infection from contaminated river water through their roots and the leakage of bacterial cells back into the river, facilitating pathogenic survival through the winter between potato growing seasons (Olsson, 1976; Elphinstone, Stanford and Stead, 1998; Pradhanang, Elphinstone and Fox, 2000). *R. solanacearum* can asymptomatically persist in the xylem vessels of secondary hosts at densities ranging from 10^4 to 10^7 CFU g^{-1} stem (Lowe-Power *et al.*, 2018). Visible symptoms of *R. solanacearum* infection on woody and black nightshade are rare and have only been reported when soil temperatures are greater than 25 °C or when inoculum densities are particularly high (van der Gaag *et al.*, 2019). In these cases, symptoms appear as brown discoloration of the vascular system on cut stems (van der Gaag *et al.*, 2019).

The most rapid method for detection of *R. solanacearum* in infected hosts is to look for bacterial streaming when submerging the infected cut plant stem in water (Tans-Kersten, Huang and Allen, 2001). Detection of *R. solanacearum* in water is optimal when water temperatures fall below 15 °C and samples are taken from within 2 m of the riverbank according to the Council Directive 98/57/EC. *R. solanacearum* can be detected in

soil using a sensitive quantitative assay based on most probable number analysis of PCR results. It involves pre-culture in a buffer containing antibiotics but no other carbon source to allow the pathogen to grow and inhibit the growth of other microorganisms within the sample. The assay can detect *R. solanacearum* at densities as low as 9.3 CFU g^{-1} soil (Inoue and Nakaho, 2014). Alternative methods of detection include observations of colony development following sample growth on semi-selective media (SMSA) agar plates, enzyme-linked immunofluorescent assays (ELISA), indirect immunofluorescent-antibody staining (IFAS), amplification of DNA by PCR and identification of *R. solanacearum* specific DNA sequences and bioassays in tomato seedlings (Elphinstone *et al.*, 1996).

1.5.2. The infectious cycle of *R. solanacearum*

The pathogen's life cycle begins with a saprophytic stage which can last for several years even in the absence of a host (Schell, 2003; Mansfield *et al.*, 2012). Physiological adaptations enable *R. solanacearum* long-term survival even in unfavourable conditions, for example the bacterium can endure starvation (Álvarez, López and Biosca, 2008) by entering a viable but nonculturable state (Overbeek *et al.*, 2007), from which the bacterium can recover and infect host plants (Grey and Steck, 2001). Additionally, *R. solanacearum* has the capacity to form biofilms, enabling protection from desiccation (Yao and Allen, 2007) and survival in the nutrient-limited environment of the host xylem (Lowe-Power, Khokhani and Allen, 2018).

R. solanacearum is believed to detect potential plant hosts through root exudates, in particular glycine amino acids, galactose sugars and citric acids (Yao and Allen, 2007). Upon detection, the pathogen uses its polar flagella to move towards plant roots as directed by chemotaxis (Tans-Kersten, Huang and Allen, 2001; Yao and Allen, 2006, 2007). *R. solanacearum* then attaches to host cells via adhesin proteins, lectins, polysaccharides and

type IV pili and forms microcolonies at sites of lateral root emergence, root elongation zones (Kang *et al.*, 2002) and physical wounds resulting from insect and nematode invasion, or damage from agricultural practices (Hayward, 1991). Inside the plant, the pathogen infects the intercellular spaces of the inner root cortex and subsequently colonises the xylem within 24 hours of infection (Vasse *et al.*, 2007), aggregating to form biofilms and obstructing water movement (Caldwell, Kim and Iyer-Pascuzzi, 2017). This causes the leaf wilt symptoms characteristic of *R. solanacearum* infection at densities of approximately 10^8 CFU g^{-1} stem in tomatoes (Huang and Allen, 2000). Whilst some *R. solanacearum* cells exist planktonically in the xylem sap, others use twitching motility to migrate along the xylem vessel walls (Liu *et al.*, 2001). The pathogen then continues to spread systemically through the plant with maximum proliferation at temperatures of approximately 27 °C (van der Gaag *et al.*, 2019).

R. solanacearum virulence depends upon the suppression of plant defences through the release of type III secretion effectors, extracellular polymeric substances (EPS), cell-wall degrading enzymes including polygalacturonase and endoglucanase, and phytohormone effectors like ethylene gas and auxin which regulate the signalling of plant defence responses (Xiao *et al.*, 1983; Peeters *et al.*, 2013; Deslandes and Genin, 2014). EPS are thought to increase the rate and extent of stem infection spreading from the root (Saile *et al.*, 2007) and further restrict water flow through xylem vessels (Garg *et al.*, 2000). Moreover, cell-wall degrading enzymes enhance virulence by facilitating invasion of roots and bacterial translocation through the xylem (González and Allen, 2007; Liu *et al.*, 2007). The expression of these virulence factors is governed by the Phc quorum sensing system (Genin and Denny, 2011) and *hrp* (hypersensitive response and pathogenicity) genes (Marenda *et al.*, 1998). *R. solanacearum* has adapted to the highly oxidative and nitrate-rich

environment of its host xylem by activating oxidative stress responses, upregulating reactive oxygen species-scavenging enzymes and using nitrate as a terminal electron acceptor (Flores-Cruz and Allen, 2009; Dalsing *et al.*, 2015). As bacterial xylem populations augment, *R. solanacearum* quorum sensing signals accumulate. After reaching a threshold level, a signal transduction cascade is triggered by PhcA activation that switches *R. solanacearum* resource allocation from growth at low cell densities, to increased expression of virulence traits such as EPS and cellulases, whilst repressing swimming motility and siderophore production (Peyraud *et al.*, 2016). During this cellular reprogramming, Phc quorum sensing also mediates the shift of *R. solanacearum* metabolism from a generalist with the ability to assimilate a broad variety of nutrients, including pectin-derived galacturonate and phenolic hydroxycinnamic acids, to a specialist that predominantly exploits just three plant sugars: sucrose, galactose and trehalose (Khokhani *et al.*, 2017). As bacterial cell densities continue to multiply to densities as high as 10^{10} CFU ml^{-1} xylem fluid, wilting symptoms worsen and, in the case of potatoes, tubers begin to stain brown at the vascular ring and often release a bacterial ooze from the eyes (Martin and French, 1985). Ultimately, *R. solanacearum* infection without treatment results in the death of the plant. Following the collapse of the plant, the infected litter acts as a source of inoculum for the subsequent growing season.

1.5.3. Current management of *R. solanacearum*

R. solanacearum was classified as a quarantine pathogen under the EC Plant Health Directive in 1998 and strict legislations are in place to prevent its spread. All infected tubers must be destroyed and reported to the Department of Agriculture, Fisheries and Food and the use of *R. solanacearum* contaminated water for field irrigation is forbidden. Additionally, legislation only allows the importation of potatoes from specific Pest Free Areas (PFAs) with thorough inspection tests and handling by certified processors only

(Defra, 2017). Despite these regulations, seven isolated outbreaks of *R. solanacearum* in potato crops have been documented in the UK since 1992 (Parkinson *et al.*, 2013). All of these have been traced back to contaminated irrigation water except for a single case in 2009 which originated from the use of infected seed potatoes from the Netherlands. To restrict its spread, watercourses throughout the UK are tested regularly for *R. solanacearum.* Storage irrigation water can also be disinfected with peroxygen compounds, chlorination and UV irradiation. Furthermore, secondary host woody nightshade (*Solanum dulcamara*) can be eradicated by treatment with glyphosate (Janse, 1996; Pradhanang and Elphinstone, 1996). Cultural practices like long (2-3 year) crop rotations can also prevent *R. solanacearum* establishment. In comparison to monocultured potato, rotations with wheat, sweet potato, maize, millet, sorghum or carrots reduced the incidence of BWD by 64-94% and increased yields up to 3-fold (Katafiire *et al.*, 2005). Soil fumigants like chloropicrin, dazomet and bromomethane have shown limited success in controlling *R. solanacearum* (Saddler, 2005), due to the pathogen's persistence in the deeper soil layers or protected in host xylem vessels (Wenneker *et al.*, 1999). In China, BWD is often controlled by the application of antibiotics like streptomycin (Diogo and Wydra, 2007). However, this method is unfavourable as it is often ineffective and in some cases can even increase BWD incidences (Farag and Fawzi, 1986), and likely contributes to the development of antibiotic resistance in soil bacteria.

1.5.4. Biocontrol of *R. solanacearum*

As alternatives to chemical control, biocontrol strategies including the enrichment of antagonistic rhizobacteria such as *Bacillus* spp. (Cao *et al.*, 2018), *Pseudomonas* spp. (Ran *et al.*, 2005; Hu, *et al.*, 2016; Sun *et al.*, 2017), *Stenotrophomonas maltophilia* (Messiha *et al.*, 2007), bacteriophage (Wang *et al.*, 2017), and arbuscular mycorrhizal fungi (Zhu and Yao,

2004) have been tested to control *R. solanacearum.* Despite the availability of these biocontrol agents, fumigants and antibiotics remain the most popular disease protection method (Zhou *et al.*, 2012), likely because biocontrol agents often shown variable effects in the field due to poor establishment and insufficient release of antimicrobials (Ran *et al.*, 2005; Wei *et al.*, 2011). Furthermore, the level of suppression achieved by biocontrol agents is often much lower than agrochemicals and sometimes requires application of large amounts of microbial inoculum (Knudsen *et al.*, 1997; Whipps, 2001).

BWD incidence could be further reduced by altering soil chemistry. For example, adjusting soil pH to above 8 or below 5 has been shown to prevent *R. solanacearum* disease development (Sturz *et al.*, 2004; Michel and Mew, 2007). Furthermore, application of pig slurry and composts have been shown to reduce BWD incidence (Islam and Toyota, 2004; Messiha *et al.*, 2007; Gorissen, Overbeek and Elsas, 2011; Youssef and Tartoura, 2013), potentially as a result of higher microbial activity (Islam and Toyota, 2004). Continuous soil organic matter amendment, for example through manure or compost application, also elevates soil substrate availability, increases soil microbial activity and shifts the soil microbiota towards one that is antagonistic to *R. solanacearum* (Hoitink and Boehm, 1999; Satoh and Toyota, 2004). Alternatively, avirulent *R. solanacearum* mutants may offer effective disease control. The most well-established *R. solanacearum* mutant, the hrp- strain, has an inactive type III secretion system (Vasse *et al.*, 2007), and likely inhibits disease by the wild-type through competition for space and nutrients. However, to date, avirulent mutants have not been shown to control bacterial wilt disease in the field (Saddler, 2005).

1.5.5. Developing resistant cultivars against BWD and integrated pest management

The identification of BWD resistant cultivars would offer the most financially economical, effective and environmentally sustainable method to control against the pathogen. Studies identifying resistant cultivars have focused on the most economically important crops including tomato, potato, tobacco, aubergine, pepper and peanut. However, breeding for resistance to BWD is difficult due to the high genetic diversity of the pathogen, the lack of stable sources of resistance and complex inheritance. Hybrids of commercial and wild potato cultivars have shown potential as BWD resistant cultivars, for example crosses of *S. chacoense* (Chen *et al.*, 2013), *S. phureja* (Sequeira and Rowe, 1969; Fock *et al.*, 2000) and *S. commersonii* (Kim-Lee *et al.*, 2005) have elevated BWD resistance compared to their wild-type ancestors. Despite the identification of brown rot disease resistant potato cultivars (Norman, Yuen and Bocsanczy, 2020), these are often poorly adapted to different field environments, resulting in reduced tuber yield and quality (Dahal *et al.*, 2010; Yuliar, Nion and Toyota, 2015), and remain susceptible to some *R. solanacearum* strains (Wang *et al.*, 2007), and asymptomatic infection (Priou, Gutarra and Aley, 1999). Alternatively, resistant cultivars could instead be derived transgenically. One study observed a 70% increase in tomato BWD resistance by transgenic expression of *Arabidopsis* NPR1 gene in tomato (Lin *et al.*, 2004). More recently, introgression of the *Arabidopsis* EF-Tu gene in the potato genome enabled enhanced resistance to *R. solanacearum* in greenhouse experiments (Boschi *et al.*, 2017) and expression of this gene also improved wheat tolerance to halo blight caused by *P. syringae* pv. *oryzae* (Schoonbeek *et al.*, 2015). Advances in transcriptome sequencing may aid in the identification of specific genes underlying *R. solanacearum* resistance in potato

(Cao *et al.*, 2020), and could facilitate the breeding or transgenic development of a high-yielding resistant cultivar.

Integrated pest management may maximise the efficiency of *R. solanacearum* control, as has been shown in studies combining the application of probiotic inoculum of *Bacillus* and *Serratia marcescens* with resistant cultivars of tomato (Barretti *et al.*, 2011). Additionally, in a field study the combination of microbial restoration substrate with an avirulent *R. solanacearum* mutant had synergistic effects in controlling against BWD (Zheng *et al.*, 2019). Moreover, the application of *P. fluorescens* alongside the plant defence priming chemical, acibenzolar-S-methyl, resulted in more effective control of BWD than single control techniques (Abo-Elyousr, Ibrahim and Balabel, 2012).

To conclude, there are no effective and sustainable control mechanisms to protect against *R. solanacearum* that provide adequate control without compromising yield. This, combined with the discovery that the pathogen is undergoing host range expansion (Genin and Boucher, 2004; Wicker *et al.*, 2009; Guidot *et al.*, 2014), with previously uncharacterised distinct populations of *Ralstonia* identified on *Mandevilla splendens* (Ruhl *et al.*, 2011), blueberry (Norman *et al.*, 2017), and European tea-rose (Tjou-Tam-Sin *et al.*, 2016; Bergsma-Vlami *et al.*, 2018), intensifies the need to develop effective and environmentally sustainable control techniques.

1.6. Biofumigation

Biofumigation may offer an effective biocontrol technique against *R. solanacearum* infection in potato. Biofumigation exploits *Brassica* crops naturally elevated in glucosinolate (GSL) and myrosinase compounds that upon tissue disruption react to release bioactive compounds including isothiocyanates (ITCs) (Cole, 1976; Fenwick and Heaney, 1983). Biofumigation has

been shown to suppress soilborne nematode (Lord *et al.*, 2011; Ngala *et al.*, 2015), fungal (Angus *et al.*, 1994; Sarwar *et al.*, 1998; Rumberger and Marschner, 2003) and bacterial pathogens (Hu *et al.*, 2015). The most common *Brassica* biofumigants are Indian mustard (*Brassica juncea*), rocket (*Eruca sativa*) and oil radish (*Raphanus sativus*).

1.6.1. Glucosinolates and their breakdown products

Glucosinolates (GSLs) are nitrogen and sulphur containing secondary metabolites produced by plants of the order Brassicaceae. Over 120 GSLs have been identified in plants (Fahey, Zalcmann and Talalay, 2001). They are found in 11 plant families but are most commonly associated with the Brassicaceae (Borek *et al.*, 1995), present in the vacuoles of most cells (Kelly, Bones and Rossiter, 1998). GSLs function as cancer-prevention agents, flavour compounds and biopesticides, but are believed to have evolved initially to function in plant defence against pathogens and insect herbivores (Wittstock and Halkier, 2002; Bednarek *et al.*, 2009). GSLs themselves are biologically inactive (Manici, Lazzeri and Palmieri, 1997; Buskov *et al.*, 2002; Lazzeri and Malaguti, 2004) before engaging in a hydrolysis reaction with the separately sequestered enzyme myrosinase (β-thioglucoside glucohydrolase). Upon *Brassica* tissue disruption, for example during insect chewing, cell integrity is compromised, allowing GSLs and myrosinases to react with each other (Bones and Rossiter, 1996; Gimsing and Kirkegaard, 2006) in a reaction known as the 'mustard oil bomb' (Kissen, Rossiter and Bones, 2009). During this reaction, myrosinase cleaves off the glucose group from the GSL which then spontaneously undergoes a Lossen rearrangement to form an unstable aglycone; thiohydroximate-O-sulfonate. The unstable aglycone then degrades into one of several different bioactive hydrolysis products, predominantly ITCs (Bending and Lincoln,

1999);Fig. 1.6.1), and in lower abundance nitriles, thiocyanates, epithionitriles and oxazolidines.

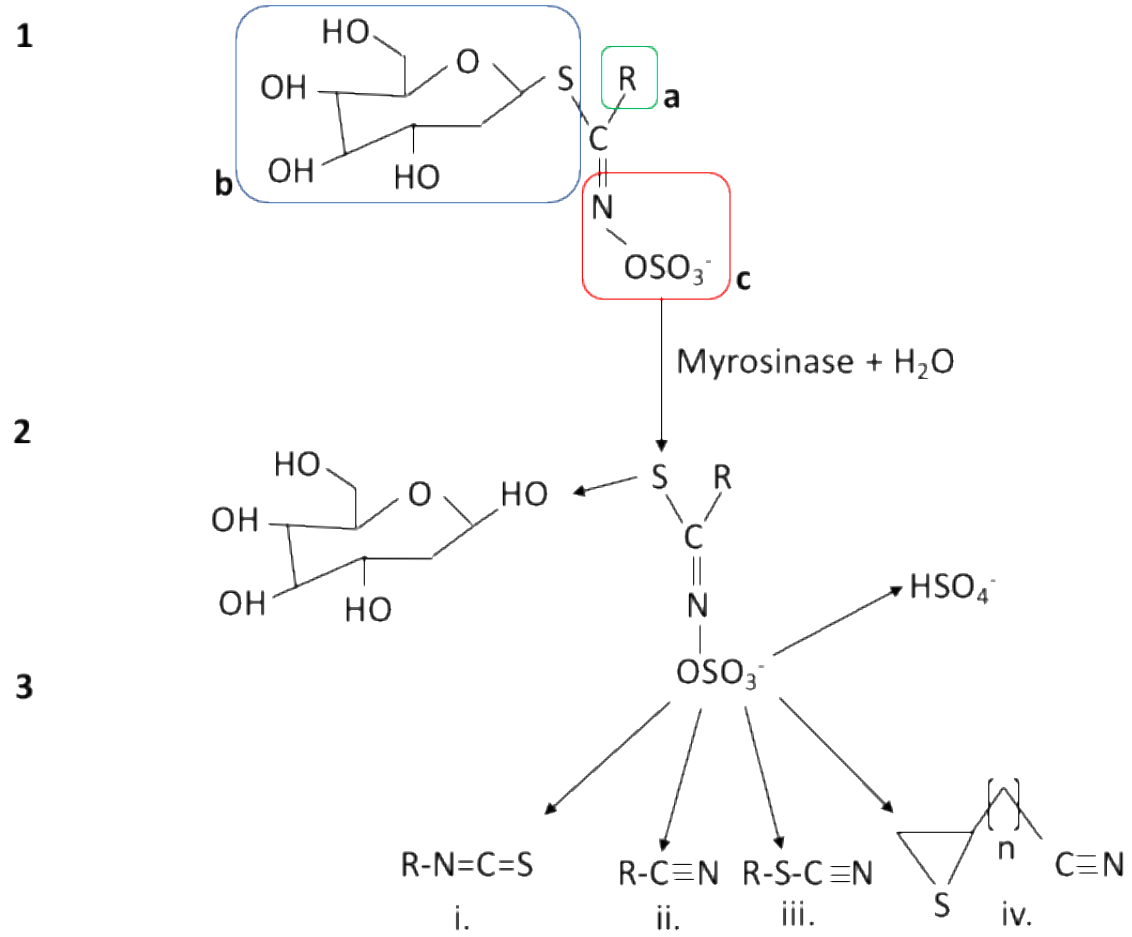


Figure 1.6.1. The general structure of glucosinolates and their hydrolysis products. (1) Glucosinolate structure, indicating the a. variable R sidechain; b. the β-thioglucoside; and c. the sulfonated oxime. (2) The myrosinase enzyme catalyses a hydrolysis reaction that converts the glucosinolate into a D-glucose molecule and an unstable aglucone. (3) Glucosinolate hydrolysis products under different conditions showing an i. isothiocyanate; ii. Nitrile; iii. thiocyanate and iv. epithionitrile.

GSLs are composed of a beta-D-glucopyranose residue linked to a (Z)-N-hydroximinosulfate ester and a variable R group by a sulphur atom derived from an α-amino

acid (Poulsen *et al.*, 2008). The R group determines the chemical classification of the compound according to their amino acid precursor as either aliphatic (derived from alanine, leucine, isoleucine, valine, methionine or glutamate), aromatic (derived from phenylalanine and tyrosine) or indole (derived from tryptophan) and the breakdown products released (Fahey, Zalcmann and Talalay, 2001) and can be modified during biosynthesis by chain-elongation, hydroxylation, methylation and oxidation (Fahey, Zalcmann and Talalay, 2001; Grubb and Abel, 2006; Sønderby *et al.*, 2010). The derivative produced during GSL hydrolysis depends on the GSL-R group, soil pH, the presence of myrosinase-binding proteins and the concentration of ferrous ions (Grubb and Abel, 2006; Kissen, Rossiter and Bones, 2009; Hanschen *et al.*, 2015b). Conversion to ITC is maximised at neutral soil pH (6-7) (Halkier and Gershenzon, 2006), whilst at acidic pH (4-7) during hydrolysis or in the presence of specific proteins, nitriles are more likely to be released (Bones and Rossiter, 2006), which are less toxic than ITCs. Thiocyanate-forming proteins lead to the generation of thiocyanates during hydrolysis of certain GSLs including sinigrin (allyl GSL) and benzyl GSL, yet other GSL-types form simple nitriles (Kuchernig *et al.*, 2011). Moreover, in the presence of epithiospecifier protein, a small protein that is present in some *Brassica*, and iron ions, GSLs with terminally unsaturated nitriles form epithonitriles as well as ITCs (Wittstock *et al.*, 2003; Holst and Williamson, 2004). However, when epithiospecifier proteins interact in the hydrolysis reaction of indolic GSLs, a nitrile is generated (Agerbirk *et al.*, 2008). Nitrile generation is also promoted in the presence of nitrile-specific proteins (Kong, Kissen and Bones, 2012).

Myrosinase (EC 3.2.1.147), the glycosylated thioglucosidase, are a class of enzymes of the β-glycosidase family found in all plant organs containing GSLs (Andersson *et al.*, 2009). They are stored in the vacuoles of myrosin cells (Bones and Rossiter, 1996) and catalyse the hydrolysis of GSL. Aside from plant cell myrosinase, the enzyme can also be

produced by soil microorganisms including fungi (Sakorn *et al.*, 2002) and bacteria (Cheng, Hashimoto and Uda, 2004; Gimsing and Kirkegaard, 2006; Hanschen *et al.*, 2015b), whose abundance in the soil can be stimulated by the presence of *Brassica* (Gimsing and Kirkegaard, 2009b). Thus, the formation of GSL-hydrolysis products has been demonstrated in the absence of *Brassica* tissue disruption (Gimsing *et al.*, 2007).

Some *Brassica* plants have a single dominant GSL whilst others contain a mixture. For instance, the model plant *Arabidopsis thaliana* contains 36 different GSLs within its tissues (Reichelt *et al.*, 2002; Brown *et al.*, 2003). *Brassica* GSL concentrations generally determine the magnitude of pathogen suppression (Fenwick and Heaney, 1983; Matthiessen and Kirkegaard, 2006), and their predominant breakdown product, ITCs, are believed to primarily account for the biocidal effects of biofumigation (Lord *et al.*, 2011). *Brassica* species differ in their tendency to release different ITCs as a result of differences in the GSL pre-cursor profiles. This is illustrated by oilseed rape (*B. napus*) which mostly forms indole GSLs that spontaneously break down into unstable ITCs to form alcohols (Holst and Williamson, 2004). Meanwhile, Indian mustard (*B. juncea*) mainly produces aliphatic GSLs and white mustard (*Sinapis alba*) mainly synthesises aromatic GSLs (Sarwar *et al.*, 1998). GSL concentrations also differ widely between *Brassica* species and cultivar. For instance, a study of 80 *Brassica* species sampled at flowering found that total GSL production ranged from 8-453 moles ha^{-1} due to differences in biofumigant biomass and GSL concentration (Sarwar *et al.*, 1998). While GSL profiles are highly dependent on the plant genotype (Satoh and Toyota, 2004), they can also differ depending on plant growth stages: younger plants tend to have lower GSL concentrations than older plants (Bellostas, Sørensen and Sørensen, 2007), and between plant organs (Shroff *et al*., 2008): roots tend to be dominated by aromatic GSLs like gluconasturtiin, which produce lower concentrations of

volatile ITCs, while shoots predominantly produce aliphatic GSLs including sinigrin and gluconapin which form highly volatile ITCs (Sarwar *et al.*, 1998; Motisi *et al.*, 2009). Seeds also have particularly high concentrations of GSLs (Holst and Williamson, 2004).

GSL concentrations can be stimulated based on seasonal cues, light availability and upon herbivore attack (Rask *et al.*, 2000; Huseby *et al.*, 2013), and higher GSL levels have been reported for *Brassica* plants grown in spring compared to those sown in autumn (Huseby *et al.*, 2013). Maximum GSL levels have been obtained from *Brassica* at 50% flowering when harvested at midday (Matthiessen and Kirkegaard, 2006; Huseby *et al.*, 2013; Doheny-Adams *et al.*, 2018) and higher soil sulphur levels have been shown to correlate with increased *Brassica* GSL concentrations (Falk, Tokuhisa and Gershenzon, 2007; Kopsell *et al.*, 2007). However, increasing soil sulphur to excessive levels does not result in GSL usage as a storage sink (Aghajanzadeh, Hawkesford and De Kok, 2014), which suggests GSLs only have a finite capacity for sulphur storage. GSL levels can also be stimulated by pathogen or herbivore attack (Agrawal and Kurashige, 2003; Reymond *et al.*, 2004), leading to a priming response (Bakhtiari, Glauser and Rasmann, 2018), which can enable plants to respond more strongly and rapidly to subsequent attack and provide systemic resistance (Pierre *et al.*, 2013).

Selective breeding has led to new varieties of Brassicaceae with different GSL contents and profiles to improve taste, maximise anti-carcinogenic effects and enhance plant defence. Previously, natural toxins like the GSL progoitrin (2-hydroxy-3-butenyl) in canola were bred out but more recently, selective breeding has been used to maximise GSL levels like 4-methylsulfinylbutyl GSL, the precursor of the putative anti-carcinogen sulforaphane (Faulkner, Mithen and Williamson, 1998; Sarikamis *et al.*, 2006), and optimise plant defences (Potter *et al.*, 2000; Kirkegaard, Rebetzke and Richards, 2001). There have

also been investigations into the potential to engineer GSLs into non-GSL producing crops (Møldrup *et al.*, 2012) to enhance disease resistance and reduce reliance upon chemical pesticides. Thus, careful selection of a *Brassica* biofumigant with elevated levels of GSLs stimulated by spring-sowing and harvest during the day could maximise biofumigant potential.

1.6.2. Pathogen-suppressing effects of isothiocyanates (ITCs)

ITCs are secondary metabolites generated as a breakdown product of GSL hydrolysis and widely recognised as largely responsible for the biocidal effects of biofumigation (Lord *et al.*, 2011). They are generally incredibly short-lived in natural soils: over 99% of ITCs are lost within 24-72 hours after biofumigation (Mazzola *et al.*, 2007a; Motisi *et al.*, 2009; Mazzola and Zhao, 2010), and after 1-2 weeks ITCs were found to be completely undetectable (Gimsing and Kirkegaard, 2006). ITC losses occur as a result of their volatility (Borek *et al.*, 1995; Dungan, Gan and Yates, 2003), sorption to organic substrates (Borek *et al.*, 1995; Gimsing and Kirkegaard, 2009b), chemical (Borek *et al.*, 1995; Dungan, Gan and Yates, 2003) and microbial degradation (Mazzola *et al.*, 2007a; Mazzola and Zhao, 2010). Highly lipophilic ITCs are more prone to sorption to organic matter than less lipophilic ITCs (Gimsing and Kirkegaard, 2009b). Chemical degradation of ITCs occurs by reactions with nucleophilic compounds like amines, thiols or water and is accelerated in warmer and alkaline soils (Hanschen *et al.*, 2012). These loss processes vary depending on soil type, water content and temperature (Gimsing and Kirkegaard, 2009b). For instance, clay soils strongly sorb ITC and limit free ITC concentrations as a result of their small pore spaces, high-water holding capacities (Frick, Zebarth and Szeto, 1998; Matthiessen and Shackleton, 2005; Gimsing and Kirkegaard, 2009b) and typically high organic matter concentrations, thus also supporting larger microbial populations which accelerates microbial degradation (Gimsing and

Kirkegaard, 2006). ITC retention in the soil can be increased by irrigation of soil immediately following biofumigant incorporation to trap ITC volatiles (Morra and Kirkegaard, 2002). Additionally, ITC persistence can be extended further by covering soil following biofumigation with a plastic film (Lord *et al.*, 2011; Hansen and Keinath, 2013), though this is not always economically viable. Thus, well-irrigated sandy soils with low organic matter and maceration of the *Brassica* at the point of flowering in spring using a hammer are optimal for biofumigation ITC release and retention.

ITC reactions are non-specific and irreversible. The mechanism of ITC toxicity is unclear although it is generally agreed that they react with sulphur-containing groups in proteins and are inhibitory to nematode, fungal and bacterial pests and pathogens. They prevent cellular respiration of nematodes resulting in paralysis or death and can also cause the premature hatching of juvenile nematodes from eggs which causes them to starve in the absence of a host. Alternatively, ITCs function against fungi by inhibiting mycelial growth (Angus *et al.*, 1994; Sarwar *et al.*, 1998) and preventing germination (Dawson *et al.*, 1993), spore (Vierheilig and Ocampo, 1990) and sporangia development (Greenhalgh and Mitchell, 1976). With bacteria, ITCs are believed to act by breaking sulphide bonds in enzymes, disrupting their tertiary structure (Brown, 1997), or by damaging the outer cell membrane and allowing cell metabolites to leak out (Lin, Preston and Wei, 2000) .

The chemical structure of the variable R sidechain of ITCs influences its biological and chemical properties, including volatility, solubility and hydrophobicity (Drobnica *et al.*, 1967; Angus *et al.*, 1994; Manici, Lazzeri and Palmieri, 1997; Sarwar *et al.*, 1998). Low molecular weight, highly mobile ITCs with short side chains have been shown to have greater toxicity than those of higher molecular weight with longer side chains (Sarwar *et al.*, 1998; Neubauer, Heitmann and Müller, 2014). Meanwhile, ITCs derived from aliphatic GSLs

have demonstrated greater toxicity in air and soil than their aromatic counterparts (Sarwar *et al.*, 1998; Matthiessen and Shackleton, 2005) but were less toxic than aromatic ITCs in agar plate experiments (Drobnica *et al.*, 1967), potentially due to their higher volatility and thus shorter persistence in this *in vitro* setting (Sarwar *et al.*, 1998; Matthiessen and Shackleton, 2005). This was proven experimentally by observing the evaporation of a drop of aliphatic allyl-ITC in just five minutes at room temperature, whilst aromatic 2-phenylethyl ITC remained stable/active for over 72 hours (Sarwar *et al.*, 1998).

GSL-ITC conversion rates are often very low. The highest conversion rate of GSL to ITC achieved in field trials is 60% (Gimsing and Kirkegaard, 2006), though ITC release efficiency levels as low as 5% have been recorded (Bending and Lincoln, 1999; Gardiner *et al.*, 1999; Morra and Kirkegaard, 2002). This variation is potentially due to insufficient breaking down of plant tissues during biofumigation (Morra and Kirkegaard, 2002), rapid volatilisation of ITCs into the atmosphere before measurement, or the reaction and breakdown of strong electrophilic ITCs with nucleophilic compounds (Drobnica *et al.*, 1967; Borek *et al.*, 1995). A number of ways to maximise ITC liberation from *Brassica* plant tissues have been suggested. For instance, GSL-ITC conversion is favoured in soils of high-water content, probably due to more rapid GSL hydrolysis, and increased stability of ITCs in these soils (Hanschen *et al.*, 2015b). In addition, GSL hydrolysis can be facilitated by maceration of *Brassica* tissues with a hammer implement, such as a flail-topper, rather than using bladed tools (Kirkegaard and Matthiessen, 2005). Cellular damage can further be increased by freezing and thawing of leaf material before incorporation which can elevate ITC concentrations 400-fold (Morra and Kirkegaard, 2002).

1.6.3. Pathogen-suppressing effects of non-GSL release products

GSL content of biofumigant material does not consistently correlate with the extent of pathogen suppression (McLeod and Steel, 1999; Mazzola, Granatstein, *et al.*, 2001; Cohen, Yamasaki and Mazzola, 2005), suggesting other factors may contribute to the biocontrol effects of biofumigation. The efficacy of *Brassica* biofumigation could be explained in part by the emission of non-GSL related bioactive compounds that are naturally elevated in *Brassica* including dimethyl sulphide, dimethyl disulphide, carbon disulphide and methanedithiol (Bending and Lincoln, 2000; Wang *et al.*, 2009). These compounds are formed as a result of the breakdown of sulphur-containing amino acids and sulphoxides (Banwart and Bremner, 1975) and are less toxic than ITCs but produced in greater abundance (Bending and Lincoln, 1999). Their toxicity has previously been demonstrated against plant parasitic nematodes (Gerik, 2005). The high vapour pressure of these compounds means they are more likely to remain in the gaseous phase and diffuse rapidly, potentially enhancing the strength of disease suppression (Wang *et al.*, 2009). Hence, the biofumigant effect is likely to result from a combination of factors including the liberation of both GSL and non-GSL hydrolysis products.

Aside from GSL and non-GSL release products, the incorporation of organic matter to the soil during biofumigation may also contribute to biocidal effects as well as affecting soil structure (Chan and Heenan, 1996), enhancing nutrient cycling (Thorup-Kristensen, Magid and Jensen, 2003) and limiting soil erosion (McGuire, 2003).

1.6.4. The response of the soil microbiome to biofumigation

The soil ecosystem is an incredibly complex and diverse environment. The zone of soil surrounding crop roots, the rhizosphere, contains up to 10^{11} microbial cells per gram root

(Egamberdieva *et al.*, 2008) and 10^4 species per gram soil (Curtis, Sloan and Scannell, 2002), including species of nematodes, fungi, bacteria and protists (Bardgett and Van Der Putten, 2014). These rhizosphere soilborne microorganisms have been shown to have fundamental impacts on biogeochemical cycling, climate regulation, their host plant's disease resistance, aboveground diversity and ecosystem functioning (Schroth and Hancock, 1982; Bender, Wagg and van der Heijden, 2016). However, decades of intensive agriculture, including monoculture cropping, pesticide application and intensive tillage, have led to soil degradation and erosion of belowground diversity (Verbruggen and Kiers, 2010; Stavi and Lal, 2015; Tsiafouli *et al.*, 2015), enabling increased opportunities for pathogen invasion (Griffiths *et al.*, 2009; Hu *et al.*, 2016), and hence, higher levels of crop disease.

Whilst many studies have focused on the potential of biofumigation to control soilborne pathogens, the effects of this biocontrol technique on beneficial and non-target microorganisms is largely overlooked. Often, the suppressive effects of biofumigation outlast the duration expected for a purely chemical mode of action (Cohen, Yamasaki and Mazzola, 2005). This may be partly due to rearrangements in soil community structure driven by biofumigation which engineer disease suppressive soils through enrichment of pathogen-antagonistic bacteria. Overall, biofumigation has been shown to stimulate microbial activity (Omirou *et al.*, 2011a). Specifically, biofumigation studies have demonstrated the enrichment of certain bacterial communities, including those considered plant growth-promoting rhizobacteria, whilst pathogenic species are suppressed (Smolinska, 2000; Cohen, Yamasaki and Mazzola, 2005; Larkin and Honeycutt, 2006; Weerakoon *et al.*, 2012; Mazzola, Hewavitharana and Strauss, 2015; Zhu *et al.*, 2020). The structure of microbial communities has been shown to change with the concentration of ITC in the rhizosphere (Rumberger and Marschner, 2003). For example, one study tested the effects of

allyl-ITC on soil bacteria and fungi and observed increased abundance of probiotic species including *Sphingomonas*, *Streptomyces*, *Acremonium* and *Pseudallescheria* (Zhu *et al.*, 2020). Thus, the long-term suppressive effects of biofumigation may be stimulated initially by ITC release which mediates a change in the soil microbial composition towards a long-term disease suppressive microbiome.

Several studies have investigated the effects of biofumigation on whole microbial communities with contradicting results. For example, some studies have shown reductions in nitrifying bacterial populations (Bending and Lincoln, 1999), whilst others have shown enhanced nitrification following biofumigation (Hollister *et al.*, 2013a). In addition, whilst some studies claim biofumigation increased bacterial diversity and decreased fungal diversity (Hollister *et al.*, 2013a; Wang *et al.*, 2014), others have reported reduction in overall soil microbial diversity (Ibekwe *et al.*, 2001; Zhang *et al.*, 2020), and others no changes following biofumigation (Wei *et al*., 2016). These conflicting findings could be explained by differences in soil type, biofumigant application techniques and biofumigant plant species between studies, highlighting the need for more detailed research into the effects of biofumigation on soil microbial communities.

Despite the perceived environmental benefits of biofumigation (Kirkegaard and Matthiessen, 2005), and often comparable suppressive effects to synthetic fumigants (Wang *et al.*, 2009), ITCs may be even more toxic than synthetic fumigants and pesticides (Gimsing and Kirkegaard, 2009b). The broad-spectrum activity of ITC could result in the suppression of non-target beneficial microorganisms (Bending and Lincoln, 1999; Ibekwe *et al.*, 2001; Rumberger and Marschner, 2003; Ibekwe and Papiernik, 2004), such as biocontrol species (Henderson *et al.*, 2009; Ramirez *et al.*, 2009) and earthworms (Fouché, Maboeta and Claassens, 2016), and lead to the disruption of trophic levels. Subsequently, this could

facilitate pathogen recolonisation (Van Bruggen and Semenov, 2000a). Furthermore, there is the potential for organisms to 'disarm the mustard oil bomb' and become tolerant to ITCs. This has been documented in some *Brassica*-specific fungi (Buxdorf *et al.*, 2013) and insects (Wittstock *et al.*, 2004). In more detail, the fungal pathogen *Sclerotinia sclerotiorum* developed ITC tolerance in less than 72 hours through the up-regulation of three glutathione S-transferase genes (Rahmanpour, Backhouse and Nonhebel, 2009). Meanwhile, the diamondback moth (*Plutella xylostella*) was found to evolve sulfatases in its gut which enabled hydrolysis of the GSL sulphate ester bond to inhibit ITC biosynthesis (Ratzka *et al.*, 2002; Ma *et al.*, 2018). Indeed, one study even showed improved growth in last instar lepidopteran larvae on GSL-producing plants (Jeschke *et al.*, 2017). Repeated exposure of microorganisms to ITC could also drive enhanced biodegradation, a process where microbes adapt to degrade and utilise ITCs as a food source (Rahmanpour, Backhouse and Nonhebel, 2009). Enhanced biodegradation has previously been documented in response to exposure to synthetic methyl ITC (Warton, Matthiessen and Shackleton, 2001) and could also lead to cross-enhancement degradation, where the microorganisms develop the capacity to breakdown other types of ITCs (Warton, Matthiessen and Shackleton, 2003a). Tolerant microorganisms may also benefit from the killing of their vulnerable counterparts by ITC by feeding upon them (Macalady, Fuller and Scow, 1998). In some cases, pathogens can even benefit from biofumigation by using *Brassica* as a food source (Stephens, Davoren and Wicks, 1999; Lu, Gilardi, Gullino, *et al.*, 2010). The varying responses of soil microorganisms to biofumigation could be explained in part by differences in their innate ITC tolerance and their ability to adapt to ITCs. Differences in the sensitivity of microbiota to biofumigation may be explained by physical differences that reduce ITC exposure including thick-walled chlamyodspores, sclerotia in *Sclerotinia* and hyphae in *Rhizoctonia* pathogens (Smolinska

and Horbowicz, 1999; Yulianti, Sivasithamparam and Turner, 2007). Hence, differential effects of ITC could result in shifts to soil microbiome structure towards a more disease suppressive or disease conducive microbiome and may drive ITC tolerance in certain microorganisms.

1.6.5. Biofumigation in agricultural practice

Biofumigants are generally grown for 8-14 weeks between July and November before integration into the soil. The field is then left vacant for approximately two weeks following biofumigation before sowing the subsequent crop, to provide sufficient time for bioactive compounds to suppress pathogens whilst minimising phytotoxic effects on crops. The ideal biofumigant is naturally elevated in GSLs, produces high levels of biomass and reaches maturity rapidly, though the optimal biofumigant species and cultivar may vary depending on the sowing window and target pest or pathogen. *Brassica* biomass production can be maximised by fertilising the soil with 60-80 kg/ha of nitrogen and 50-60 kg/ha of sulphur (AHDB, 2019). When selecting the biofumigant, growers should also consider the potential for clubroot, a fungal disease that infects species of *Brassica*, and cabbage root fly, and use resistant *Brassica* varieties if available. Several seed companies supply commercial biofumigant species, for example *B. juncea* cultivar ISCI 99 has been bred to produce high concentrations of sinigrin GSL in its tissues and high biomass, reaching 40-70t ha^{-1} in summer.

Aside from the environmental benefits of biofumigation, there may also be some financial savings from using the technique. Biofumigant seed costs approximately £60-100/ha for Indian mustard, rocket and oilseed radish and requires an additional £400-500/ha for crop sowing and maintenance (Lord *et al.*, 2011). Additionally, generally farmers

already possess the machinery necessary for maceration and rolling of the biofumigant into the soil. In comparison, chemical fumigants like methyl bromide combined with chloropicrin at recommended application rates costs the equivalent of approximately £600/ha. Biofumigation can occur through several methods, as outlined below:

(i) **Incorporation of biofumigants as a green manure.** This is the most widely used method in *in vivo* field trials (Lord *et al.*, 2011; Valdes, Viaene and Moens, 2012). It involves the growth of *Brassica* crops during the intercrop period between two commercial crops, usually until flowering where GSL levels peak (Rumberger and Marschner, 2003; Gimsing and Kirkegaard, 2006), and thorough maceration using a mulching device (Matthiessen and Shackleton, 2005). The crop residues are then incorporated to the soil using a rotary flail device to a depth of 10-20 cm. Incorporation should occur within 30 seconds of biofumigant maceration. The soil surface can be sealed by gentle rolling using a rotavator hood or soil irrigation to ensure complete GSL hydrolysis, trap toxic volatiles and maximise the duration of disease suppressive effects (Morra and Kirkegaard, 2002; Kirkegaard and Matthiessen, 2005). This method of biofumigation simultaneously enriches soil nutrients including nitrogen, potassium and phosphorus as a result of organic matter incorporation and has proven successful in controlling against soilborne potato diseases in numerous field trials (McGuire, 2003; Larkin and Griffin, 2007; Snapp *et al.*, 2007).

(ii) **Biofumigation using *Brassica* seed meals.** Defatted seed meals produced as a by-product of processing high-GSL *Brassica* seeds for oil, for example in mustard crops, can serve as an effective biofumigant (Mazzola *et al.*, 2007a; Matteo *et al.*, 2018). Seed meals offer a convenient method of biofumigation since the timing of

application can be flexible even during periods of frost when growth of biofumigants is limited. Hence, this method of biofumigation can be easily integrated with crop rotations. GSL concentrations are highest in the seed (Popova and Morra, 2014) and the drying of plant material as part of the seed meal manufacturing process preserves GSL-myrosinase activity (Michel, 2014). Upon incorporation to the soil and watering, bioactive products including ITCs are released from seed meals. *Brassica* seed meals have shown promise in the suppression of soilborne fungal (Cohen, Yamasaki and Mazzola, 2005; Mazzola *et al.*, 2007a; Weerakoon *et al.*, 2012) and nematode pests (Lazzeri *et al.*, 2009; Zasada *et al.*, 2009). The use of *Brassica* seed meals has also been shown to be conducive to suppressive soils (Cohen and Mazzola, 2006a; Mazzola, Hewavitharana and Strauss, 2015). Seed meals are also commercially available in the form of pellets (Lazzeri *et al.*, 2009; Morales-Rodriguez and Wanner, 2015). Despite this, seed meals are not as widely used as green manures for biofumigation, potentially due to their limited availability and cost (Rahman and Somers, 2005).

(iii) **Rotations or intercropping with biofumigants (partial biofumigation).** This involves the growth of *Brassica* plants as a 'living mulch' during the break period and allows the harvest of aboveground plant material to enhance cash-crop yield. This method has been shown to be effective against nematode (Ngala *et al.*, 2015; Park *et al.*, 2019) and fungal pathogens (Motisi *et al.*, 2009; Larkin and Lynch, 2018). The suppression of soilborne pathogens via partial biofumigation relies on GSLs, ITCs or other bioactive compounds released through root exudates (Rumberger and Marschner, 2003; Van Dam *et al.*, 2009), and negates the requirement to incorporate the biofumigant into the soil. Canola (*B. napus*) and Indian mustard (*B.*

juncea) growth as break crops has been shown to improve the yield of subsequent crops in the rotation (Angus *et al.*, 1994; Kirkegaard *et al.*, 2000; Morra and Kirkegaard, 2002), potentially as a result of 2-phenylethyl ITC release from *Brassica* roots (Sarwar *et al.*, 1998; Smith and Kirkegaard, 2002). Additionally, soil microorganisms, such as *Aspergillus* spp., capable of producing myrosinase tend to be enriched in regions of *Brassica* growth (Borek *et al.*, 1995; Gimsing and Kirkegaard, 2006). This means that GSL hydrolysis by myrosinase can occur even in the absence of plant myrosinase exudation. Whilst only low concentrations of ITC (1-2 nmol g^{-1} soil) are released to the soil during *Brassica* growth (Schreiner and Koide, 1993), continuous exposure can affect microbial community composition and influence disease susceptibility (Rumberger and Marschner, 2003). However, in some cases there were no yield improvements following *Brassica* intercropping, potentially due to the insufficient levels of ITC released from intact roots (Smith, Kirkegaard and Howe, 2004). Despite the convenience of this technique, generally, optimal disease suppressive effects of biofumigation are achieved with complete biofumigation, including incorporation of the *Brassica* crop to soil (Motisi *et al.*, 2009).

1.7. Thesis Chapter Outline

This thesis includes the following chapters, presented in the form of research papers which aim to address three central research questions. Firstly, which ITCs are effective *against R. solanacearum* and can tolerance evolve; secondly, what are the effects of ITCs on non-target microorganisms; and finally, how does ITC exposure influence host health?

Chapter 2: Plant pathogenic bacterium can rapidly evolve tolerance to an antimicrobial plant allelochemical

In this chapter, we compared the efficacy of three different ITCs commonly released by biofumigation with Indian mustard against *R. solanacearum* when applied alone or in combination. We found there was no additive suppressive effect in combining ITCs and in all cases, allyl-ITC applied alone was most suppressive to the plant pathogenic bacterium. Focusing only on the most suppressive allyl-ITC, we tested whether repeated exposure during an *in vitro* serial transfer experiment could select for tolerant *R. solanacearum* strains. We exposed the pathogen to allyl-ITC at three different rates, high, medium and low, (daily, every two-day and every three-day; respectively) and observed ITC tolerance evolution only in the three-day exposure treatment. Interestingly, we also observed ITC tolerance in *R. solanacearum* strains previously grown in control conditions (no ITC) in the three-day transfer treatment. We attributed this to media adaptation. Genome sequencing identified insertion mutations in genes linked with metabolism and antibiotic resistance (dehydrogenase-like protein) and transmembrane protein movement (Tat pathway signal protein). Moreover, insertion sequence movement at one position on the chromosome (acyltransferase gene associated with lipid storage and toxin production) and three positions on the megaplasmid (genes associated with iron storage (2-Fe-2S-binding protein), stress responses (H-NS histone like protein) and calcium ion sequestration (calcium-binding protein)) were observed that may explain this ITC tolerance evolution.

Chapter 3: Allyl-ITC selectively suppresses a pathogenic bacterium in model bacterial communities

Following on from Chapter 2, we developed model communities to determine the effects of allyl-ITC on *R. solanacearum* and non-target PGPR bacteria, *Pseudomonas,* using liquid and

soil *in vitro* and *in vivo* tomato mesocosm experiments. We also evaluated the potential for *R. solanacearum* to develop ITC tolerance. We found that *Pseudomonas* were less susceptible to ITC suppression *than R. solanacearum* pathogen and growth in co-culture amplified the suppressive effects of ITC on *R. solanacearum* growth. Additionally, no evidence for ITC tolerance or adaptation to *Pseudomonas* supernatant was observed. Whilst ITC exposure reduced tomato BWD symptoms, phytotoxic effects were also found, including reduced dry weight and inhibition of flowering when *Pseudomonas* were inoculated to the soil. In contrast to our *in vitro* liquid findings, we found much weaker signs of tolerance evolution in soil microcosm experiments, suggesting ITC tolerance evolution might be less common in natural field conditions.

Chapter 4: Collateral effects of model biofumigation on soil microbiome composition and diversity

In this chapter, we identified the effects of ITCs on *R. solanacearum* pathogen and non-target microbiota in natural soil communities derived from the kale and potato rhizosphere using 16S rRNA amplicon sequencing approaches. We found that ITC was suppressive to *R. solanacearum* in these natural communities but ITC reduced soil microbiome richness, suppressed rare taxa to undetectable levels and reduced network connectivity. There were differential effects of ITC on taxa and whilst Actinobacteria were enriched, Firmicutes and 7 other phyla were suppressed. Meanwhile, the presence of the pathogen and the rhizosphere microbiome origin (kale or potato) had relatively smaller effects compared to ITC exposure. ITC application could thus drive rearrangement and reassembly of soil microbiomes, having potential implications for future disease susceptibility.

Chapter 5: Collateral effects of model biofumigation on the crop and non-target microbiota

Following on from Chapter 4, we used an *in vivo* tomato model system to evaluate the efficacy of ITC exposure on *R. solanacearum* and surrounding microbiome in the presence of the tomato plant host. We found that, similar to the previous experiments, ITC was also suppressive to *R. solanacearum* in these conditions. Similar to Chapter 4, weekly ITC exposure reduced soil microbiome richness and eliminated rare taxa, but in this case, increased network connectivity and reduced negative associations between taxa. However, ITC exposure alleviated BWD symptom severity in tomato, yet reduced dry weight. Furthermore, we found evidence for *R. solanacearum* ITC tolerance evolution.

Chapter 6: General discussion

A general discussion considering the results and contexts of all chapters together. The potential future directions that could advance this area of research and translate biocontrol outcomes to agricultural environments are discussed.

Chapter 2. Plant pathogenic bacterium can rapidly evolve tolerance to an antimicrobial plant allelochemical

2.1. Abstract

Crop losses to plant pathogens are a growing threat to global food security and more effective control strategies are urgently required. Biofumigation, an agricultural technique where *Brassica* plant tissues are mulched into soils to release antimicrobial plant allelochemicals called isothiocyanates (ITCs), has been proposed as an environmentally friendly alternative to agrochemicals. While biofumigation has been shown to suppress a range of plant pathogens, its effects on plant pathogenic bacteria remain largely unexplored. Here we used a laboratory model system to compare the efficacy of different types of ITCs against *Ralstonia solanacearum* plant bacterial pathogen. Additionally, we evaluated the potential for ITC-tolerance evolution under high, intermediate and low transfer frequency ITC exposure treatments. We found that allyl-ITC was the most efficient compound at suppressing *R. solanacearum* growth, and its efficacy was not improved when combined with other types of ITCs. Despite consistent pathogen growth suppression, ITC tolerance evolution was observed in the low transfer frequency exposure treatment, leading to cross-tolerance to ampicillin beta-lactam antibiotic. Mechanistically, tolerance was linked to insertion sequence movement at four positions in genes that were potentially associated with stress responses (H-NS histone like protein), cell growth and competitiveness (acyltransferase), iron storage ((2-Fe-2S)-binding protein) and calcium ion sequestration (calcium-binding protein). Interestingly, pathogen adaptation to the growth media also indirectly selected for increased ITC tolerance through potential adaptations linked with

metabolism and antibiotic resistance (dehydrogenase-like protein) and transmembrane protein movement (Tat pathway signal protein). Together, our results suggest that *R. solanacearum* can rapidly evolve tolerance to allyl-ITC plant allelochemical which could constrain the long-term efficiency of biofumigation biocontrol and potentially shape pathogen evolution with plants.

2.2. Introduction

Plant pathogens are a growing threat to global food security, accounting for up to 40% of crop losses annually (Savary *et al.*, 2012). The phasing out of environmentally toxic chemical fumigants, such as methyl bromide, has directed attention towards alternative biocontrol strategies (Qin *et al.*, 2004). Plant-derived antimicrobial allelochemicals, such as phenolic acids, terpenes and volatile isothiocyanates (ITCs), are naturally exuded by the roots of legumes (Wink, 2013; Mondal, Asaduzzaman and Asao, 2015), cereals (Mazzola and Gu, 2002; Larkin and Halloran, 2015) and other crops such as *Brassica* (Kirkegaard, Wong and Desmarchelier, 1996; Sarwar *et al.*, 1998). These compounds could potentially be used to control pathogens by biofumigation, which involves mulching plant tissues into soils to release biocidal allelochemicals. While biofumigation has previously been shown to suppress the growth of soil-borne fungal (Angus *et al.*, 1994; Sarwar *et al.*, 1998; Rumberger and Marschner, 2003), nematode (Lord *et al.*, 2011; Ngala, Woods and Back, 2015) and bacterial pathogens (Ji *et al.*, 2007; Hu *et al.*, 2015), outcomes are still varied, ranging from clear pathogen suppression (Matthiessen and Kirkegaard, 2006; Larkin and Griffin, 2007) to having no effect (Kirkegaard *et al.*, 2000; Stirling and Stirling, 2003; Hartz *et al.*, 2005). A better understanding of the antimicrobial and biocidal effects of plant allelochemicals on pathogens is thus required.

The success of biofumigation is influenced by various factors including soil conditions, the biofumigant plant species, timing of application and the half-life of biocidal compounds (Matthiessen and Kirkegaard, 2006). The biocidal effects of *Brassica*-based biofumigation are believed to result primarily from the release of toxic ITCs from their glucosinolate (GSL) pre-cursors (Gimsing & Kirkegaard, 2009; Lord *et al.,* 2011; Matthiessen

& Kirkegaard, 2006). Moreover, other allelochemicals such as dimethyl sulfide and methyl iodide might contribute to the biocidal activity of biofumigant plants (Wang *et al.*, 2009; Vervoort *et al.*, 2014). Even though ITC-liberating GSL levels can potentially reach as high as 45.3 mM/m^2 following initial mulching of plant material into the soil (Kirkegaard and Sarwar, 1998), their concentrations often decline rapidly due to high volatility, sorption to organic matter, leaching from the soil and microbial degradation (Frick et al., 1998; Gimsing et al., 2007; Hanschen et al., 2015; Matthiessen & Kirkegaard, 2006; Warton et al., 2001). As ITCs often have short half-lives of up to sixty hours (Borek et al., 1995; Gimsing & Kirkegaard, 2006), it is important to identify ITCs that are highly effective against pathogens even during short-term exposure.

The antimicrobial activity of different types of ITCs can vary depending on their mode of action and the species and genotype of the target pathogen. In the case of bacterial pathogens, several antimicrobial mechanisms have been suggested. For instance, ITCs could damage the outer cell membrane of Gram-negative bacteria leading to changes in cell membrane potential (Sofrata *et al.*, 2011) and leakage of cell metabolites (Lin, Preston Iii and Wei, 2000). Further, it has been suggested that ITCs could bind to bacterial enzymes, such as thioredoxin reductases and acetate kinases and disrupt their tertiary structure and functioning (Luciano and Holley, 2009). It is also possible that some ITCs, such as allyl-ITC, could have multiple targets, making them relatively more toxic to pathogenic bacteria (Luciano and Holley, 2009). However, antimicrobial activity and potential tolerance evolution to ITCs are still poorly understood in plant pathogenic bacteria.

Antibiosis is an important mechanism underlying bacterial competition in soils and soil bacteria often produce and are resistant to several antimicrobials, enabling them to outcompete surrounding bacteria for space and nutrients (Hibbing *et al.*, 2010).

Antimicrobial tolerance is also important for plant-bacteria interactions, as it can help bacteria to tolerate antimicrobials secreted by plants, such as coumarins, giving them a selective advantage in the plant rhizosphere microbiome (Stringlis *et al.*, 2018). Such tolerance has recently been shown to evolve *de novo* in *Pseudomonas protegens* CHA0 bacterium against the antimicrobial scopoletin secreted by *Arabidopsis thaliana* (Li et al., 2020). Prolonged exposure to plant allelochemicals could thus select for more tolerant plant pathogen genotypes also during biofumigation and will likely be affected by the strength and duration of ITC exposure, which is important in determining whether potential tolerance or resistance mutations have enough time to sweep through pathogen populations. If the mutations enabling ITC tolerance are costly, their selective benefit could be further reduced by competition or growth trade-offs, leading to loss of tolerance mutations in the absence of ITCs. While ITC concentrations are known to reach antimicrobial levels during biofumigation in the field (Sarwar *et al.*, 1998), no direct experimental evidence for ITC tolerance evolution in plant pathogenic bacteria exists.

To study these questions, we developed a model laboratory system where we tested the growth-inhibiting effects of ITCs produced by Indian mustard (*Brassica juncea*) on *Ralstonia solanacearum* plant pathogenic bacterium, which is the causative agent of bacterial wilt and potato brown rot diseases and a globally important pathogen, affecting over 200 different plant species including various important crops (Yabuuchi *et al.*, 1995; Elphinstone, 2005). Disease control techniques such as crop rotation, the use of clean and certified seeds or resistant plant cultivars, have shown only limited success in controlling *R. solanacearum* (Ciampi-Panno *et al.*, 1989; Chellemi *et al.*, 1997; Ramesh, Joshi and Ghanekar, 2009). Indian mustard was chosen as a model biofumigant plant due to its well-established allelochemical properties (Sarwar *et al.*, 1998; Bending and Lincoln, 1999;

Kirkegaard and Matthiessen, 2005; Mazzola, Hewavitharana and Strauss, 2015), which are predominantly caused by the release of allyl, sec-butyl and 2-phenylethyl ITCs (Olivier *et al.*, 1999; Bangarwa *et al.*, 2011; Yim *et al.*, 2016). As these ITCs might vary in their biocidal activity, we first tested to what extent they suppress *R. solanacearum* growth when applied either alone or in combination at concentrations relevant to field biofumigation (Gimsing et al., 2007; Hanschen et al., 2012; Kirkegaard & Sarwar, 1998; Matthiessen & Kirkegaard, 2006; Rudolph et al., 2015). Subsequently, we explored whether long-term exposure to the most effective ITC type could select for resistant or more ITC-tolerant pathogens in the lab, and if ITC tolerance is associated with competitive costs or cross-tolerance to other antimicrobials. It was found that allyl-ITC was the most suppressive allelochemical. However, long-term exposure selected for ITC-tolerant pathogen mutants that also had increased cross-tolerance to the beta-lactam antibiotic ampicillin. At the molecular level, adaptations were associated with a few parallel mutations and loss of insertion sequences mainly in the megaplasmid. Together these results suggest that while Indian mustard could be used as a biofumigant plant against *R. solanacearum* due to the high antimicrobial activity of allyl-ITC, its long-term efficacy could be constrained by rapid ITC tolerance evolution.

2.3. Materials and Methods

(a) Pathogen strain and culture media

We used a *Ralstonia solanacearum* strain (21415687) which was originally isolated from the river Loddon (phylotype II sequevar 1) in the UK as our ancestral pathogen strain (Source: John Elphinstone, Fera Science, 2014). This strain was chosen as river water is the most common environmental source of potato brown rot outbreaks in the UK (Elphinstone *et al*.,

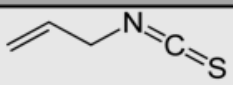
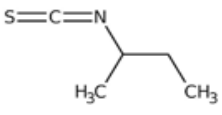
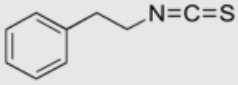
1998), and hence highly relevant for UK *R. solanacearum* epidemics. The strain was cultured in CPG broth (1 g casamino acids, 10 g peptone and 5 g glucose per litre of ddH_2O) for 48 hours at 28 °C to create cryostocks (20% w/v glycerol) that were preserved at -80 °C. CPG was also used as the main growth media in all experiments except for fitness assays, where lysogeny broth (LB: 10 g tryptone, 5 g yeast, 10 g NaCl per litre of ddH_2O) was also used as a 'naïve' growth media to control the effects of *R. solanacearum* adaptation to CPG media during the selection experiment. These media are both widely used and nutritionally rich yet differ in their vitamin and amino acid compositions which enables comparisons of growth in different conditions. Specifically, CPG media has glucose as a carbon source while LB media has catabolisable amino acids, not sugars.

(b) Comparing the effects of different types of ITCs for pathogen suppression

To determine antimicrobial activity of ITCs, we first identified concentrations that caused a significant reduction in *R. solanacearum* growth relative to the no-ITC control treatments. To this end, we conducted short-term growth assays where *R. solanacearum* was exposed to allyl, sec-butyl and 2-phenylethyl ITCs at 63, 125, 250, 500, 1000, 2500 and 5000 μM concentrations in CPG media (Table 2.3.1.; Appendix Fig. A2). For this experiment, *R. solanacearum* was revived from cryostocks by growing with shaking (250 rpm) for 48 hours at 28 °C before normalising bacterial density to an optical density (OD) reading of 0.1 (600 nm; Tecan, Sunrise), equalling ~10^7 cells per ml. This method was consistently used to revive and adjust bacterial densities in all growth experiments. *R. solanacearum* was grown in 200 μl CPG media in different ITC concentrations for 48 hours and bacterial densities were measured every 24 hours (OD600 nm). We found that allyl-ITC concentrations as low as 125 μM inhibited *R. solanacearum* growth, while relatively higher concentrations of 500 μM of

sec-butyl and 2-phenylethyl ITC were required to inhibit pathogen growth (Appendix Fig. A1). Based on this data, 500 μM and 1000 μM ITC concentrations were selected because they showed pathogen growth suppression in the case of all measured ITCs (Appendix Table A.1). Furthermore, these concentrations are known to be achievable at least transiently during biofumigation in the field (Gimsing et al., 2007; Hanschen et al., 2012; Kirkegaard & Sarwar, 1998; Matthiessen & Kirkegaard, 2006; Rudolph et al., 2015). To explore the effects of ITCs on pathogen growth alone and in combination, different ITCs were mixed in all possible two-way and three-way combinations using equal concentrations of each ITC within combinations (two-way 50:50%; three-way 33:33:33%) to achieve final low (500 μM) and high (1000 μM) ITC concentrations in 200 μl of CPG media in 96-well microplates. Microplates were cultured at 28 °C (N= 8 for all treatments) and the experiment was run for three days (72 hours), with population density measurements recorded every 24 hours as optical density at 600 nm.

Table 2.3.1. The chemical properties of the three different ITCs predominantly released from Indian mustard biofumigant plant (*Brassica juncea*).

Isothiocyanate	IUPAC name	Molar mass (g mol^{-1})	Structure
Allyl	3-isothiocyanato-1-propene	99.15	
Sec-Butyl	2-isothiocyanatobutane	115.19	
2-Phenylethyl (PEITC)	(2-Isothiocyanatoethyl) benzene	163.24	

(c) Determining pathogen ITC and beta-lactam tolerance evolution in response to repeated allyl-ITC exposure

To investigate the potential for ITC tolerance evolution, we set up a 16-day selection experiment where we exposed *R. solanacearum* to 500 μM of allyl-ITC, which has the strongest effect on pathogen growth suppression of all tested ITCs (Fig. 1A; Appendix Fig. A1). We also manipulated the frequency of ITC exposure using high (1-day), intermediate (2-day) and low (3-day) serial transfer frequency treatments. At each serial transfer, a subset of evolved bacteria (5% of the homogenised bacterial population) was serially transferred to fresh CPG media in the absence (control) and presence of allyl-ITC. ITC treatments thus manipulated both resource renewal and exposure to fresh ITC. We estimated the ancestral *R. solanacearum* clone doubling times in the presence and absence of allyl-ITC (500 μM) during three growth periods of the transfer frequency treatments (0-24h, 24-48h and 48-72h) using cell density data (CFU per mL, N=8) and the following formula: Doubling time= Duration of time x Ln(2) / Ln (Final concentration/Initial concentration). Based on this information, we estimate that the evolved populations experienced approximately 274, 157 and 120 generations in the absence and 216, 122 and 96 generations in the presence of ITC in high, intermediate and low frequency transfer treatments, respectively (Appendix Table A.2). As a result, the number of bacterial generations differed between transfer frequency treatments. The selection experiment was set-up following the same protocols described earlier and following this, separate fitness assays were conducted to directly compare the growth of ancestral and evolved populations (and individual colonies) in the absence and presence of 500 μM allyl-ITC. In addition to testing potential ITC tolerance evolution, we quantified changes in the growth of evolved bacteria in the absence of ITCs to reveal

potential adaptations to the CPG growth media. All fitness assays were also repeated in 'naïve' LB media to control the potential effects of pathogen adaptation to the CPG growth media during the selection experiment. In all assays, bacteria were revived and prepared as described earlier, and grown in 96-well microplates in different media (CPG or LB) in the absence or presence of 500 μM allyl-ITC for 72 hours. Changes in ITC tolerance were quantified as bacterial growth relative to the ancestral and control treatments based on optical density at 600 nm (48-hour time point). Fitness assays were also conducted for individual bacterial colonies at the final time point where a single ancestral colony and one colony from each replicate selection line per treatment were selected resulting in a total of 49 clones. All evolved colonies detected on agar plates showed ancestral, fluidic colony morphology.

To explore potential ITC-tolerance mechanisms, we tested if ITC tolerance correlated with tolerance to ampicillin beta-lactam antibiotic (growth assays), which is commonly produced by various soil bacteria (Ranjan *et al.*, 2021). Moreover, we specifically tested for ampicillin tolerance as we identified potential antibiotic-linked insertion sequence movement in our evolved clones, which has previously been shown to confer beta-lactam antibiotic tolerance in clinical settings (Poirel, Decousser and Nordmann, 2003; Boutoille *et al.*, 2004). Ampicillin tolerance was tested using the sequenced isolated clones from the final time point of the selection experiment (intermediate transfer frequency no-ITC, low transfer frequency no-ITC and low transfer frequency ITC exposure treatments) and the ancestral strain (total of 24 evolved clones and 8 replicate ancestral clones per treatment). Clones were prepared as described earlier and grown in 96-well microplates in CPG media in the absence or presence of 15 or 30 μg/ml ampicillin. Ampicillin tolerance was quantified as

bacterial growth relative to the ancestral clones based on optical density at 600nm (48-hour time point).

(d) Genome sequencing of evolved bacterial clones

A subset of evolved clones were whole genome sequenced to identify potential single nucleotide polymorphisms (SNPs), genomic rearrangements (small insertions and deletions) and potential changes in prophage and insertion sequence movement linked with *R. solanacearum* adaptation. Based on phenotypic data, we chose eight clones (1 per replicate selection line) from the low transfer frequency treatments that had evolved in the absence or presence of ITC (16 clones). Moreover, we sequenced the ancestral strain (1 clone) and eight clones from the intermediate transfer frequency no-ITC treatment (8 clones), that showed no evidence of ITC tolerance adaptation (a total of 25 clones), as controls. Genomic DNA was extracted using the Qiagen DNeasy UltraClean Microbial Kit according to the manufacturer's protocol. DNA was quantified using the NanoDrop microvolume spectrophotometer and quality checked by gel electrophoresis imaging. DNA yields of all samples were diluted with EB buffer to 30 ng/μl concentrations and DNA samples were sent to MicrobesNG for sequencing (Illumina 30x coverage; http://www.microbesng.uk). MicrobesNG conducted library preparation using Nextera XT Library Prep Kit (Illumina, San Diego, USA) following the manufacturer's protocol with the following modifications: 2 ng of DNA were used as input, and PCR elongation lasted 1 min. Hamilton Microlab STAR automated liquid handling system was used for DNA quantification and library preparation. Pooled libraries were quantified using the Kapa Biosystems Library Quantification Kit for Illumina on a Roche light cycler 96 qPCR machine. Libraries were sequenced on the Illumina HiSeq 2500 using a 250 bp paired end protocol. Reads were adapter trimmed using Trimmomatic 0.30 with a sliding window quality cut-off of Q15 (Bolger, Lohse and Usadel,

2014). Assembly was performed on samples using SPAdes v.3.7 (Bankevich *et al.*, 2012) and contigs were annotated using Prokka v.1.11 (Seemann, 2014). Genomes were analysed using a standard analysis pipeline (Guarischi-Sousa *et al.*, 2016), where reads were first mapped to a high quality and well annotated UY031 reference genome (NCBI accession: NZ_CP012687) which showed 99.95% similarity with our ancestral *R. solanacearum* strain at the chromosome level and 97.87% similarity at the mega-plasmid level. Variant calling was performed using Snippy v.3.2, a rapid haploid variant calling pipeline (Seemann, 2015). When comparing the sequenced genomes, the SNPs identified in both the ancestral strain and the evolved clones were first filtered out as these likely represent pre-existing phylogenetic differences between the reference genome and our ancestral *R. solanacearum* strain. We also compared the control treatment clones isolated from low and intermediate transfer frequency treatments (no ITC exposure) to identify potential mutations linked with CPG media adaptation. The software IMSindel v.1.0.2 (Shigemizu *et al.*, 2018) was used to identify potential intermediate indels with options "—indelsize 10000" and using UY031 as a reference. After running IMSindel, putative indels in all isolates were combined. Putative short indels that were < 50 bp in length were removed. To investigate potential insertion sequences underlying ITC tolerance and media adaptation, insertion sequences were detected in the UY031 with ISEScan v.1.7.2.3; (Xie and Tang, 2017) using default parameters. Potential false positives were determined by blasting insertion sequences against the ISFinder database (https://isfinder.biotoul.fr/) and removing hits with an E-value > e-04. Experimental isolates were then screened for the insertion sequences identified with ISEScan using ISMapper v.2.0; (Hawkey *et al.*, 2015) with default settings. In line with a previous study (Hawkey *et al.*, 2020), ISMapper was run using an IS-removed UY031 assembly to improve insertion site precision. The genes flanking putative IS sites were

determined by annotating the UY031 assembly using the stand-alone NCBI prokaryotic genome annotation pipeline 2021-07-01.build5508 (Tatusova *et al.*, 2016). Additionally, we determined isolate prophage content and positions to identify potential phenotypic changes via mobile genetic elements. Isolate draft assemblies were generated using Unicycler Illumina-only assembly v.0.4.7 (Wick *et al.*, 2017). Prophages were then identified in draft assemblies using the PHASTER (PHAge Search Tool Enhanced Release) web server (Arndt *et al.*, 2016). Prophage movement was detected by parsing out the 5kb (or to end of contig) flanking regions either side of the prophages in the draft assemblies and mapping them to a closely related complete UY031 genome sequence. Prophage movement was detected if the flanking regions map to different parts of the UY031 genome between isolates. Prophage movement analyses were conducted using custom R and Python scripts available at (https://github.com/SamuelGreenrod/Prophage_movement). All genomes including the ancestral strain have been deposited in the European Nucleotide Archive database under the following accession number: PRJEB42551.

(e) Statistical analysis

Repeated measures ANOVA was performed to analyse all the data with temporal sampling structure and pairwise differences were determined using *post-hoc* t-test with Bonferroni correction. All other statistical analyses (ITC tolerance and cost of tolerance in CPG and LB media and cross-tolerance in ampicillin) were conducted focusing on the 48-hour measurement time point (where ITC was still actively suppressive to *R. solanacearum* as the compound had not yet entirely evaporated from the liquid media, Appendix Fig. A2) and two-way ANOVA was used to explain variation in bacterial growth between different treatments. Tukey *post-hoc* tests were used to compare differences between subgroups ($p < 0.05$). Where data did not meet the assumptions of a parametric test, non-parametric

Kruskal-Wallis test and *post-hoc* Dunn test were used. All statistical analyses and graphs were produced using R (R Foundation for Statistical Computing, R Studio v.3. 5. 1) using ggplot2 (Wickham, 2011), ggpubr (Kassambara, 2018) and lme4 (Bates *et al.*, 2018) packages.

2.4. Results

(a) Only allyl-ITC suppressed pathogen growth irrespective of the presence of other ITCs

We first determined the effects of different ITCs on *R. solanacearum* growth alone and in combination. Overall, there was a significant reduction in *R. solanacearum* densities in the presence of ITCs (ITC presence: $F_{1, 120}$= 6.33, p< 0.01; Tukey: p< 0.05; Fig. 2.4.1B). However, this effect was mainly driven by the allyl-ITC, which significantly reduced bacterial densities compared to the no-ITC control treatment (ITC type: $F_{7, 114}$= 49.45, p< 0.001; Tukey: p< 0.05), while other ITCs had no significant effect on the pathogen (p> 0.05; Fig. 2.4.1B). Increasing the ITC concentration from low to high (500 to 1000 μM) had no effect on inhibitory activity in either single or combination ITC treatments (ITC concentration in single ITC treatment: $F_{1, 43}$= 2.0, p= 0.17; combination ITC treatment: $F_{1, 59}$= 0.68, p= 0.41; Fig. 2.4.1B). However, a significant interaction between ITC type and ITC concentration in both single and combination treatments was found (ITC concentration × ITC type in single ITC treatment: $F_{2, 39}$= 4.67, p< 0.05; in combination ITC treatment: $F_{3, 53}$= 4.94, p< 0.01; Fig. 2.4.1B), which was driven by the increased inhibitory activity of allyl-ITC at high concentration (Tukey: p< 0.05). As a result, ITC combinations were less inhibitory than single ITC treatments (Number of ITCs: $F_{2, 103}$= 3.82, p<0.05; Fig. 2.4.1B), which was due to reduced allyl-ITC concentration in combination treatments (total ITC concentrations were kept the

same between treatments). Similarly, ITC combinations that included allyl-ITC significantly reduced bacterial densities relative to the control treatment (Allyl-ITC presence: $F_{1, 57}$= 36.21, p< 0.001; Fig. 2.4.1B), and the presence of allyl-ITC had a clearer effect at the high ITC concentration (Allyl-ITC presence × ITC concentration: $F_{1, 57}$= 7.51, p< 0.01; Fig. 2.4.1B). Together these results suggest that allyl-ITC was the most inhibitory compound and its antimicrobial activity was not enhanced by the presence of other ITCs.

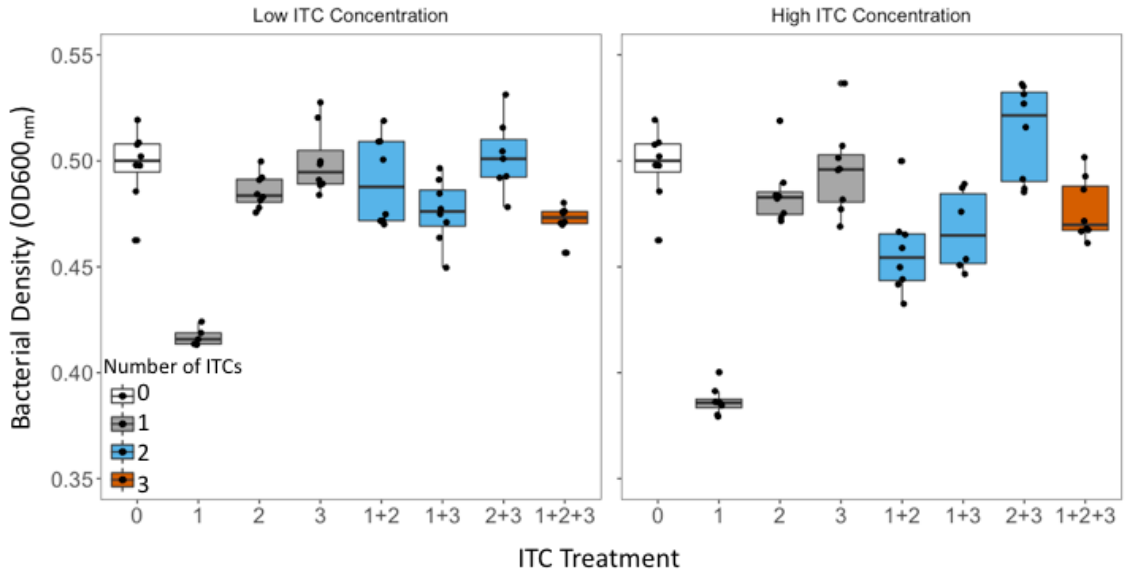


Figure 2.4.1. The antimicrobial activity of different ITCs against *Ralstonia solanacearum* pathogen when applied alone and in combination. The effects of three different ITCs (allyl, sec-butyl and 2-phenylethyl) on *R. solanacearum* growth after 48h exposure when applied alone and in combination in liquid microcosms at low (500 μM) and high (1000 μM) concentrations. Boxplot colours represent different ITC treatments that are labelled on X-axes as follows: (0): no-ITC (control); (1) allyl-ITC; (2) sec-butyl ITC and (3) 2-phenylethyl ITC. Individual data points show bacterial densities for each technical replicate (N=8). The boxplots show the minimum, maximum, interquartile range and the median (black line).

(b) Pathogen growth was more clearly suppressed in high and intermediate ITC exposure treatments during an experimental evolution experiment

To study the evolutionary effects of ITCs, we exposed the ancestral *R. solanacearum* strain to allyl-ITC at the low concentration (500 μM) and manipulated the frequency of exposure to ITC by transferring a subset of evolved bacterial population to fresh ITC-media mixture everyday (high), every second day (intermediate) and every third day (low) for a total of 16 days. As a result, this manipulation also affected the resource renewal rate. Overall, bacteria reached the highest population densities in the low transfer frequency treatments and the second highest in the intermediate transfer frequency treatments (Transfer frequency: $F_{2, 45}$= 4.66, p< 0.001; p< 0.05 for pairwise comparison; Fig. 2.4.2). While allyl-ITC exposure significantly reduced bacterial densities in all ITC-containing treatments (ITC presence: $F_{1, 46}$= 30.68, p< 0.001; Fig. 2.4.2), bacterial growth was least affected in the low transfer frequency treatment (ITC presence × Transfer frequency: $F_{2, 42}$= 4.36, p< 0.05; p< 0.001 for all pairwise comparisons; Fig. 2.4.2). The inhibitory activity of allyl-ITC also varied over time: while relatively more constant suppression was observed in the high and intermediate transfer frequency treatments, pathogen growth suppression became clear in the low transfer frequency treatment only towards the end of the selection experiment potentially due to media growth adaptation in the no-ITC control treatment (Time × Transfer frequency × ITC presence: $F_{2, 673}$= 7.33, p< 0.001; Fig. 2.4.2). Together these results suggest that the long-term ITC activity varied temporally and depended on the ITC exposure and serial transfer frequency.

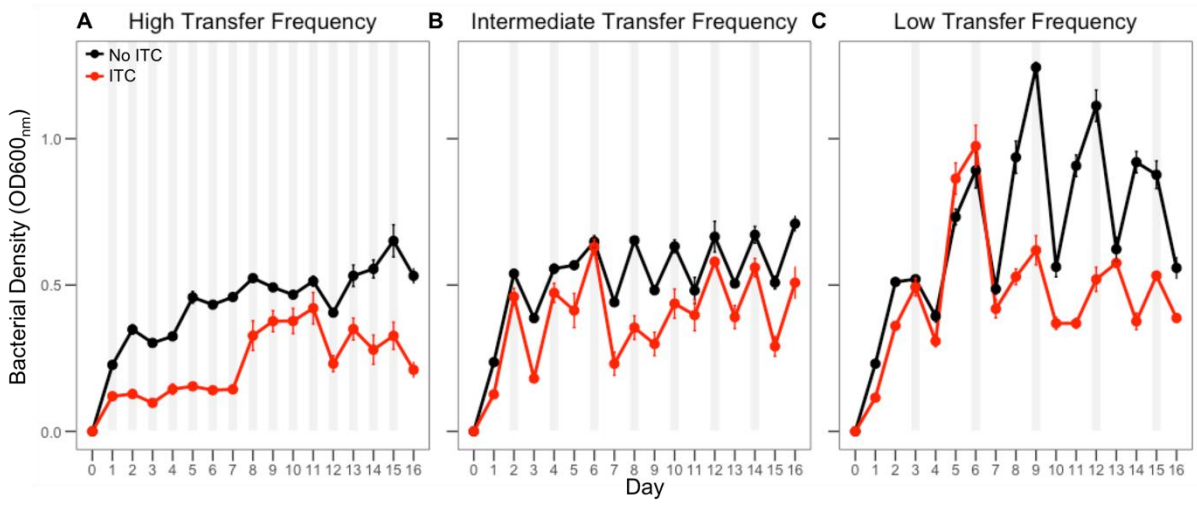


Figure 2.4.2. *R. solanacearum* density dynamics ($OD600_{nm}$) during the evolution experiment in the absence and presence of allyl-ITC in high, intermediate and low transfer frequency treatments. In all panels, black and red lines correspond to *R. solanacearum* densities in the absence and presence of 500 μM allyl-ITC, respectively. Panels A-C correspond to high (1-day), intermediate (2-day) and low (3-day) transfer frequency treatments, respectively. Grey shaded areas indicate the time point of serial transfers, while optical density reads were taken at 24-hour intervals in all treatments. Each time point shows the mean of eight biological replicates and bars show ±1 error of mean.

(c) ITC tolerance evolution was observed only in the low transfer frequency ITC exposure treatment

Fitness assays were conducted at the end of the selection experiment to compare the growth of the ancestral strain and evolved populations from different treatments in the presence and absence of allyl-ITC (experimental concentration: 500 μM). The ancestral strain reached lower densities in the presence of ITC compared to evolved populations regardless of the ITC treatment they had evolved in during the selection experiment (Evolutionary history: $F_{2, 45}$= 5.39, p< 0.01; Tukey: p< 0.05; Fig. 2.4.3A). However, ITC

tolerance was mainly observed in the low transfer frequency ITC exposure treatment, while populations that had evolved in the high or intermediate transfer frequency treatments did not significantly differ from the ancestral strain (Transfer frequency within ITC-exposed populations: $F_{2, 19}$= 24.72, p< 0.001; Tukey: p< 0.05; Fig. 2.4.3A). Surprisingly, even the control populations that had evolved in the absence of ITCs in the low transfer frequency treatment showed an increase in ITC tolerance (p< 0.05; Fig. 2.4.3A). One potential explanation for this is that these populations adapted to grow better in CPG media, which could have helped to compensate for the mortality imposed by allyl-ITC during the fitness assays. To test this, we compared the growth of ancestral and evolved populations in the absence of allyl-ITC in the CPG media (Fig. 2.4.3B). We found that all control populations showed improved growth in the CPG media compared to ITC-exposed populations regardless of the transfer frequency treatment (Evolutionary history: $F_{1, 40}$= 20.00, p< 0.001; Transfer frequency: $F_{2, 40}$= 2.66, p= 0.08, in all pairwise comparisons, Tukey: p< 0.05; Fig. 2.4.3B). In contrast, none of the ITC-exposed populations showed improved growth in CPG media relative to the ancestral strain (Tukey: p< 0.05; Fig. 2.4.3B), which suggests that ITC exposure constrained *R. solanacearum* adaptation to the growth media.

To disentangle the effects due to adaptation to the media and allyl-ITC, we repeated fitness assays in 'naïve' LB growth media which the bacteria had not adapted to. ITC tolerance was observed only when bacterial populations had previously been exposed to allyl-ITC (Evolutionary history: $F_{2, 49}$= 18.82, p< 0.001; Tukey: p< 0.05; Fig. 2.4.3C), and this effect was driven by adaptation in the low transfer frequency ITC exposure treatment (no ITC tolerance was observed in the high and intermediate transfer frequency treatment; Transfer frequency: $F_{2, 49}$= 4.37, p< 0.01; Tukey: p< 0.05; Fig. 2.4.3C). Crucially, CPG-adapted control populations showed no signs of ITC tolerance, but instead, suffered reduced growth

in LB media relative to the ancestral strain and ITC-exposed populations (Evolutionary history: $F_{2, 49}$= 94.89, p< 0.001; Fig. 2.4.3D), which was clearest in the low transfer frequency exposure treatment (Evolutionary history × Transfer frequency: $F_{2, 49}$= 23.17, p< 0.001; Fig. 2.4.3D).

We further validated our population level fitness results using individual clones (one randomly chosen clone per replicate population per treatment). In line with previous findings, ITC-exposed clones showed increased ITC tolerance compared to the control and ancestral bacterium in the LB media (Evolutionary history: $F_{2, 49}$= 14.20, p< 0.001; Fig. 2.4.4A), and tolerance evolution was the greatest in the low transfer frequency ITC exposure treatment (Transfer frequency: $F_{2, 49}$= 11.15, p< 0.001; Tukey: p< 0.05; Evolutionary history × Transfer frequency: $F_{2, 49}$= 3.04, p< 0.05; Fig. 2.4.4A). Together, our results suggest that ITC tolerance, which evolved in the low transfer frequency ITC exposure treatment was robust and independent of the growth media it was quantified in. Moreover, while all control populations adapted to grow better in the CPG media, this adaptation had a positive effect on ITC tolerance only when quantified in CPG media and when the clones had evolved in the low transfer frequency treatment.

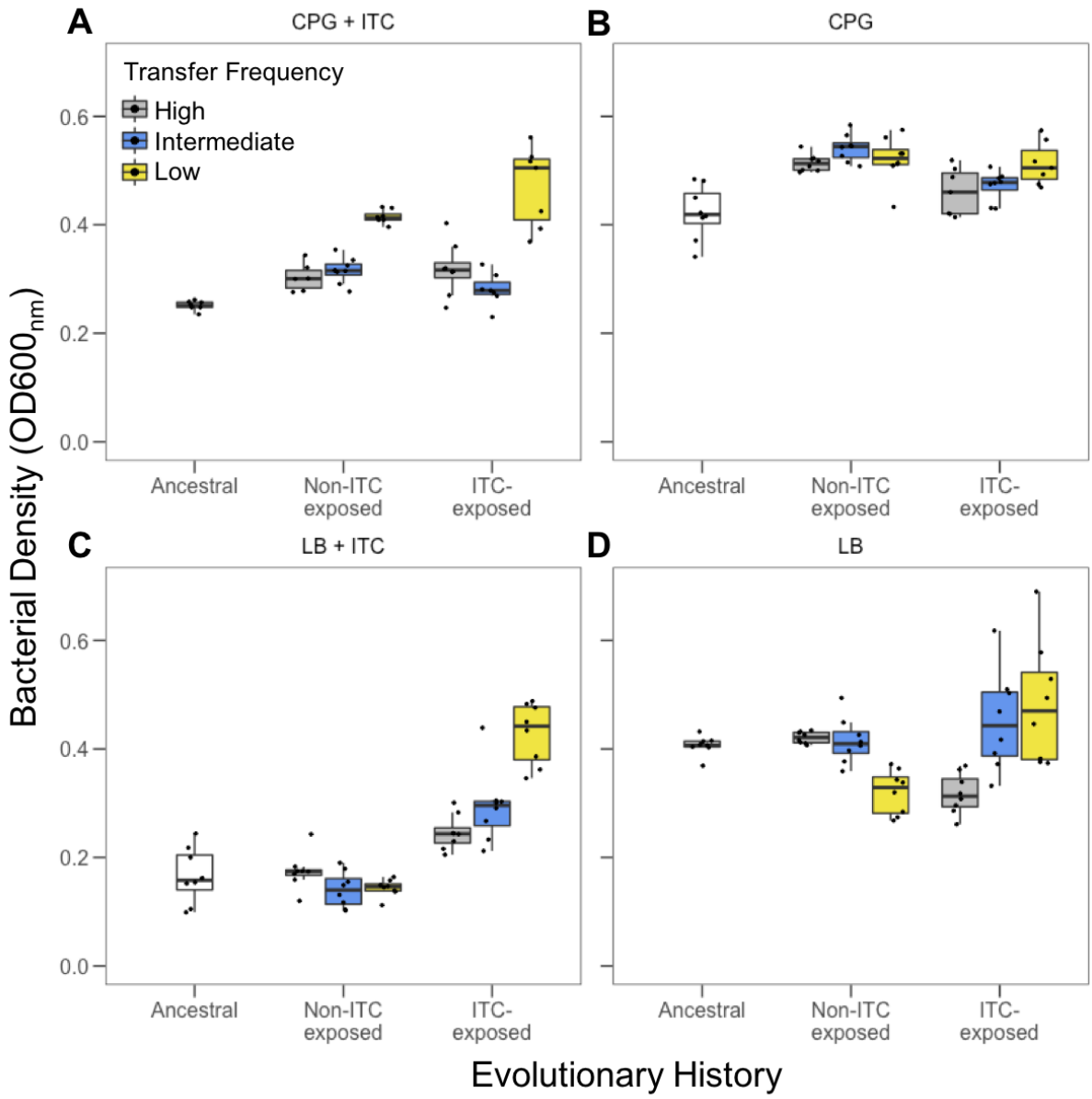


Figure 2.4.3. Comparison of *R. solanacearum* ITC tolerance between the ancestral clone and evolved populations from high, intermediate and low transfer frequency treatments at the end of the evolution experiment in CPG and LB media. ITC tolerance was determined as *R. solanacearum* growth ($OD600_{nm}$) after 48h exposure to 500 μM allyl-ITC in CPG (A) and LB (C) media. Growth was also measured in the absence of allyl-ITC in both CPG (B) and LB (D) media. High (1-day), intermediate (2-day) and low (3-day) transfer frequency treatments are shown in grey, blue and yellow boxplots, respectively, and boxplots show

the minimum, maximum, interquartile range and the median (black line). Individual data points show bacterial densities for each biological replicate population (N=8).

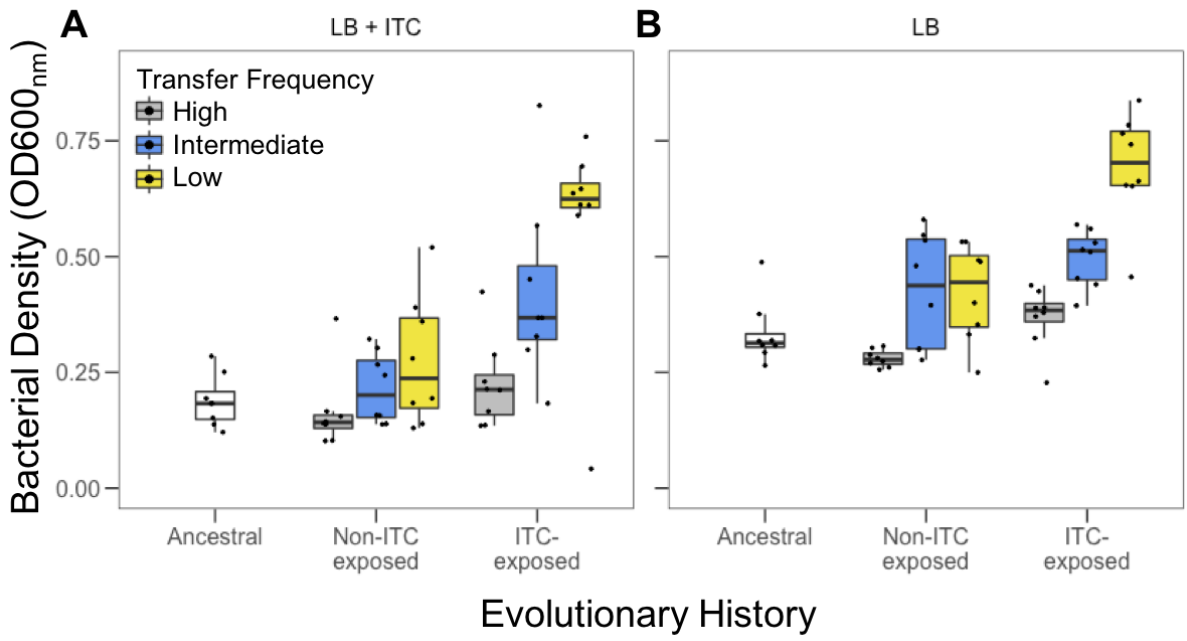


Figure 2.4.4. Comparison of *R. solanacearum* ITC tolerance between the ancestral and evolved clones from high, intermediate and low transfer frequency treatments at the end of the evolution experiment in LB media. ITC tolerance was determined as *R. solanacearum* growth ($OD600_{nm}$) after 48h exposure to 500 μM allyl-ITC in LB media (A). Growth was also measured in the absence of allyl-ITC (B). High (1-day), intermediate (2-day) and low (3-day) frequency treatments are shown in grey, blue and yellow, respectively, and boxplots show the minimum, maximum, interquartile range and the median (black line). Individual data points show bacterial densities for each biological replicate population (N=8).

(d) Evolution of ITC-tolerance confers cross-tolerance to ampicillin beta-lactam antibiotic

We also tested if exposure to allyl-ITC could have led to cross-tolerance to other antimicrobials such as the beta-lactam antibiotic ampicillin. Overall, both low (15 μg/ml) and high (30 μg/ml) ampicillin concentrations had negative effects on *R. solanacearum* growth relative to the no-ampicillin control treatment (Ampicillin concentration: $F_{2, 93}$= 50.12, p< 0.001; Tukey: p< 0.05; high concentration was relatively more inhibitory, Appendix Fig. A3). However, the evolved clones from the low transfer frequency ITC exposure treatment reached significantly higher bacterial densities than the ancestral strain (Evolutionary history: $F_{3, 92}$= 3.51, p< 0.05; Tukey: p< 0.05; Appendix Fig. A3), while evolved clones derived from low and intermediate transfer frequency control treatments (no prior ITC exposure) did not differ from the ancestral strain (Tukey: p> 0.05; Appendix Fig. A3). Ampicillin tolerance was only observed in the high ampicillin concentration (High ampicillin concentration: $F_{3, 28}$= 8.22, p< 0.001; Appendix Fig. A3C; Low ampicillin concentration: $F_{3, 28}$= 1.551, p= 0.223; Appendix Fig. A3B). Together these results suggest that ITC tolerance conferred cross-tolerance to ampicillin for clones that had evolved in the low transfer frequency ITC exposure treatment.

(e) Media adaptation and ITC tolerance are linked to a few mutations and loss of insertion sequences

A subset of clones which were phenotyped regarding ITC and ampicillin tolerance were selected for genome sequencing as well as the ancestral strain (N=25). All isolated colonies showed ancestral, fluid colony morphotype with no evidence for spontaneous evolution of small colony types as observed previously (Khokhani *et al.*, 2017; Perrier *et al.*, 2019).

Specifically, we focused on comparing parallel small mutations and indels, intermediate indels (>50 bp) and prophage and insertion sequence (IS) movement between populations that had evolved in the absence and presence of ITC in the low transfer frequency treatments (evidence of ITC tolerance evolution) with ancestral and control populations from the intermediate transfer frequency treatment (no ITC tolerance evolution observed). Potential genetic changes were investigated in both the chromosome and megaplasmid of the bipartite genome.

Only a few mutations were observed in 1 to 6 different genes, which was expected considering the relatively short duration of the selection experiment (16 days). Of these mutations, 8 were non-synonymous and 4 synonymous (Table 1). Some mutations were observed across all treatments, indicative of adaptation to the culture media or other experimental conditions. For example, parallel non-synonymous mutations in *hisH1* gene controlling imidazole glycerol phosphate synthase were observed in 6/8 to 8/8 replicate clones in all treatments (Table 2.4.1; Fig. 2.4.5). Similarly, non-synonymous mutations in serine/threonine protein kinase genes (between 5/8 to 8/8 replicate clones) and synonymous mutations in putative deoxyribonuclease *RhsC* gene (between 1/8 to 5/8 replicate clones) were found across all treatments (Table 2.4.1; Fig. 2.4.5). A single clone that had evolved in the absence of allyl-ITC in the intermediate transfer frequency treatment had a unique non-synonymous mutation in the gene encoding the putative HTH-type transcriptional regulator *DmlR* and another clone originating from this treatment had a mutation in the IS5/IS1182 family transposase encoding gene (Table 2.4.1; Fig. 2.4.5). Additionally, we observed mutations exclusively in the low transfer control clones in genes encoding the dehydrogenase-like uncharacterised protein (3/8 replicate clones) and Tat pathway signal protein (2/8 replicate clones; Table 2.4.1; Fig. 2.4.5), which may explain ITC

tolerance via media adaptation. However, no clear parallel mutations exclusive to the low frequency ITC-exposed populations were found.

In terms of putative intermediate indels (>50 bp), we identified 122 and 116 indel sites in the chromosome (Chr) and megaplasmid (MP), respectively. Almost all of these were insertions (Chr, 119/122; MP, 113/116) and the majority were singletons (Chr, 101/122; MP, 95/116) or doubletons (Chr, 14/122; MP, 13/116). The number of intermediate indels did not differ between evolutionary treatments either in the chromosome (Kruskal-Wallis: x^2= 3.65; df= 2; p= 0.161) or megaplasmid (Kruskal-Wallis: x^2= 3.46; df= 2; p= 0.178). As a result, this genetic variation was likely non-adaptive and driven by random drift.

Target gene	Location	Locus tag	Position	Type	Effect	Ref	Alt	Int No ITC	Low No ITC	Low ITC
Putative deoxyribonuclease RhsC	Chromosome	RSUY_02640	293900	snp	Synonymous variant	G	A	5/8	4/8	2/8
			294413	snp	Synonymous variant	C	T	1/8	0/8	0/8
Imidazole glycerol phosphate synthase subunit HisH1	Chromosome	RSUY_05230	585646	insertion	Frameshift variant	T	TCGTGCTG	8/8	7/8	6/8
HTH-type transcriptional regulator DmlR	Chromosome	RSUY_11710	1257034	snp	Missense variant	G	C	1/8	0/8	0/8
Hypothetical transcription regulator protein (86.1% similarity)	Chromosome	RS_RS11675	1258240	snp	Missense variant	C	G	3/8	0/8	0/8
Hypothetical F-box domain-containing protein (83.3% similarity)	Chromosome	RSUY_21390	2302809	snp	Synonymous variant	C	T	2/8	1/8	0/8
Hypothetical serine/threonine protein kinase (84.6% similarity)	Chromosome	RSUY_26530	2830656	insertion	Frameshift variant	A	ACAGCAACGG	5/8	8/8	7/8
Hypothetical dehydrogenase-like uncharacterised protein (90.9% similarity)	Chromosome	ATK36_5281	2874048	insertion	Frameshift variant	C	CGGGCACT	0/8	3/8	0/8
Hypothetical Tat pathway signal protein (90.9% similarity)	Chromosome	BSE24067_05643	3123064	insertion	Frameshift variant	A	AC	0/8	2/8	0/8
Hypothetical DNA-3-methyladenine glycosidase II (100% similarity)	Megaplasmid	NA8A_21102	44512	deletion	Missense variant	TCGTGAGCGGCAAGCCGGCACATCGCAA	T	0/8	0/8	1/8
Hypothetical transmembrane protein (100% similarity)	Megaplasmid	RSIPO_03141	105610	deletion	Frameshift variant	TG	T	0/8	0/8	1/8
Hypothetical IS5/IS1182 family transposase (100% similarity)	Megaplasmid	RSUY_33140	124931	snp	Missense variant	G	A	1/8	0/8	0/8
Intergenic (hypothetical protein and acyltransferase)	Chromosome	Intergenic	2302900	Insertion sequence				2/8	5/8	2/8
Calcium binding protein	Megaplasmid	RSUY_33720	209500	Insertion sequence				8/8	8/8	7/8
Intergenic (type III effector HopG1 and (2Fe-2S)-binding protein)	Megaplasmid	RSUY_33970	243500	Insertion sequence				4/8	1/8	4/8
Intergenic (Hypothetical protein and H-NS histone family protein)	Megaplasmid	RSUY_34060	253900	Insertion sequence				4/8	3/8	2/8

Table 2.4.1. Annotated *R. solanacearum* genes and annotated gene functions observed in intermediate and low transfer frequency control (no-ITC), and ITC-exposed low transfer frequency treatments. Gene function predictions were derived based on BLAST using UNIPROT and percentage (%) sequence similarity is included for putative (hypothetical) proteins. Filled cells denote for the presence of mutations in given clones and white cells denote for the absence of given mutations. Replicates are named by treatments, IntNoITC= Intermediate transfer frequency, no ITC; LowNoITC= Low transfer frequency, no ITC; LowITC= Low transfer frequency, ITC.

To identify other potential molecular mechanisms, variation in prophages and insertion sequences (ISs) was investigated. Two prophages were found in all sequenced isolates: Inoviridae prophage ϕRS551 and a novel, unclassified prophage (Appendix Table A.3). Prophage genome positions were almost identical between all sequenced isolates (Appendix Table A.3). Therefore, no evidence for systematic prophage movement was observed in the evolved isolates relative to the ancestral strain. In contrast, ISs appeared to be highly mobile regarding 15 variable positions in the chromosome and 15 variable positions in the megaplasmid (Appendix Fig. A3). In most variable positions (7 in the chromosome and 9 in the megaplasmid), the gain or loss of ISs was infrequent, occurring in up to three clones per treatment (Appendix Fig. A.3), which is indicative of non-adaptive, random IS movement. However, the remaining IS positions showed higher frequency of gain or loss, indicating of potentially adaptive IS movement which was also in some cases treatment-specific. For example, an IS element in position 2302900 on the chromosome absent in the ancestral strain was observed in 2 clones in the intermediate transfer frequency control and 2 low transfer ITC treatment clones, while it was gained by 5 clones in low transfer control treatment. The IS element in this position was found to be close to the start codon (~50 bp) of an acyltransferase. In two of the low transfer control clones, the IS was found to disrupt the gene (Fig. 2.4.5), potentially knocking out acyltransferase gene expression after inserting into this position. Moreover, three IS elements in the megaplasmid were almost exclusively lost in the low transfer frequency treatment (Fig. 2.4.5). In one of the positions (209500), the IS disrupted a putative calcium-binding protein in the intermediate transfer control clones but was absent in 4/8 low transfer control and 4/8 low transfer ITC treatment clones. In the other two positions (243500 and 253900), the ISs were intergenic (positioned 450 bp and 104 bp (243500) and 301 bp and 46 bp (253900)

from their left- and right-flanking genes; Fig. 2.4.5). The right-flanking genes closest to the ISs included a (2Fe-2S)-binding protein (243500) and an H-NS histone family protein (253900), whilst the left-flanking genes included the type III effector *HopG1* (243500) and an unknown hypothetical protein (253900). The frequency of IS absence in these positions (243500 and 253900) differed between low transfer treatments. Specifically, in position 243500, the IS was absent in 7/8 low transfer control and 5/8 low transfer ITC treatment clones. Meanwhile, in position 253900, the IS was absent in 4/8 low transfer control and 6/8 low transfer ITC treatment clones. However, despite these patterns, the extent of IS loss did not differ statistically between low transfer control and ITC-exposed clones when analysed individually (Mann-Whitney: 209500: w= 32, n1= 8, n2= 8, p= 1; 243500: w= 40, n1= 8, n2= 8, p= 0.29; 253900: w= 24, n1= 8, n2= 8, p= 0.35) or in combination (Mann-Whitney: w= 32, n1= 8, n2= 8, p= 1). Together, these results suggest that media adaptation and ITC tolerance was potentially driven by parallel mutations in a few genes and more frequent loss of IS elements in the low transfer frequency treatments.

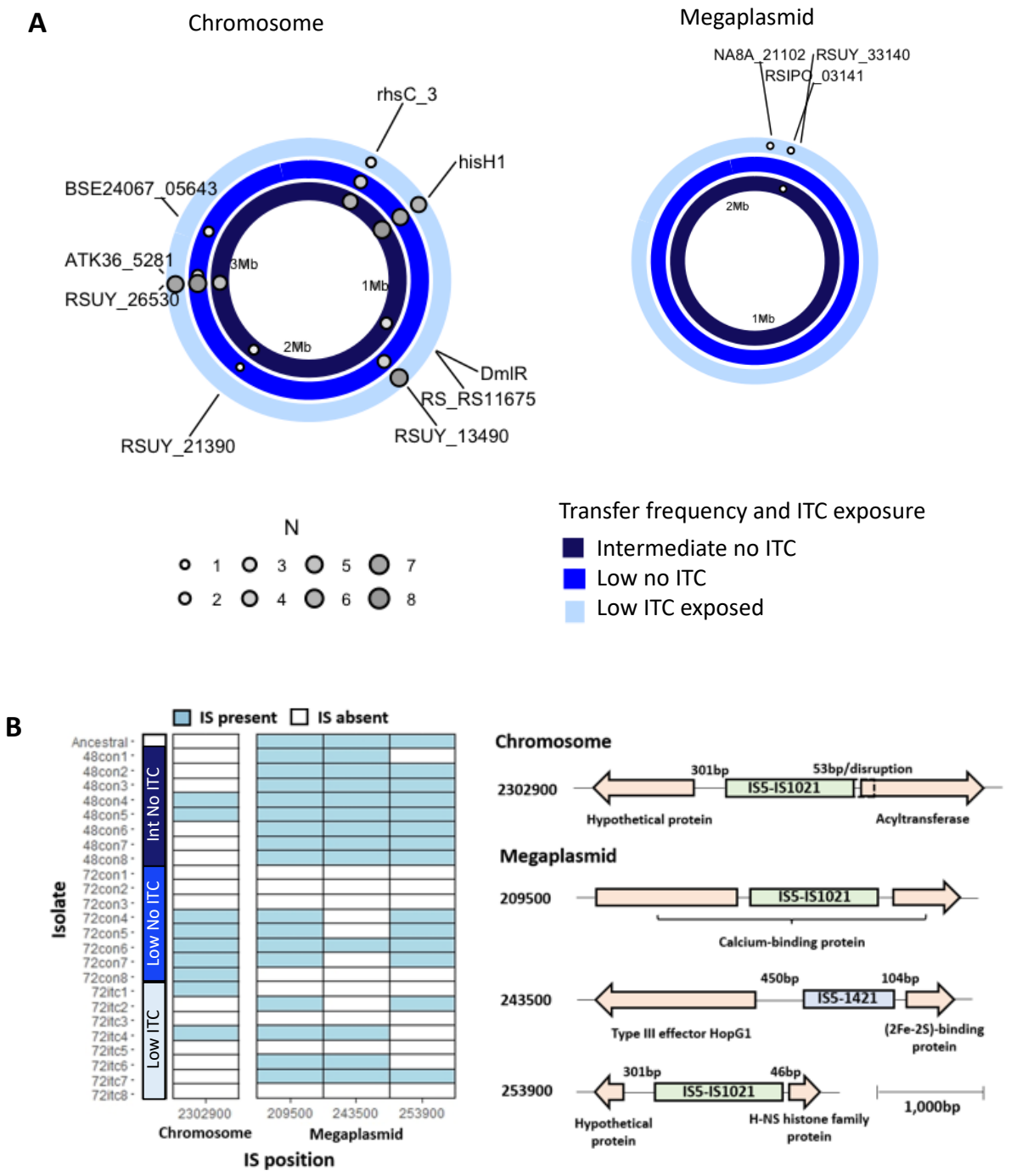


Figure 2.4.5. Mutations (A) and insertion sequences (IS; B) associated with evolved *R. solanacearum* clones. Each ring in panel A represents the *R. solanacearum* genome (Chromosome on the left and Megaplasmid on the right). Rings are grouped by the sequenced treatments) in different colours (see key) and dots represent mutations at different loci. Dots are sized and coloured by the number of replicates that had the same

mutations (N=8) in the indicated locus. Labels show the gene name, when named, or the numbered locus tag. Distance marker is shown as Mb within each ring. In panel B, tile plot shows presence (filled tiles) and absence (unfilled tiles) of insertion sequences (ISs) in each isolate. The X-axis of the tile plot shows the IS position rounded to the nearest 100 bp. Gene schematics on the right show insertion sequence at each position and nearby genes. Gene annotation and distance between insertion sequence and genes are shown, with gene size and distance proportional to the scale bar (bottom right).

2.5. Discussion

Here we studied the effects of *Brassica*-derived ITC allelochemicals for the suppression and tolerance evolution of plant pathogenic *R. solanacearum* bacterium in a model biofumigation experiment. We found that only allyl-ITC suppressed *R. solanacearum* growth, while no reduction in pathogen densities were observed when sec-butyl and 2-phenylethyl ITCs were applied alone or in combination. By using experimental evolution, we further showed that long-term allyl-ITC exposure selected for ITC tolerance in the low transfer frequency ITC exposure treatment and was associated with cross-tolerance to ampicillin. At the genetic level, tolerance evolution was associated with the loss of IS elements. Together, our results suggest that allyl-ITC derived from Indian mustard is effective at suppressing the growth of the *R. solanacearum* pathogen *in vitro*. However, prolonged exposure could select for increased ITC tolerance, potentially reducing the efficiency of ITC-based biocontrol.

Only allyl-ITC suppressed pathogen growth and its effects were not enhanced by the presence of other ITCs. This contradicts previous studies which demonstrated *R. solanacearum* sensitivity to 2-phenylethyl ITC at concentrations as low as 330 μM (Smith

and Kirkegaard, 2002). However, in the previous experiment *R. solanacearum* was exposed to 2-phenylethyl ITC in agar instead of liquid media, which has been shown to increase the toxicity of ITCs (Sarwar *et al.*, 1998). Moreover, it is possible that different *R. solanacearum* strains respond differently to ITCs, potentially through pre-existing tolerance mechanisms (i.e. efflux pumps, reduced cell membrane permeability, enzymes involved in toxin degradation) which could also explain discrepancy between ours and other studies. While the suppressive effects of sec-butyl ITC have previously been demonstrated against dust mites (Yun *et al.*, 2012) and fungi (Bainard, Brown and Upadhyaya, 2009), no antimicrobial activity has been observed in bacteria. Variation in the antimicrobial activity of ITCs could be explained by differences in chemical side-chain structure and molecular weight which govern ITC volatility and hydrophobicity (Sarwar *et al.*, 1998). Previous studies have shown greater pathogen suppression by ITCs with aliphatic compared to aromatic sidechains in fungal pathogens (Sarwar *et al.*, 1998; Kurt, Güneş and Soylu, 2011), insect pests (Matthiessen and Shackleton, 2005), and weeds (Vaughn *et al.*,1999). With bacteria, the toxicity of allyl-ITC could be attributed to its high volatility, very short R-side chains and high reactivity (Manici, Lazzeri and Palmieri, 1997; Kirkegaard and Sarwar, 1998; Neubauer, Heitmann and Müller, 2014). These properties could enable rapid diffusion through the liquid media before ITC is lost in the gaseous phase (Wang *et al.*, 2009). This is supported by a study by Sarwar *et al.* (Sarwar *et al.*, 1998), where a droplet of aliphatic allyl-ITC was shown to volatilise at room temperature in 5 minutes, whilst aromatic 2-phenylethyl ITC remained in the liquid for over 72 hours. Together, our result suggests that high volatility and reactivity could be important properties determining the antibacterial effects of ITCs.

The evolution of ITC tolerance was mainly observed in the low transfer frequency ITC exposure treatment. However, we also found that low transfer frequency control

populations showed improved tolerance measured in CPG media even though they had not been exposed to allyl-ITC during the experiment. As all treatments were kept separate from each other using tightly sealed bags, this effect is unlikely explained by 'cross selection' due to ITC volatilisation. Alternatively, ITC tolerance evolution could have been linked to certain metabolic adaptations in this transfer frequency treatment. In support of this, we found that evolved control bacterial populations showed improved growth in the CPG media relative to ancestral and ITC-exposed populations, indicative of media adaptation. While similar media adaptations were observed in all control treatment populations, it is not clear why ITC tolerance did not evolve under one- and two-day transfer frequency treatments. Surprisingly, the clearest ITC-adaptation was observed in the low transfer frequency environment where bacteria experienced the lowest number of generations (Appendix Table A.2), which suggests that the length of the experiment did not constrain bacterial adaptation in the high and intermediate frequency treatments. One potential explanation for this could be growth-dependent effects on mutation rates. For example, prior studies have shown that bacterial mutation rates can be elevated at stationary phase (Loewe, Textor and Scherer, 2003; Navarro Llorens, Tormo and Martínez-García, 2010), which could have promoted ITC tolerance and media adaptation in the low transfer frequency treatment where bacteria had spent the relatively longest time at stationary phase (Appendix Fig. A2). Alternatively, stationary phase growth conditions could have triggered expression of stress tolerance genes, enabling selection for mutants with relatively higher ITC tolerance (Navarro Llorens, Tormo and Martínez-García, 2010). For example, expression of *RpoS* sigma factor in *P. aeruginosa* has previously been linked to elevated antibiotic resistance and biofilm formation at stationary phase (Murakami *et al.*, 2005; Olsen, 2015). While more work is needed to elucidate these mechanisms, it is likely that the periodic 3-day growth cycle was

important for driving ITC tolerance evolution in our experimental conditions. Interestingly, the ITC tolerance that evolved in the absence of allyl-ITC exposure was specific to CPG media and disappeared when measured in 'naïve' LB media. This result suggests that ITC tolerance observed in control populations was likely driven by adaptation to CPG growth media. Such adaptation may have helped to offset the suppressive effects of allyl-ITC by boosting pathogen growth to compensate increased mortality. Alternatively, it is possible that the glucose availability in the CPG media indirectly favoured the evolution of ITC tolerance via metabolic adaptations, which has previously been shown to occur both in the absence (Knöppel, Näsvall and Andersson, 2017) and presence of clinical antibiotics (Zampieri *et al.*, 2017). Together, our results suggest that prior exposure to allyl-ITC was required for the evolution of robust ITC tolerance, which was independent of the growth media.

At the genetic level, ITC tolerance was not associated with any clear parallel mutations or indels in the low transfer frequency treatments. Three clones from the low transfer frequency control treatment had unique mutations in a gene encoding a dehydrogenase-like uncharacterised protein. Dehydrogenase genes have previously been associated with both metabolism and antibiotic resistance (Marshall, Zolli and Wright, 1999). For instance, in *Escherichia coli*, a mutation in a glucose dehydrogenase gene has been shown to function in lipopolysaccharide modification and calanic acid biosynthesis, which enabled resistance to polymyxin and other antimicrobial peptides (Lacour *et al.*, 2008; Rodionova *et al.*, 2020), and may have contributed to ITC tolerance in these clones. Additionally, two clones from the low transfer control treatment had mutations in a gene encoding a Tat pathway signal protein which is involved in protein translocation across membranes (Palmer, Sargent and Berks, 2005), and may have enabled improved growth in the CPG media. Three clones from

the intermediate transfer frequency treatment had unique mutations in a gene encoding a probable transcription regulator protein. While there is little information available regarding this gene, it is located beside the IS2 transposase *TnpB* gene, potentially affecting its regulation in DNA replication, recombination and repair activity (Pasternak *et al.*, 2013). Instead of treatment-specific parallel mutations, certain mutations were found across all treatments. For example, mutations in genes encoding putative serine/threonine protein kinases, amino acid biosynthesis (*hisH1* gene) and DNA replication, recombination and repair (putative *RhsC* gene) were common for clones isolated from all treatments. Mutations observed in serine/threonine protein kinase genes could have potentially affected ITC tolerance if these enzymes were targeted by the ITCs as has been shown before in the fungus *Alternaria brassicicola* (Calmes *et al.*, 2015), and bacterial pathogen *E. coli* (Luciano and Holley, 2009). However, as these mutations were not specific to ITC-treatment clones, they were probably associated with bacterial growth and metabolism.

In *R. solanacearum*, insertion sequences (ISs) have been shown to affect host virulence and phenotypic plasticity by inserting into and disrupting type III effectors and global virulence regulators (Jeong and Timmis, 2000; Gonçalves *et al.*, 2020). Therefore, we investigated whether IS movement may be the cause of *R. solanacearum* ITC tolerance adaptation. We identified one IS position in the chromosome and three positions in the megaplasmid which showed treatment specific patterns. The gain of IS at position 2302900 was primarily observed with low transfer control isolates and was situated either ~50 bp from the start codon or inside of a putative acyltransferase. Acyltransferases have a broad range of functions including lipid storage (Ohlrogge and Browse, 1995), phospholipid biosynthesis (Li et al., 2017), and the production of toxins (Greene *et al.*, 2015) and

antibiotics (Kozakai *et al.*, 2020). Whilst many of these functions are critical to cell growth, some such as the production of toxins would be redundant when grown in media. Therefore, gene disruption by ISs in the low transfer control may increase fitness by allowing energy and nutrients to be re-directed towards promoting cell growth and competitivity, potentially at the expense of reduced virulence *in planta*. We also found loss of two ISs in the intergenic region of the megaplasmid in the low transfer control and ITC treatments. While these were intergenic, they were close (~50-100 bp) to the start codons of their right flanking genes and could have affected gene expression. In position 243500, the IS was situated close to a (2Fe-2S)-binding protein gene. Iron-sulfur clusters have been implicated in cellular metabolism, protein structural stabilisation, iron storage, and the regulation of gene expression (Johnson *et al.*, 2005). In the other position (253900), the IS was situated close to an H-NS histone like protein gene and while non-significant, was lost more frequently across low transfer ITC treatment clones (6/8) than low transfer control isolates (4/8). H-NS histone like proteins are transcriptional repressors generally involved in adaptation to environmental challenges like temperature stress and osmolarity gradients (Atlung and Ingmer, 1997). Further, H-NS histone like proteins have been shown to stabilise the sigma factor *RpoS* (Hommais *et al.*, 2001) which acts as a master regulator of the bacterial stress response. Whilst the H-NS histone-like protein could affect ITC tolerance by mediating the bacterial stress response, the impact of the (2Fe-2S)-binding protein is less clear. Notably, in *Campylobacter jejuni*, genes containing iron-sulfur clusters have been found to be upregulated in response to ITCs, potentially due to their susceptibility to oxidative stress caused by ITC exposure (Dufour *et al.*, 2013). Therefore, by altering the expression of the (2Fe-2S)-binding protein, IS loss could increase the pool of cellular iron-sulfur cluster proteins and compensate for losses caused by ITC oxidative stress. In the final

megaplasmid IS position (209500), we identified a loss of IS from a calcium-binding protein gene, which had likely disrupted gene expression or protein function in this gene with the ancestral strain. In human breast cancer cells, ITCs, including phenethyl- (Tusskorn *et al.*, 2013) and allyl-ITC (Bo *et al.*, 2016) have been found to induce mitochondrial calcium ion mobilisation resulting in cytotoxicity through a reduction in mitochondrial membrane potential. Whilst further work is required to determine the causes of ITC cytotoxicity in *R. solanacearum*, upregulation of calcium-binding protein gene expression could have increased ITC tolerance by facilitating the sequestration of free calcium ions. However, like other genetic changes, loss of this IS did not occur statistically more often in the presence of ITC selection. As a result, specific genetic mechanisms underlying ITC tolerance remain elusive.

In conclusion, our findings demonstrate that allyl-ITC could potentially be used to suppress the growth of *R. solanacearum* plant pathogen. However, repeated ITC exposure could select for mutants with increased ITC tolerance, potentially weakening the long-term efficiency of ITCs and biofumigation. Future work should focus on validating these findings in more complex natural environments. For example, it is currently not clear if *R. solanacearum* ITC tolerance evolves in the plant rhizosphere in the presence of other microbes that could constrain mutation supply rate via resource and direct competition. Moreover, different resistance mechanisms could be selected depending on soil physiochemical properties and nutrient and plant root exudate availability, while it is not clear if the ITC concentrations used in this experiment are achievable through biofumigation and whether they might have negative effects on beneficial soil microbes. More efficient ITC application could be attained by drilling the biofumigant plants into fields at the time of

flowering when GSL levels are highest using finely chopped plant material, which maximises cell disruption and ITC release to the soil (Back, Barker and Evans, 2019). In addition, the efficacy of *Brassica*-based biofumigation could potentially be improved by using plant cultivars with elevated levels of sinigrin, the GSL precursor to allyl-ITC. Comprehensive *in vivo* work is thus required to validate the potential of allyl-ITC for *R. solanacearum* biocontrol in the field. It would also be interesting to study if ITC tolerance leads to life-history traits in *R. solanacearum,* potentially affecting its virulence or competitiveness in the rhizosphere.

Chapter 3. Allyl-ITC selectively suppresses a pathogenic bacterium in model bacterial co-cultures

1.1. Abstract

Biofumigation, a biocontrol strategy involving the integration of *Brassica* plant tissues into the soil that contain biocidal isothiocyanates (ITCs), could offer an environmentally sustainable alternative to agrochemicals. Despite the success of biofumigation against a range of plant pathogens, its effects on beneficial and other non-target soil microorganisms remain poorly understood. Here we used three model systems (*in vitro* liquid and soil microcosms and *in vivo* tomato rhizosphere mesocosms) to test the effects of allyl-ITC on plant-pathogenic *Ralstonia solanacearum* in the absence and presence of two plant growth-promoting *Pseudomonas* bacteria. We found that ITC exposure successfully inhibited *R. solanacearum* growth in both *in vitro* and *in vivo* systems, while *Pseudomonas* species were unaffected or inhibited to a much lesser extent. Co-culturing suppressed the growth of both *R. solanacearum* and *Pseudomonas* species, and this competition further magnified the negative effects of ITC on *R. solanacearum* growth. In contrast, *Pseudomonas* species were mostly unaffected by ITC exposure in co-cultures, indicative of potential pre-existing ITC tolerance. No evidence was observed for *R. solanacearum* ITC tolerance evolution in the tomato rhizosphere *in vivo*. However, ITC exposure had negative effects on tomato aboveground dry weight and constrained flowering when inoculated with *Pseudomonas* bacteria. Together, these results show that biofumigation could potentially be used to selectively target pathogens in bacterial communities, while careful balance is required to prevent harmful effects on crop yield.

1.2. Introduction

Pests and diseases account for the loss of a third of annual crop production worldwide (Savary *et al.*, 2019). Restrictions against traditional, pathogen-inhibiting synthetic agrochemicals, such as methyl bromide, metam sodium and 1,3-dichloropropene fumigants, are becoming stricter. This is due to their environmentally damaging effects in depleting the ozone layer, degrading soil biodiversity and often inhibiting non-target soil microbiota (Schreiner, Ivors and Pinkerton, 2001). As such, environmentally sustainable crop protection methods are urgently required. Biofumigation, the incorporation of *Brassica* plant material into soils to release biocidal allelochemicals could offer an effective biocontrol solution (Angus *et al.*, 1994). *Brassica* plants are naturally elevated in secondary metabolites, including glucosinolates (GSLs) and myrosinase enzymes which are hydrolysed upon contact with each other to release bioactive compounds, principally isothiocyanates (ITCs) (Matthiessen and Kirkegaard, 2006; Gimsing and Kirkegaard, 2009a; Lord *et al.*, 2011). Widespread adoption of biofumigation has not however been achieved yet, partly due to variation in its efficiency. For instance, whilst some studies have identified disease-suppressive effects (Matthiessen and Kirkegaard, 2006; Larkin and Griffin, 2007; Hanschen *et al.*, 2015), others have reported neutral effects (Kirkegaard *et al.*, 2000; Stirling and Stirling, 2003; Hartz *et al.*, 2005), and in some cases biofumigation has even been shown to enhance growth of the target pathogen (Yulianti, Sivasithamparam and Turner, 2007; Lu, Gilardi, Lodovica Gullino, *et al.*, 2010). These inconsistencies may be explained by the research focus on pathogenic species with less emphasis on the effects on the surrounding, often beneficial microbiome, which could also suffer collateral damage during biofumigation.

The broad-spectrum effects of biofumigation have been documented against fungal (Olivier *et al.*, 1999), nematode (Ji *et al.*, 2007; Lord *et al.*, 2011) and bacterial pathogens, yet can also result in collateral damage to beneficial soil arthropods (van Ommen Kloeke *et al.*, 2014; Zuluaga *et al.*, 2015) and interfere with the activity of beneficial entomopathogenic nematodes (Henderson *et al.*, 2009; Ramirez *et al.*, 2009). Additionally, biofumigation could negatively alter microbial community composition and diversity, disrupting ecosystem stability (Griffiths *et al.*, 2000), driving the collapse of food webs (Bending and Lincoln, 1999) and potentially facilitating the re-colonisation of pathogens (Van Bruggen and Semenov, 2000b; Friberg *et al.*, 2009a). More research on the effects of biofumigation at the level of the whole soil microbiome is thus required to ensure their safe use.

Here, we focused on determining the effects of allyl-ITC biofumigant on the plant pathogenic *Ralstonia solanacearum* pathogen in the absence and presence of two plant growth-promoting bacteria from the *Pseudomonas* genera: *Pseudomonas protegens* CHA0 and *Pseudomonas fluorescens* SBW25. *R. solanacearum* is the causative agent of bacterial wilt disease, capable of infecting over 200 plant species across 50 families, including agronomically important crops like tomato and potato (Yabuuchi *et al.*, 1995; Elphinstone, 2005). The two *Pseudomonas* species were chosen because they have different mechanisms of pathogen suppression and are known plant growth-promoting rhizobacteria (PGPRs). *P. protegens* CHA0 shows direct inhibition of *R. solanacearum* growth (Ran *et al.*, 2005), predominantly due to the release of the broad-spectrum antibiotics DAPG, pyrrolnitrin, hydrogen cyanide and pyoluteorin (Duffy and Défago, 1999; Ramette *et al.*, 2011), as well as lipopeptide biosurfactants like orfamides (Jang *et al.*, 2013). Meanwhile, the suppression of *R. solanacearum* by *P. fluorescens* SBW25 has been attributed to the production of

pyoverdine siderophores which exacerbate iron competition amongst neighbouring soil microorganisms (Moon *et al.*, 2008), release of bioactive lipopeptides (De Bruijn *et al.*, 2007; Mazzola, 2007), and L-furanomycin compounds with antimicrobial properties (Trippe *et al.*, 2013). Whilst field level biofumigation studies have been conducted previously (Kirkegaard *et al.*, 2000; Ramirez *et al.*, 2009; Yim *et al.*, 2016), systematic studies exploring ITC effects on the pathogen and surrounding microbiome are lacking. To achieve this, we employed three different model systems to causally explore the effect of biofumigation on the pathogen and two plant-growth promoting *Pseudomonas* species. Specifically, we used (a) *in vitro* liquid microcosms, (b) *in vitro* soil microcosms and (c) *in vivo* tomato rhizosphere mesocosms. Allyl-ITC was chosen as the model biofumigant in this study as it is the predominant GSL-hydrolysis product released from Indian mustard (*Brassica juncea*), which is a common and highly effective biofumigant plant (Kirkegaard and Sarwar, 1998; Bending and Lincoln, 1999; Kirkegaard and Matthiessen, 2005; Mazzola, Hewavitharana and Strauss, 2015).

Based on previous findings, we predicted that allyl-ITC would be relatively more harmful to *R. solanacearum* (Smith and Kirkegaard, 2002) than *Pseudomonas* species due to potentially pre-existing tolerance to antimicrobials (Kirkegaard *et al.*, 2004), which could lead to competitive exclusion of the pathogen (Hu *et al.*, 2015). Moreover, probiotic microorganisms have been shown to develop more intense pathogen inhibitory activity following biofumigation (Wiggins and Kinkel, 2005). We hence predicted that the suppressive effects of ITC could be magnified in the presence of antagonistic *Pseudomonas* species if they show ITC tolerance and can further suppress the pathogen growth via resource competition or production of antimicrobials (Duffy and Défago, 1999; Ramette *et al.*, 2011). In support for our hypotheses, ITC was relatively more suppressive to *R.*

solanacearum pathogen both in *in vitro* and *in vivo* study systems and only very mild negative effects of ITC exposure were observed with both *Pseudomonas* species. Moreover, the inhibitory effects of ITC were magnified when grown in the presence of a *Pseudomonas* species. However, ITC exposure also reduced tomato dry weight, while *Pseudomonas* species failed to alleviate bacterial wilt disease symptoms *in vivo* despite clearly reducing *R. solanacearum* abundances in the rhizosphere. While these results suggest that allyl-ITC could potentially be used to specifically target the *R. solanacearum* bacterial pathogen, more work is needed to validate these results in more diverse rhizosphere microbiomes.

1.3. Materials and methods

(a) Bacterial strains and culture conditions

We used the same *Ralstonia solanacearum* strain (21415687) used in our previous experiments (Source: John Elphinstone, Fera Science, 2014). This strain was selected as it was isolated from river water, the most common source of inoculum in UK fields through the use of contaminated river water for irrigation (Prior *et al.*, 1998). We used two species of *Pseudomonas* plant growth-promoting bacteria. The first one was *P. protegens* strain CHA0 (formerly *P. fluorescens*), which was isolated from a tobacco plant in Switzerland (Natsch *et al.*, 1994). The second strain was *P. fluorescens* strain SBW25, which was isolated from the leaf-surface of sugar-beet in the UK (De Leij *et al.*, 1995). Both *Pseudomonas* strains are known to have beneficial effects on plants in the rhizosphere and often coexist in terrestrial soils (Cho and Tiedje, 2000). All bacterial strains were cultured in CPG broth (1 g casamino acids, 10 g peptone and 5 g glucose per litre of ddH_2O) at 28 °C to create cryostocks (20% w/v glycerol) that were preserved at -80 °C.

(b) Comparing the effects of ITC on pathogenic and non-pathogenic bacteria in *in vitro* liquid and soil microcosms

To test the effects of ITC on *R. solanacearum* and *Pseudomonas in vitro,* we used both liquid and soil microcosms. We established populations of *R. solanacearum, P. protegens* CHA0 and *P. fluorescens* SBW25 either in monoculture or in two-species *R. solanacearum Pseudomonas* co-cultures in sterile liquid and soil microcosms.

(i) Liquid microcosm experiments

R. solanacearum and *Pseudomonas* (CHA0 and SBW25) bacterial cultures were normalised to an optical density reading of 0.1 (OD 600 nm; Tecan Sunrise), equalling ~10^7 cells per ml and inoculated to wells of a 96-well microplate in CPG media (200 µl media, 10 µl bacteria in monoculture or 5 µl:5 µl in two-species co-culture, mixed by vortexing). Bacteria were exposed to allyl-ITC at 0.5 µmol g^{-1} soil concentrations, and in liquid microcosms equivalent concentrations were applied by mixing in CPG medium. This ITC concentration was selected as it is in line with those used in our previous studies and relevant to field biofumigation (Borek *et al.*, 1995; Sarwar *et al.*, 1998; Warton, Matthiessen and Shackleton, 2003b; Hanschen *et al.*, 2012; Rudolph *et al.*, 2015). The bacterial densities were measured at 24-hour intervals throughout the 72-hour ITC exposure experiment (OD 600 nm) and microplates were kept at 28 °C between measurements (sealed in plastic bags to prevent cross-volatilisation of ITC). All treatments were replicated eight times (N=8). To compare differences in bacterial species abundances, the samples from the end of the experiment (72 hours following set-up) were serially diluted and spotted onto semi-selective agar plates containing specific nutrients, antibiotics and fungicide, including crystal violet, polymyxin B sulfate, tyrothricin, chloromycetin and casamino acids, to select for *R. solanacearum* (SMSA plates (Elphinstone *et al.*, 1996)) and *Pseudomonas* colonies (Mohan, 1987)). Agar plates

were stored at 28 °C and colony counts were taken at 24-hour intervals for a period of 72 hours following spotting onto agar plates. For CFU counts in each of the three study systems, all treatment replicates were spotted four times (technical replication) to determine average CFU mL^{-1} for each replicate.

(ii) Soil microcosm experiments

To test ITC effects in an environment more closely resembling field conditions with spatial structure and low-resource availability, soil microcosms were set up using loosely lidded Universal bottles (30 ml) based on protocols described earlier (Hall *et al.*, 2016). 10 g of twice-autoclaved John Innes #2 potting soil with 25% w/v water content was added to sterile Universal bottles and allowed to equilibrate for 1 hour before use. Bacterial densities of *R. solanacearum* and *Pseudomonas* (CHA0 and SBW25) liquid cultures were normalised to an OD of 0.5 (600 nm), resulting in ~10^9 CFU/ml and spun by centrifugation (14,000 rpm) to form a pellet. The supernatant was then removed, and the pellet was re-suspended in 1 ml M9 buffer (128 g sodium phosphate dibasic, 30 g monopotassium phosphate, 5 g sodium chloride, 10 g ammonium chloride per litre ddH_2O; recipe for 10x stock concentration). 100 µl of this liquid suspension was added to sterile microcosms and homogenised by vortexing (final concentration of cells: ~4 x 10^9 cells per g soil). In ITC treatments, liquid allyl-ITC was mixed with CPG media, resulting in a final concentration of 0.5 µmol g^{-1} soil (the same concentration used in the liquid microcosm experiment). For the negative control treatment, an equal volume of sterile CPG media was added. Microcosms were then stored stationary at 28 °C for four days before the experiment was ended. All treatments were replicated four times (N=4). To determine bacterial abundances, CFU counts were quantified by adding 10 ml sterile M9 buffer to each soil microcosm with 20 sterile glass

beads and mixing thoroughly by vortexing to homogenise soil. Soil washes were then serially diluted and spotted onto selective agar plates to determine CFUs as described earlier.

(c) Comparing the effects of ITC on *R. solanacearum* and *Pseudomonas* species *in vivo* in the tomato rhizosphere

We also determined ITC effects on the *R. solanacearum* and *Pseudomonas* species *in vivo* in the tomato rhizosphere and recorded the effects of bacteria and ITC application on tomato flowering, growth and bacterial wilt symptoms. *In vivo* experiments were set up by sowing seeds of the cultivar Micro Tom tomato (*Solanum lycopersicum*) in 30 g twice-autoclave-sterilised John Innes #2 potting soil. Tomatoes were inoculated with mono- or co-culture mixes of *R. solanacearum* and *Pseudomonas* species both in the absence and presence of ITC similar to *in vitro* experiments (N=10 per treatment, total number of mesocosms=120).

To remove the potential for unequal nutrient addition between treatments, *Pseudomonas* cultures were washed from their nutrient media by centrifugation (14,000 rpm for 10 minutes) and resuspended in sterile ddH_20. 14 days following the sowing of seeds in plant trays, tomatoes were inoculated with 6 ml of *Pseudomonas* liquid culture to the base of the stem to a concentration of approximately 5×10^7 CFU of bacteria g^{-1} soil (OD 0.25). This volume was sufficient to drench the soil and ensure even spread of bacteria throughout the root system. After 7 days of *Pseudomonas* inoculation, the roots of all tomato seedlings were cut using a sterile scalpel to mimic natural pathogen entry points in field conditions and to increase the consistency of infection between replicates (Personal communication: John Elphinstone, Fera protocol, UK). *R. solanacearum* inoculum density was normalised to 0.4 (OD 600 nm) after washing and resuspension in sterile ddH_20 and 1 ml of liquid pathogen suspension was inoculated directly to the tomato root wound at a concentration of approximately 10^8 CFU of bacteria g^{-1} soil. With all ITC treatments, ITC was

mixed with CPG media and 1 ml of this solution was added to the soil surface 2 days after pathogen inoculation, resulting in final concentrations of 0.5 μmol g^{-1} soil, consistent with our *in vitro* liquid and soil microcosm experiments. In the case of no-ITC treatments, equal volume of sterile CPG media was inoculated directly to the root wound to ensure the same levels of nutrient addition between treatments.

Tomato wilt disease symptoms were monitored based on visual observations every 2 days following *R. solanacearum* inoculation using an index of 0-4 according to Roberts *et al.* (1988), with a score of 0 meaning no leaves wilted, 1 meaning 25% of leaves wilted, 2 meaning 26-50% of leaves wilted, 3 meaning 51-75% of leaves wilted and 4 meaning 76-100% of leaves wilted. The presence of flowers was also monitored qualitatively (1=flowers, 0=no flowers). All seedlings were watered regularly using sterilised water and plants were kept in growth chambers with 12-hour dark/light cycles at 28 °C during the day and 20 °C at night for a total of 49 days. Seedling plates were rearranged randomly within the growth chamber every two days.

At the end of the experiment (28 days following *R. solanacearum* inoculation), plants were cut at the base and the aboveground sections of the plant were dried in a drying oven at 70 °C for 4 days before recording aboveground dry weight measurements. Tomato rhizosphere soil samples were collected from all replicate plants by washing the soil surrounding the roots in 20% w/v glycerol. Soil suspensions were allowed to settle for 30 minutes before 1.25 ml from each sample was frozen at -80 °C in 50% glycerol for later experiments. To distinguish *R. solanacearum* and *Pseudomonas* bacterial abundances, semi-selective agar plate CFU counts were used as described earlier. Agar plates were stored at 28 °C and colonies counted at 24-hour intervals for a total period of 72 hours.

(d) Determining evolutionary changes in *R. solanacearum* tolerance to ITC and *Pseudomonas* antimicrobials (supernatant)

To investigate the potential for *R. solanacearum* to evolve tolerance to ITC and *Pseudomonas* antimicrobials (or other metabolites), we set up fitness assays that compared the densities of ancestral and evolved control (non-ITC exposed) and ITC-exposed *R. solanacearum* populations derived from the final time point of the tomato growth experiment (28 days post inoculation) when re-exposed to ITC or ancestral *Pseudomonas* supernatants (Gu, *et al.*, 2016). Supernatant contained all metabolites produced by *Pseudomonas* species and the production of any specific antibiotics was not quantified. Four ancestral and six evolved *R. solanacearum* clones per each 5 replicate populations were isolated from SMSA agar plates using sterile loops, after all clones were inoculated into 200 μl CPG media in 96-well microtiter plates (Total of 30 evolved clones and 4 ancestral clones). After 24 hours growth at 28 °C, bacterial densities were normalised to an OD reading of 0.1 (600 nm) and 10 μl of each replicate colony was inoculated into liquid microcosms containing 200 μl CPG or CPG media mixed with ITC at 500 μM concentrations similar to our previous liquid experiments. Bacterial density measurements were recorded at 24-hour intervals for a period of 72 hours based on OD readings (600 nm). Relative growth inhibition by ITC was determined by comparing growth in the absence of ITC (CPG only) with growth in the presence of ITC. Therefore, a value of 0 indicates the strain grows equally well in the absence and presence of ITC, while values below 0 indicate the suppressive effect of ITC.

To test if *R. solanacearum* adapted to *Pseudomonas* antimicrobials (or other metabolites), we compared the ancestral and evolved *R. solanacearum* clones' growth in the presence of ancestral *Pseudomonas* strain's supernatant. Supernatants were derived by

growing ancestral *P. protegens* CHA0 and *P. fluorescens* SBW25 strains in CPG liquid media for 24 hours at 28 °C, after bacterial densities were normalised to an OD reading of 0.1 (600 nm), followed by centrifugation (14,000 rpm) for 10 minutes and filter sterilising using 0.2 μm filters. To determine *Pseudomonas* effects, we grew the ancestral and evolved *R. solanacearum* clones in 80% *Pseudomonas* supernatant, 20% CPG mixture. As a control treatment, evolved *R. solanacearum* clones were grown in 50% ancestral *R. solanacearum* supernatant, 50% CPG mixture. In all cases, 10 μl of each *R. solanacearum* clone was cultured in 200 μl of solution in 96-well microtitre plates at 28 °C for 72 hours. Bacterial densities were recorded (OD 600 nm) at 24-hour intervals. Supernatant adaptation was determined as bacterial growth relative to when grown in the absence of *Pseudomonas* supernatant in control conditions as described earlier.

(e) Statistical analyses

Two-way ANOVA was used to analyse differences in bacterial growth and CFU mL^{-1} between treatments and Tukey *post-hoc* tests were used to compare differences between subgroups ($p< 0.05$). In the case of comparison of single bacterial species growth in the presence or absence of ITC, a pairwise t-test was used. Where ordinal and categorical data were analysed, non-parametric Kruskal-Wallis test was used to determine significances between treatments. In the case of binomial tomato flowering data, poisson glm with Chi-square test was used to determine significances between treatments. To avoid pseudoreplication, means of technical replicates were used in statistical analyses. All analyses were conducted using the final time point of the experiments and all statistical analyses and graphs were produced using R (R Foundation for Statistical Computing, R Studio Version (4. 0. 3), Packages: ggplot2 (Wickham, 2011), ggpubr (Kassambara, 2018)).

1.4. Results

(a) *R. solanacearum* growth was suppressed by both ITC and *Pseudomonas* competitors in liquid and soil microcosms

(i) Liquid microcosm experiment

We first determined the effect of ITC exposure on *R. solanacearum, P. protegens* CHA0 and *P. fluorescens* SBW25 in liquid microcosm monocultures. ITC had negative effects on bacterial growth overall ($F_{1, 42}$= 4.89, p< 0.05; Fig. 3.4.1A), and this differed between species ($F_{2, 42}$= 24.49, p< 0.001; Fig. 3.4.1A). Specifically, we found that the suppressive effects of ITC were mainly driven by the relatively higher sensitivity of *R. solanacearum* to ITC in monoculture (ITC × *Pseudomonas* presence: $F_{2, 42}$= 3.50, p< 0.05; Tukey: p< 0.05, Fig. 3.4.1A). In contrast to monocultures, ITC had no effect on bacterial densities in co-cultures ($F_{1, 28}$= 1.06, p= 0.31; Fig. 3.4.1A). However, all bacteria reached significantly lower bacterial densities when grown in co-cultures ($F_{1, 78}$= 5.67; p< 0.05; Fig. 3.4.1A), and this effect depended on the co-culture treatment ($F_{1, 28}$= 9.97, p< 0.01; Fig. 3.4.1A). Specifically, *R. solanacearum* and *P. fluorescens* SBW25 co-cultures had lower growth compared to *R. solanacearum* and *P. protegens* CHA0 co-cultures in the presence of ITC (ITC × *Pseudomonas* presence: $F_{1, 28}$= 4.75, p< 0.05; Tukey: p< 0.05; Fig. 3.4.1A).

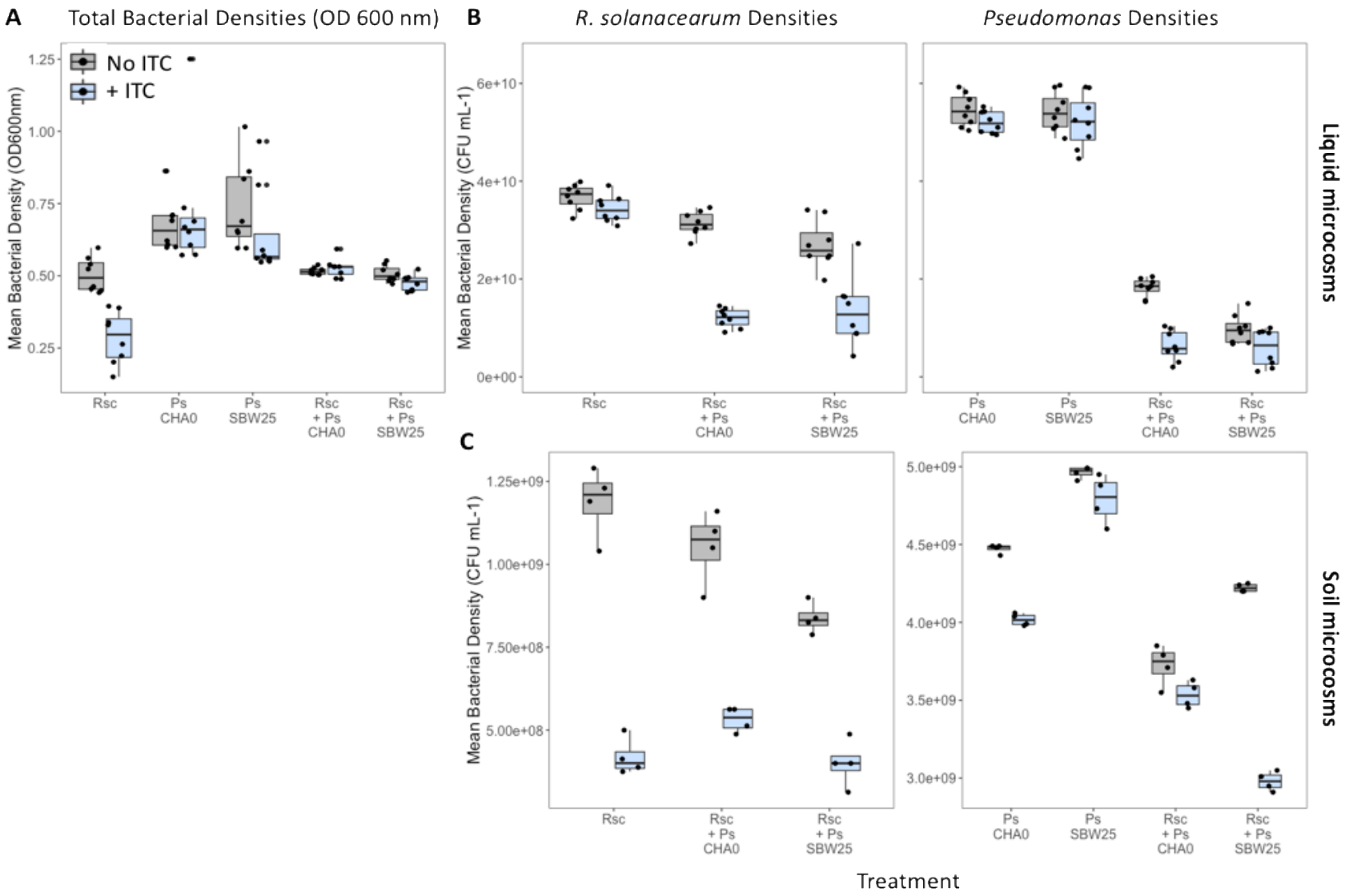

A
Total Bacterial Densities (OD 600 nm)
No ITC
+ ITC
Mean Bacterial Density (OD600nm)
1.25
1.00
0.75
0.50
0.25
Rsc
Ps CHA0
Ps SBW25
Rsc + Ps CHA0
Rsc + Ps SBW25
B
R. solanacearum Densities
Pseudomonas Densities
Mean Bacterial Density (CFU mL-1)
6e+10
4e+10
2e+10
0e+00
Liquid microcosms
C
Mean Bacterial Density (CFU mL-1)
1.25e+09
1.00e+09
7.50e+08
5.00e+08
5.0e+09
4.5e+09
4.0e+09
3.5e+09
3.0e+09
Soil microcosms
Treatment

Figure 3.4.1. Effects of ITC on pathogenic *Ralstonia solanacearum* and plant growth-promoting *Pseudomonas* bacterial densities in mono- and co-cultures. (A) Total bacterial densities (OD 600 nm) for *R. solanacearum*, *P. protegens* CHA0 and *P. fluorescens* SBW25 bacteria in mono- and co-cultures after 72 hours growth in liquid CPG media in 96-well microtitre plates (N=8). (B) Densities (CFU mL^{-1}) of *R. solanacearum, P. protegens* CHA0 and *P. fluorescens* SBW25 bacteria in mono- and co-cultures after 72 hours growth in liquid microcosms (N=8). (C) Densities (CFU mL^{-1}) of *R. solanacearum, P. protegens* CHA0 and *P. fluorescens* SBW25 bacteria in mono- and co-cultures after 4 days of growth in soil microcosms (N=4). Grey boxplots denote for no-ITC and blue boxplots ITC treatments (see key in A). Boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates.

To determine ITC effects on different species in co-cultures, we used selective agar plates to quantify *R. solanacearum* and *Pseudomonas* frequencies. Colony count data did not fully align with optical density data. For example, *R. solanacearum* abundances were unaffected by ITC in monoculture (t= 1.32, *df*= 13.81, p> 0.05; Fig. 3.4.1B), but there was a suppressive effect of ITC in co-culture (t= 9.90, *df*= 29.36, p< 0.001; Fig. 3.4.1B). Moreover, *R. solanacearum* growth was suppressed also by the presence of *Pseudomonas* in the absence ($F_{1, 22}$= 20.51, p< 0.001) and presence of ITC ($F_{1, 22}$= 126.3, p< 0.001; Fig. 3.4.1B). Interestingly, *R. solanacearum* growth was suppressed relatively more by *P. fluorescens* SBW25 than *P. protegens* CHA0 in the absence of ITC ($F_{1, 14}$= 5.04, p< 0.05; Fig. 2.4.1B), while similar growth suppression by both *Pseudomonas* species was observed in the presence of ITC (p> 0.05; Fig. 3.4.1B). Importantly, *R. solanacearum* growth was suppressed the most when exposed to ITC in the presence of *Pseudomonas*, indicative of synergistic suppression by these two factors (ITC × Co-culture: $F_{1, 44}$= 30.28, p< 0.001; Fig. 3.4.1B).

With *Pseudomonas* strains, we found no significant effect of ITC on *P. protegens* CHA0 (t= 1.72*, d.f.*= 12.35, p> 0.05; Fig. 3.4.1B), or *P. fluorescens* SBW25 (t= 0.81, *d.f.*= 12.83, p> 0.05; Fig. 3.4.1B) abundances in monocultures. However, the suppressive effect of ITC became clearer in co-cultures with both *Pseudomonas* strains (CHA0: t= 11.63, *d.f.*= 11.63, p< 0.001; SBW25: t= 2.27, *d.f.*= 13.12, p< 0.05; Fig. 3.4.1B), and overall, the presence of *R. solanacearum* constrained the growth of both *Pseudomonas* strains (CHA0: $F_{1, 30}$= 503.7, p< 0.001; SBW25: $F_{1, 30}$= 896.4, p< 0.001; Fig. 3.4.1B). However, while ITC and co-culturing with *R. solanacearum* had a synergistic suppressive effect on *P. protegens* CHA0 (ITC × Co-culture: $F_{1, 28}$= 24.39, p< 0.001; Fig. 3.4.1B), no synergistic effect was found with *P. fluorescens* SBW25 (ITC × Co-culture: $F_{1, 28}$= 0.43, p> 0.05; Fig. 3.4.1B). Together, liquid

microcosm results show that *R. solanacearum* was relatively more sensitive to suppression by ITC and that this effect was magnified in the presence of *Pseudomonas* species.

(ii) Soil microcosm experiment

ITC exposure reduced *R. solanacearum* growth in monoculture (t= 12.75, *d.f.*= 5, p< 0.001; Fig. 3.4.1C) and co-culture soil microcosms (ITC: t= 8.23, *d.f.*= 12, p< 0.001; Fig. 3.4.1C). Overall, the presence of *Pseudomonas* had no effect on *R. solanacearum* abundance ($F_{1, 22}$= 048, p> 0.05; Fig. 3.4.1C), and only *P. fluorescens* SBW25 showed a slight suppressive effect on *R. solanacearum* in co-culture in the absence of ITC (t= 3.48, *df*= 19, p< 0.01; Fig. 3.4.1C), while *P. protegens* CHA0 had no effect (t= 1.28, *df*= 21, p> 0.05; Fig. 3.4.1C). The presence of *P. fluorescens* SBW25 did not magnify the negative effects of ITC on *R. solanacearum* (t= 0.41, *df*= 6, p= 0.70; Fig. 3.4.1C), while the presence of *P. protegens* CHA0 slightly reduced the inhibitory effect of ITC on *R. solanacearum* (t= -3.34, *df*= 5, p< 0.05; Fig. 3.4.1C). As a result, the suppressive effect of ITC were relatively stronger compared to the effects of *Pseudomonas* species on *R. solanacearum* in soil microcosms.

With *Pseudomonas*, we observed significant growth reduction by ITC in monocultures of *P. protegens* CHA0 (t= 18.91, *df*= 6, p< 0.001; Fig. 3.4.1C), but no effect on *P. fluorescens* SBW25 abundance (t= 2.14, *df*= 3, p> 0.05; Fig. 3.4.1C). Overall, the growth of *Pseudomonas* strains was reduced in co-cultures with *R. solanacearum* (CHA0: t= 6.13, *df*= 11, p< 0.001; SBW25: t= 5.30, *df*= 7.62, p< 0.001; Fig. 3.4.1C). Moreover, while the negative effect of *R. solanacearum* on *P. protegens* CHA0 was not magnified in the presence of ITC (t= 2.45, *df*= 5, p> 0.05; Fig. 3.4.1C), the growth of *P. fluorescens* SBW25 was further reduced in the presence of both *R. solanacearum* and ITC (t= 36.81, *df*= 4; p< 0.001; Fig. 3.4.1C).

To summarise, ITC had a suppressive effect on pathogen growth in both liquid and soil microcosms, while *Pseudomonas* species were less affected by ITC. While the

suppressive effect of ITC on *R. solanacearum* was magnified in the presence of *Pseudomonas* competitors in liquid microcosms, the ITC effect was relatively stronger in soil microcosms.

(b) ITC exposure reduced bacterial wilt disease symptoms in tomatoes

We next assessed the effects of ITC exposure on *R. solanacearum* and *Pseudomonas* bacterial abundances in the rhizosphere of a tomato using a plant growth chamber mesocosm experiment. Plants were first inoculated with *Pseudomonas* strains (14 days after sowing seeds), followed by *R. solanacearum* infection 7 days later. ITC was applied once 2 days after pathogen inoculation and wilting symptoms were quantified every 2 days for 4 weeks. We found that ITC exposure significantly reduced the severity of disease symptoms when plants were inoculated with *R. solanacearum* (X^2= 14.21, *d.f.*= 4, p< 0.01; Fig. 3.4.2). However, *Pseudomonas* species had no effect on tomato disease symptoms (X^2= 1.29, *d.f.*= 8, p= 0.52; Fig. 3.4.2), and while *R. solanacearum* infections typically reduced tomato plant aboveground dry weight ($F_{1, 118}$= 23.47, p< 0.001; Fig. 3.4.3A), ITC application did not alleviate these negative effects ($F_{1, 78}$= 0.05, p> 0.05; Fig. 3.4.3A). Instead, ITC exposure reduced tomato dry weight compared to the non-ITC treated control plants ($F_{1, 118}$= 5.67, p< 0.05; Tukey: p< 0.05; Fig. 3.4.3A) but to a lesser degree than *R. solanacearum* infections. Moreover, while ITC had no effect on flowering in control conditions (Flowering: N= 10 for control and N= 10 for ITC; Fig. 3B), ITC exposure reduced flowering in the presence of *Pseudomonas* bacteria (X^2= 4.85, *df*= 1, p< 0.05; Fig. 3B). However, ITC exposure in *R. solanacearum* infected plants had no effect on flowering (X^2= 0.05, *df*= 1, p> 0.05; Fig. 3.4.3B).

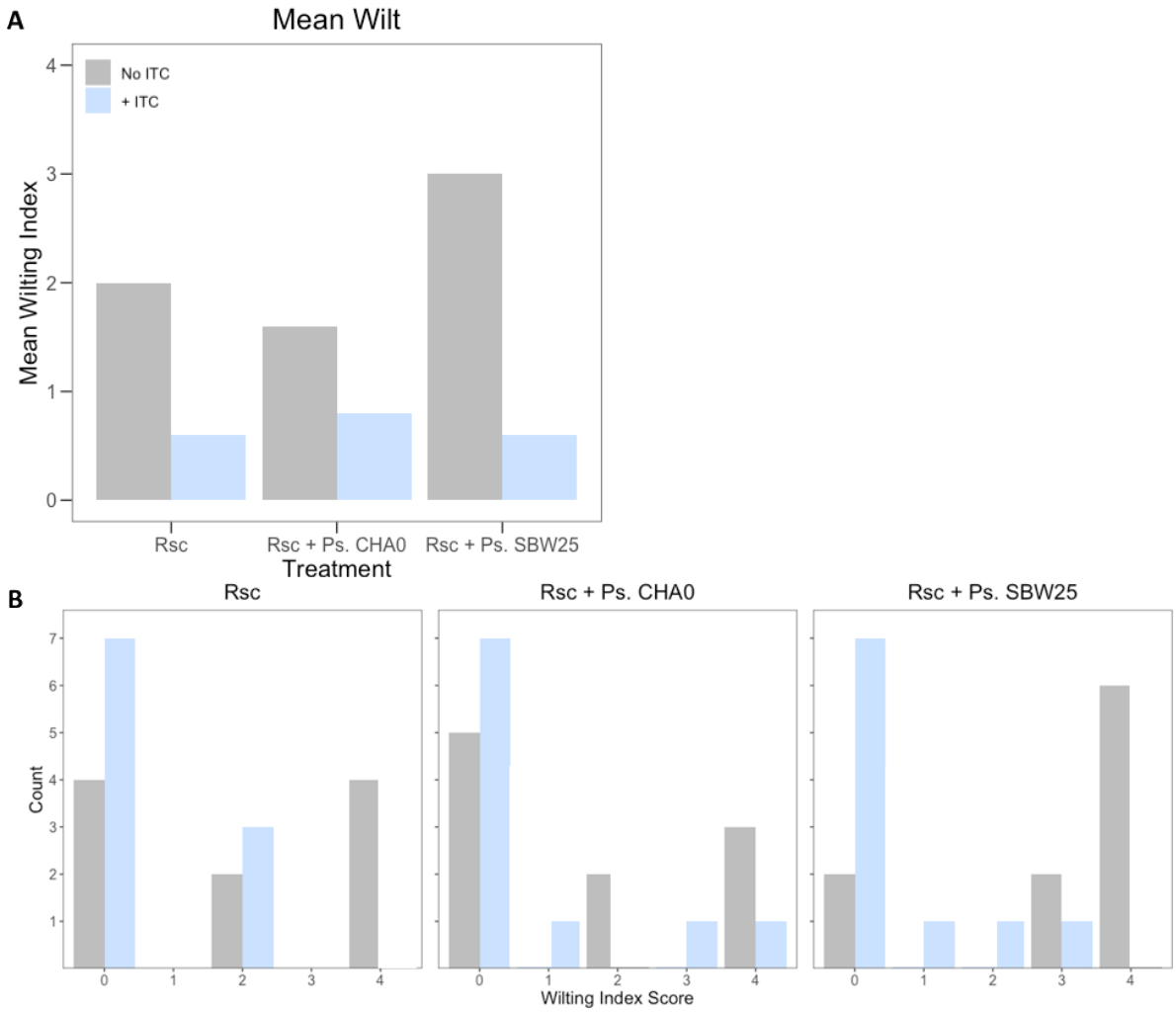


Figure 3.4.2. **Tomato disease symptom severity when exposed to *R. solanacearum* pathogen and plant growth-promoting *Pseudomonas* species in the presence and absence of ITC.** Panel (A) shows the effect of ITC on mean wilting index in tomato and panel (B) shows the effects of *R. solanacearum* pathogen alone and *R. solanacearum* grown in co-culture with *P. protegens* CHA0 (Ps. CHA0) and *P. fluorescens* SBW25 (Ps. SBW25) in the presence (blue bars) and absence (grey bars) of ITC on the number of wilted tomatoes (N=10 for all treatments). Bacterial wilt disease index was measured every second day for 2 weeks after pathogen inoculation based on Roberts *et al*., (1988) on a scale, where 1-4: 0=no leaves wilted, 1=25% of leaves wilted, 2=26-50% of leaves wilted, 3=51-75% of leaves wilted, 4=76-100% of leaves wilted.

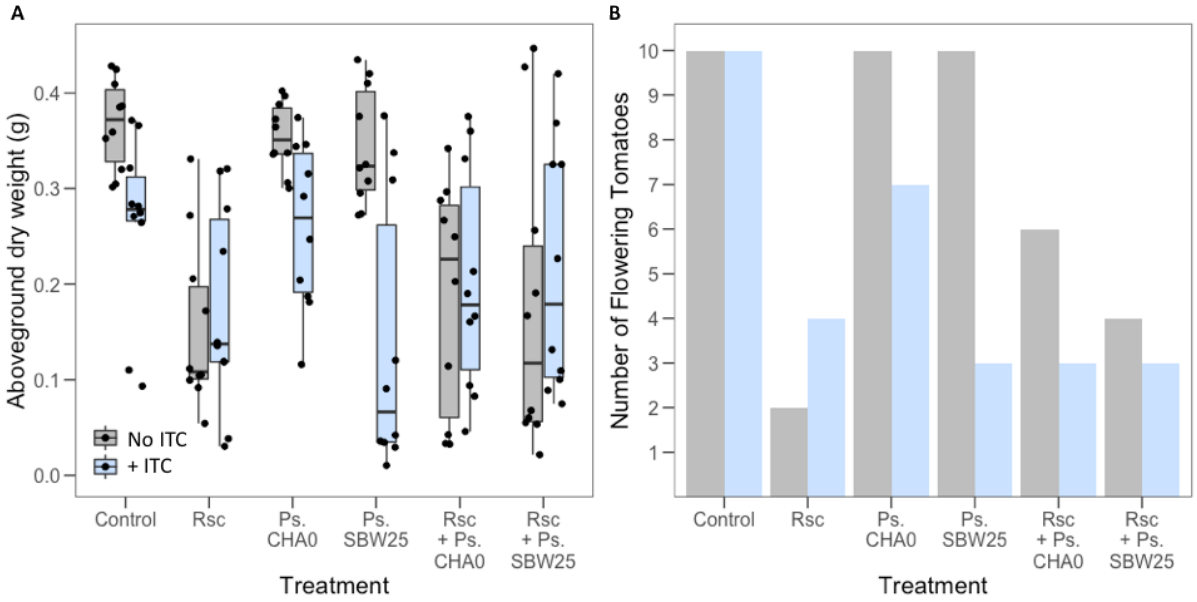


Figure 3.4.3. Differences in tomato aboveground dry weight and number of flowering plants when exposed to *R. solanacearum* and plant growth-promoting *Pseudomonas* species in the presence and absence of ITC. Panel (A) shows tomato plant dry weight when grown in sterile soil (control) or in the presence of *R. solanacearum*, *P. protegens* CHA0 or *P. fluorescens* SBW25 mono- and co-cultures in the presence (blue) and absence (grey) of ITC (N=10 for all treatments). Boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates. In panel (B), bars show the number of plants that developed flowers in different treatments in the presence (blue) and absence (grey) of ITC (N=10).

In line with disease symptom data, ITC exposure reduced *R. solanacearum* abundances in rhizosphere monoculture ($F_{1, 18}$= 27.76, p< 0.001; Fig. 3.4.4A). Moreover, even though the presence of *Pseudomonas* species did not protect plants from wilting, they had strong suppressive effects on *R. solanacearum* growth ($F_{2, 57}$= 111.1, p< 0.001; Fig. 3.4.4A). ITC reduced *R. solanacearum* abundances only in *P. protegens* CHA0 co-cultures (ITC × *Pseudomonas* strain: $F_{1, 36}$= 6.39, p< 0.05; CHA0: t= 3.12, *df*= 13.01, p< 0.001; SBW25: t= -0.122, *df*= 15.27, p> 0.05; Fig. 3.4.4A).

With *Pseudomonas,* a significant, inhibitory effect of ITC exposure was observed only with *P. protegens* CHA0 (t= 2.45, df= 13.25, p< 0.05; Fig. 4B). While co-culturing *with R. solanacearum* significantly reduced both *Pseudomonas* species abundances (CHA0: t= 8.41, df= 27.77, p< 0.001; SBW25: t= 13.97, df= 23.50, p< 0.001; Fig. 4B), ITC effects were non-significant in these co-cultures (CHA0: t= -0.314, df= 14.25, p> 0.05; SBW25: t= 3.20, df= 17.82, p> 0.05; Fig. 3.4.4B).

In summary, our *in vivo* results show that ITC application reduced bacterial wilt disease severity in tomato, while *Pseudomonas* species presence did not alleviate disease symptoms. Moreover, ITC exposure reduced tomato dry weight and inhibited flowering in bacterial co-cultures, indicative of negative biofumigant effects on plants.

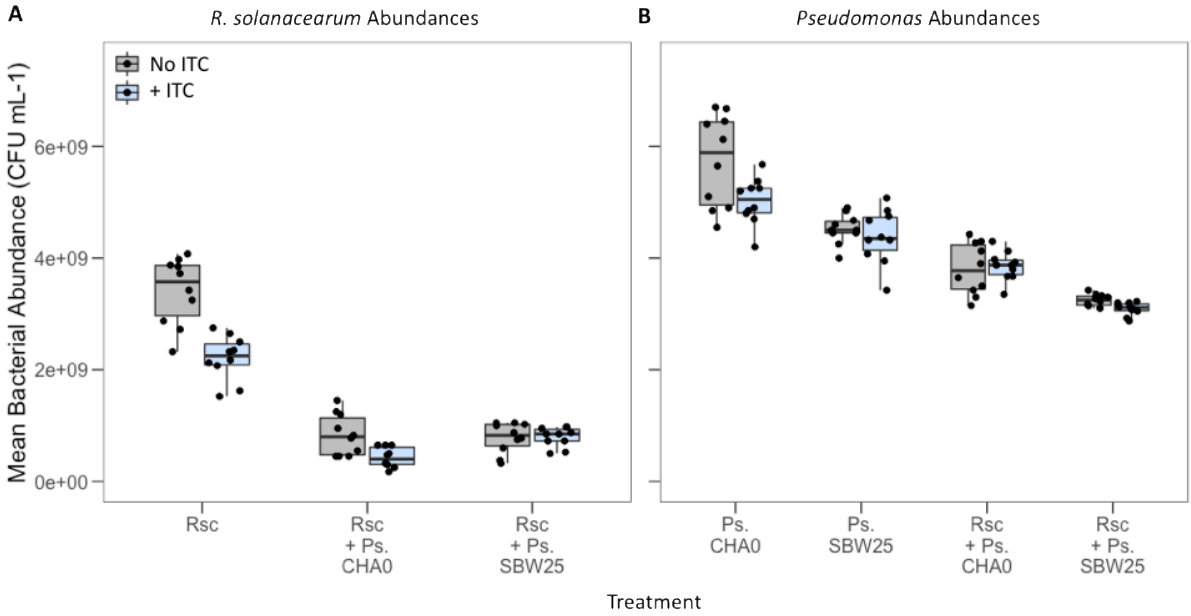


Figure 3.4.4. Effects of ITC on pathogenic *R. solanacearum* and plant growth-promoting *Pseudomonas* bacterial densities in mono- and co-cultures in the tomato rhizosphere. Panels show the densities (CFU mL^{-1}) of plant-pathogenic *R. solanacearum* (A) and plant growth-promoting *P. protegens* CHA0 and *P. fluorescens* SBW25 species (B) in mono- and co-culture treatments in the absence (grey) and presence of ITC (blue) at the end of the tomato rhizosphere mesocosm experiment. The boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=10).

(c) Quantifying *R. solanacearum* evolutionary responses to ITC and *Pseudomonas* during the tomato growth experiment

(i) ITC tolerance

To test for potential ITC tolerance evolution in the rhizosphere, evolved *R. solanacearum* isolates were re-exposed to ITC in a separate liquid microcosm experiment. Previous ITC exposure during the tomato growth experiment had no significant effect on the growth of

evolved *R. solanacearum* isolates ($F_{2, 31}$= 0.035, p= 0.966; Fig. 3.4.5) and isolates derived from any experimental treatments were inhibited to the same extent as their ancestral strain. However, we observed increased susceptibility to ITC in *R. solanacearum* isolates that had evolved in co-culture with *P. fluorescens* SBW25 in the absence of ITC ($F_{3, 30}$= 9.20; p< 0.001; Fig. 3.4.5B).

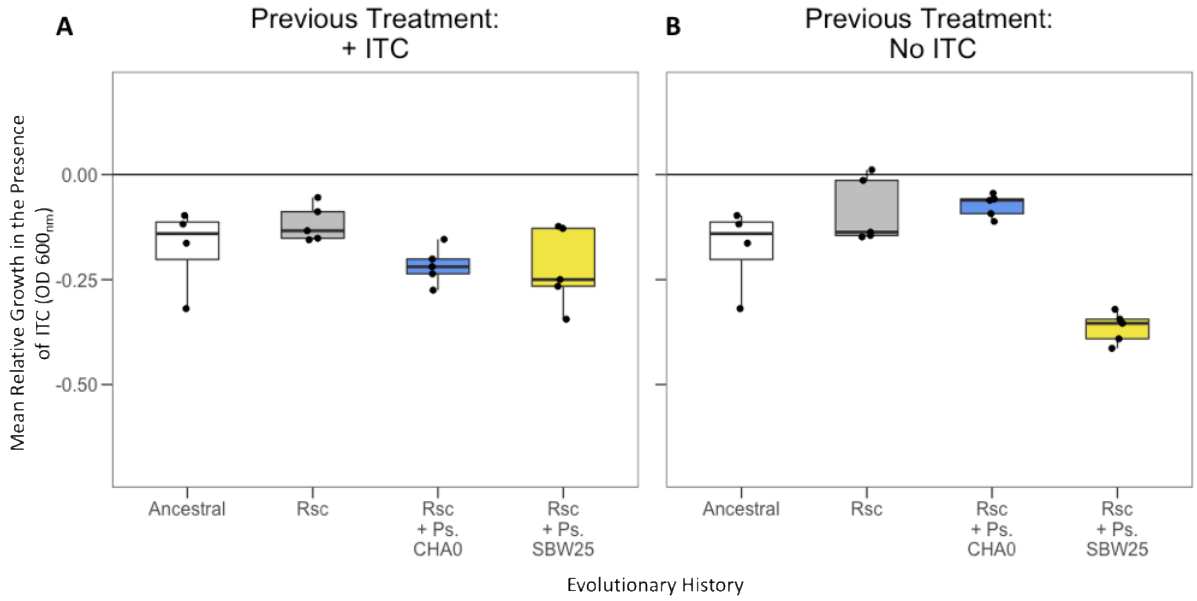


Figure 3.4.5. *R. solanacearum* tolerance to ITC measured at the end of the tomato mesocosm experiment. ITC tolerance was measured as the relative growth of bacteria in the presence versus absence of ITC for the ancestral clone (white) and evolved clones from the ITC exposed (A) and non-ITC exposed control (B) *R. solanacearum* isolates. The black horizontal line indicates no effect of ITC on bacterial growth, while observations below the line denote for negative effects of ITC on *R. solanacearum* growth. The boxplots show the minimum, maximum, interquartile range and the median (black line). Individual data points show bacterial densities for each biological replicate (N=4 for the ancestral and N=5 for all evolved clones).

(ii) Adaptation to Pseudomonas species

We also determined if *R. solanacearum* strains adapted to the presence of *Pseudomonas* by growing ancestral and evolved *R. solanacearum* isolates in the supernatants of ancestral *P. protegens* CHA0 and *P. fluorescens* SBW25 species (supernatants included all secreted metabolites; *R. solanacearum* own supernatant was used as the negative control). Despite a significant effect of *R. solanacearum* evolutionary history on relative growth in both *Pseudomonas* supernatants (CHA0: $F_{3, 30}$= 8.91, p< 0.001; SBW25: $F_{3, 30}$= 8.247, p< 0.001; Fig. 3.4.6), previous ITC exposure had no effect on the relative growth of *R. solanacearum* in either *Pseudomonas* supernatant (CHA0: $F_{1, 29}$= 0.007, p> 0.05; SBW25: $F_{1, 22}$= 1.10, p= 0.307; Fig. 3.4.6). However, *R. solanacearum* isolates that had evolved in the absence of ITC as a monoculture became more sensitive to the *P. protegens* CHA0 supernatant ($F_{3, 27}$= 6.50, p< 0.01; Tukey: p< 0.05; Fig. 3.4.6A). Additionally, the supernatant of *P. fluorescens* SBW25 had a positive effect on *R. solanacearum* growth overall ($F_{1, 59}$= 116.7, p< 0.001; Fig. 3.4.6B). Together, these results suggest that *R. solanacearum* did not show consistent signs of adaptation to either *Pseudomonas* species.

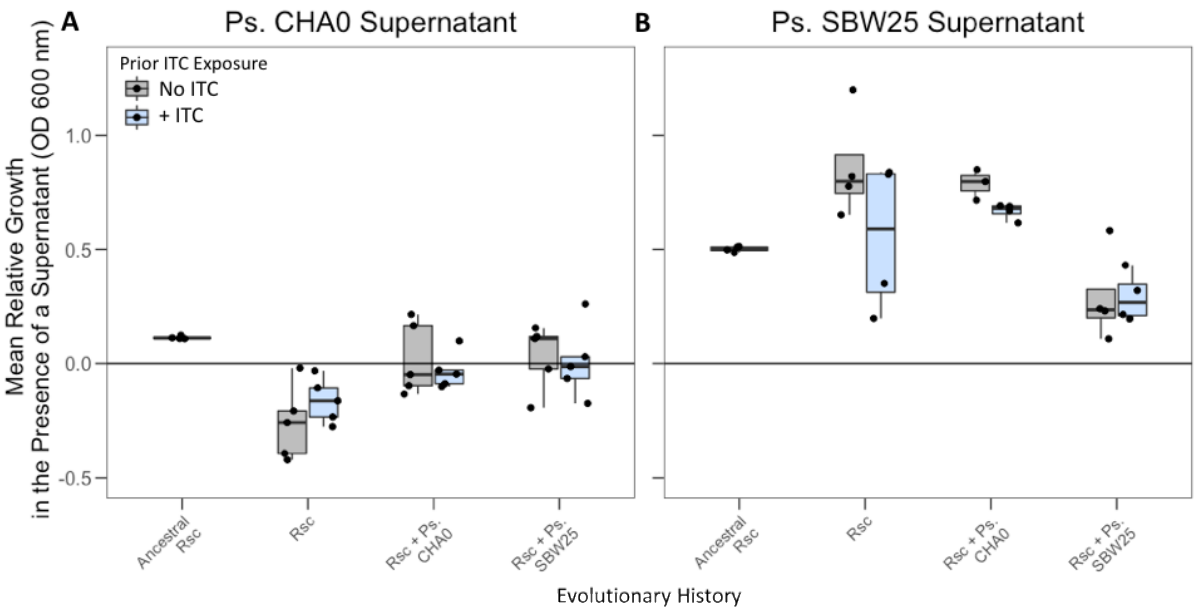


Figure 3.4.6. *R. solanacearum* adaptation to *P. protegens* CHA0 and *P. fluorescens* SBW25 supernatants measured at the end of the tomato mesocosm experiment. Adaptation was measured as the relative growth of *R. solanacearum* in the presence versus absence of *P. protegens* CHA0 (A; Ps. CHA0) and *P. fluorescens* SBW25 (B; Ps. SBW25) supernatants for the ancestral clone (white) and evolved clones from ITC-exposed (grey) and non-ITC exposed control (blue) *R. solanacearum* isolates. The black horizontal line indicates no effect of supernatant on *R. solanacearum* growth relative to control (own supernatant), while observations below and above the line denote for negative and positive effects of ITC on *R. solanacearum* growth, respectively. The boxplots show the minimum, maximum, interquartile range and the median (black line). Individual data points show bacterial densities for each biological replicate clone (N=4 for ancestral and N=5 for all evolved clones).

1.5. Discussion

Here we studied the effects of ITC exposure on pathogenic *R. solanacearum* and plant growth-promoting *P. protegens* CHA0 and *P. fluorescens* SBW25 soil bacteria in mono- and co-culture communities in three model systems. Firstly, we found that ITC was significantly more suppressive to *R. solanacearum* than *Pseudomonas* species in *in vitro* liquid and soil microcosms and *in vivo* mesocosms. Secondly, *Pseudomonas* had antagonistic effects on *R. solanacearum* when grown in co-culture. Whilst ITC was efficient at reducing bacterial wilt symptoms, it also reduced tomato aboveground dry weight. Furthermore, whilst *Pseudomonas* retained its antagonistic effects on *R. solanacearum* densities in tomato mesocosms, this did not translate to a reduction in tomato bacterial wilt disease symptom severity or elevated tomato dry weight. Finally, no clear evidence for pathogen ITC tolerance evolution or adaptation to *Pseudomonas* secretions (supernatant) was observed at the end of the tomato rhizosphere mesocosm experiment.

We observed a consistent reduction in *R. solanacearum* abundances when exposed to ITC in all experimental systems. This finding is in line with previous studies that document the sensitivity of *R. solanacearum* to ITC directly in *in vitro* assays (Smith and Kirkegaard, 2002), as well as pot and field trials (Arthy *et al.*, 2005). Whilst ITC was also slightly inhibitory to *Pseudomonas* species in some experiments, ITC was consistently more inhibitory to the *R. solanacearum* pathogen. This contradicts previous studies that demonstrate the broad- spectrum biocidal effects of ITC on microorganisms (Mojtahedi, 1993; Motisi *et al.*, 2009), while our results suggest that the bacterial species tested here differed in their innate susceptibility to ITC exposure. Smith and Kirkegaard (2002) have established that *P. fluorescens* can continue to grow at ITC concentrations up to 3.34 mM,

whilst *R. solanacearum* sensitivity to ITC has been documented at 2 μmol g^{-1} soil (Olivier *et al.*, 2006). Moreover, *R. solanacearum* was proven to be the most sensitive to ITC suppression of 41 tested bacterial isolates in an *in vitro* study, including several species of *Pseudomonas* (Smith and Kirkegaard, 2002). *Pseudomonas* tolerance to ITCs released by the model plant *Arabidopsis thaliana* has been attributed to *sax* genes (Fan *et al.*, 2011). These genes encode putative multi-drug resistance (MDR) efflux pumps which enable exudation of antimicrobials from the cell (Fan *et al.*, 2011) and may explain the higher innate tolerance of *Pseudomonas* to ITCs. The relatively higher sensitivity of *R. solanacearum* to ITC may thus indicate that biofumigation could be a feasible strategy for the control of bacterial wilt disease.

In liquid microcosms and tomato rhizosphere mesocosms, *R. solanacearum* growth was reduced in *Pseudomonas* co-cultures. These effects were particularly evident in co-culture with *P. fluorescens* SBW25 compared to *P. protegens* CHA0. We also observed enhanced inhibitory effects of ITC on *R. solanacearum* when grown in co-culture. This is likely driven by the relatively higher *Pseudomonas* ITC tolerance combined with resource competition (Wei, Yang, Friman, *et al.*, 2015a), as well as direct antagonistic effects through antibiotic and siderophore production (Duffy and Défago, 1999; De Bruijn *et al.*, 2007; Moon *et al.*, 2008), that magnified the suppressive effects of ITCs on *R. solanacearum*. We also observed that *R. solanacearum* reduced *Pseudomonas* densities in co-cultures across all three study systems, while the exact mechanism for this remains unclear. This finding aligns well with previous reports on the high invasiveness of *R. solanacearum* in microbiomes (Schell, 2003; Wei *et al.*, 2018) and might be due to its ability to compete with known biocontrol strains in the rhizosphere for example via use of plant exudates and other resources (Vasse, Frey and Trigalet, 1995; Tans-Kersten, Huang and Allen, 2001; Yao and Allen, 2006).

ITC suppressed bacterial wilt disease symptoms in tomato via *R. solanacearum* growth suppression. Whilst we also observed the reduction in *R. solanacearum* abundances in co-culture with *Pseudomonas* in *in vivo* tomato rhizosphere mesocosms, this did not correspond with a reduction in bacterial wilt disease symptoms. Previous studies have also found that bacterial wilt disease symptoms do not always correlate with pathogen densities in the rhizosphere (Angot *et al.*, 2006; Guidot *et al.*, 2014). The mismatch may be because *R. solanacearum* densities were determined from soil samples instead of from the plant stem, where *R. solanacearum* densities are normally much higher when wilting symptoms are evident (Peeters *et al.*, 2013). It is also possible that the presence of *Pseudomonas* species could have affected *R. solanacearum* virulence gene expression (Peyraud *et al.*, 2016; Khokhani *et al.*, 2018) and the likelihood of successful colonisation of the plant xylem. Thus, *Pseudomonas* antagonism against *R. solanacearum* observed in the lab may not directly translate to high antagonism in the rhizosphere.

Of particular concern for field biofumigation, we found that ITC application *in vivo* reduced tomato dry weight. This may indicate that biofumigation could limit the reproductive output of plants and hence reduce crop yields. In support of our finding, the allelopathic effects of allyl-ITC have previously been demonstrated on the growth of *Medicago sativa* plant (Choesin and Boerner, 1991). The herbicidal effects of ITCs on plants could occur by reacting with sulfur-containing groups of proteins, as occurs during ITC exposure in microorganisms (Brown and Morra, 1995). It is also possible that the negative effects of ITC on tomato were partly due to the manual root wounding treatment, which may have led to phytotoxic effects and disrupted the growth of the tomato plant itself. Our result contradicts previous studies that have correlated ITC exposure with increased crop yields (Angus *et al.*, 1994; Triky-Dotan *et al.*, 2007), and it is possible that biofumigation

might not have such detrimental phytotoxic effects when ITCs are incorporated into soil as *Brassica* plant material instead of as a synthetic compound. Moreover, plant dry weight is only a crude measure of crop yield instead of total biomass and number of tomato fruits. Interestingly, we observed a reduction in the number of flowering tomatoes when inoculated with any of the two *Pseudomonas* species alongside ITC exposure. Potentially, ITC effects on *Pseudomonas* species could have induced stress reactions and production of phytotoxic chemicals which inhibited flowering. In support of this, one study found that *P. protegens* CHA0 was initially antagonistic to *A. thaliana* due to the production of a variety of compounds, before evolving into mutualists (Li *et al.*, 2020). In the case of *P. fluorescens* SBW25, siderophore production by the bacteria could have exacerbated iron competition with the tomato, though this has generally been found to have the opposite effect (Nagata, Oobo and Aozasa, 2013). More research is thus needed to understand ITC effects on tomato and *Pseudomonas.*

The micro- and mesocosm experiments presented here provide pivotal information on the effects of ITC release on pathogenic and plant growth-promoting bacteria in the absence of confounding external factors that are difficult to control in the field. While ITC effects on *R. solanacearum* were consistent across all employed systems, we also found some inconsistencies. For example, whilst ITC had a relatively larger effect on *R. solanacearum* abundances in *in vitro* soil microcosms, *Pseudomonas* presence was more important than ITC in reducing *R. solanacearum* abundance in the tomato rhizosphere. Despite this, even the relatively simple, liquid media *in vitro* experiments had some predictive power over what might happen in the soil and plant rhizosphere systems. Encouragingly, we did not observe ITC tolerance evolution in *R. solanacearum* when re-introduced following previous exposure, implying the disease suppressive effects of

biofumigation could be long-lasting. This contradicts our previous work which identifies *R. solanacearum* ITC tolerance after multiple exposures (Alderley, Greenrod and Friman, 2021). The lack of ITC tolerance evolution in the present study may have been because ITC was added only once at the beginning of the experiment, meaning the selective pressure for tolerance was relatively weak. Hence, ITC release at a single instance during biofumigation might be sufficient to achieve pathogen suppression without concomitant evolution of ITC tolerance.

To conclude, our work indicates that allyl-ITC is a promising candidate ITC against *R. solanacearum* and that biofumigation might have relatively higher suppression of pathogenic compared to plant growth-promoting bacteria. Further work is however needed to test the effects of biofumigation in complex, natural microbiomes using real biofumigation approaches instead of addition of synthetic bioactive compounds.

Chapter 4. Collateral effects of model biofumigation on soil microbiome composition and diversity

4.1. Abstract

Biofumigation, which involves the integration of *Brassica* tissues into soils to release isothiocyanates (ITCs) could offer an environmentally sustainable alternative to agrochemicals to protect crops against diseases. While the effects of ITCs against pathogenic microbes are well established, their effects towards non-target microbiota are less well understood. Here we used a short-term model biofumigation experiment to evaluate the efficacy of allyl-ITC against *Ralstonia solanacearum* plant bacterial pathogen and the surrounding microbiome originating from kale and potato rhizosphere soil microcosms. We found that ITC was efficient at suppressing *R. solanacearum* growth. However, ITC also reduced the soil microbiome richness and eliminated certain rare taxa. Additionally, ITC changed microbiome composition by enriching Actinobacteria but reducing the relative abundances of many phyla, including Firmicutes, Thaumarchaeota and Verrucomicrobia. ITC exposure also reduced the connectedness of bacterial co-occurrence networks, while the pathogen presence or the rhizosphere microbiome origin (kale or potato) had relatively small effects on microbiomes overall. Our results suggest that while ITC can successfully inhibit *R. solanacearum* growth, it can also have collateral effects on the diversity and composition of non-target soil microbiota. Further research is hence required to weigh the benefits of biofumigation against the potential loss of soil biodiversity and ecosystem functioning.

4.2. Introduction

Annual crop losses due to disease account for a third of global food production (Oerke and Dehne, 2004; Savary *et al.*, 2019). Pathogen control methods, such as application of the methyl bromide fumigant, often act as a broad-spectrum biocide, resulting in the loss of beneficial bacterial species including plant growth-promoting rhizobacteria (PGPR) (Davis *et al.*, 1996; Imfeld and Vuilleumier, 2012; Toyota *et al.*, 2012), as well as having ozone-depleting effects (Mellouki *et al.*, 1992). As a result, short-term benefits of elimination of the pathogen may lead to reduced soil biodiversity and hence, loss of soil fertility.

Biofumigation has been suggested as an environmentally friendly alternative to broad-spectrum agrochemicals. The efficacy of biofumigation is based on pathogen-suppressing plant allelochemicals secreted for example by *Brassica* crops, including Indian mustard (*Brassica juncea*), rocket (*Eruca sativa*) and oilseed radish (*Raphanus sativus*), and is commonly integrated with farming via crop rotation, seed meal or green manure crops (Kirkegaard and Sarwar, 1998). *Brassica* contain the separately sequestered compounds glucosinolates (GSLs) and myrosinase enzymes in their tissues (Brown and Morra, 1995). Upon cellular disruption, these compounds engage in a hydrolysis reaction, releasing volatile biocidal chemicals, principally isothiocyanates (ITCs) (Matthiessen and Kirkegaard, 2006), and in lower concentrations thiocyanates, nitriles and oxazolidine-thiones (Borek *et al.*, 1995). ITCs differ in their chemical structure which influences volatility and toxicity (Hanschen *et al.*, 2015) and have very short half-lives in the soil (Warton, Matthiessen and Shackleton, 2003b; Gimsing and Kirkegaard, 2009a), often reaching undetectable levels within 24 hours of *Brassica* tissue incorporation (Brown *et al.*, 1991), although persistence

of ITCs up to 45 days has also been observed (Poulsen *et al.*, 2008; Gimsing and Kirkegaard, 2009a).

The suppressive effects of biofumigation against pathogens have been well-established (Arthy *et al.*, 2005; Gouws-Meyer, McLeod and Mazzola, 2020). In bacteria, ITCs potentially disrupt disulphide bonds and damage the tertiary structure of enzymes (Luciano and Holley, 2009), or harm the integrity of the outer cell membrane, leading to leakage of cell metabolites and ultimately cell death (Lin, Preston and Wei, 2000; Sofrata *et al.*, 2011). As these potential modes of action are not bacterial species-specific, biofumigation could also affect the surrounding non-target soil microbiota. In support of this, application of ITCs has been shown to influence the soil microbial community composition (Rumberger and Marschner, 2003; Bressan *et al.*, 2009; Hollister *et al.*, 2013a; Reardon, Strauss and Mazzola, 2013) by favouring *Brassica*-compatible microbes (Bressan *et al.*, 2009). Furthermore, ITC effects vary between microbial species and even between isolates of the same species (Mari *et al.*, 1993; Smith and Kirkegaard, 2002). At the level of microbial communities, fungi tend to be more susceptible to biofumigants compared to bacteria (Hollister *et al.*, 2013a; Reardon, Strauss and Mazzola, 2013; Zhu *et al.*, 2020). Moreover, certain groups of microorganisms that are known for their antibiotic activity and pathogen suppression, including *Trichoderma* (Weerakoon *et al.*, 2012), *Pseudomonas* (Mazzola, Granatstein, *et al.*, 2001; Hollister *et al.*, 2013a), *Streptomyces* (Cohen, Yamasaki and Mazzola, 2005; Cohen and Mazzola, 2006a; Mazzola *et al.*, 2007b; Hollister *et al.*, 2013a), *Bacillus* (Hollister *et al.*, 2013a) and actinomycetes (Mazzola, Granatstein, *et al.*, 2001), have been shown to be particularly resilient to ITCs (Larkin and Honeycutt, 2006; Friberg *et al.*, 2009a). As a result, these innately tolerant microorganisms could benefit from the killing of their ITC-sensitive counterparts, increasing in abundance during biofumigation due to the elimination of their

competitors (Macalady, Fuller and Scow, 1998; Van Bruggen and Semenov, 2000b; Smith and Kirkegaard, 2002; Friberg *et al.*, 2009a). It is also possible that ITCs could have suppressive effects on plant growth-promoting bacteria (Bending and Lincoln, 1999; Rumberger and Marschner, 2003; Ibekwe *et al*., 2004), such as biocontrol species (Henderson *et al.*, 2009; Ramirez *et al.*, 2009), and earthworms (Fouché, Maboeta and Claassens, 2016), which could lead to dysbiosis of soil microbiomes (Ramirez *et al.*, 2009). Biofumigation could thus have negative consequences for ecosystem functioning, including carbon cycling and decomposition of organic residues and pollutants (Troncoso-Rojas *et al.*, 2009), and cascading downstream effects on the homeostatic balance of microbial community composition, potentially influencing microbiome-mediated host disease resistance (Hacquard *et al.*, 2015; Shin, Whon and Bae, 2015). Despite the potential collateral damage to soil biodiversity, systematic studies testing the effects of biofumigation on wider microbial communities are scarce.

Biofumigation is most commonly integrated as part of pre-existing crop rotation systems with multiple different crops (Sarwar *et al.*, 1998). ITC effects on microbiota are thus likely to differ depending on the rotated crops (Mazzola, Granatstein, *et al.*, 2001; Cohen and Mazzola, 2006a; Mazzola *et al.*, 2007b; Yulianti, Sivasithamparam and Turner, 2007; Friberg *et al.*, 2009a; Mazzola, Reardon and Brown, 2012). Different plant genotypes secrete a unique profile of root exudates (Paterson *et al.*, 2007), which can determine the recruitment and composition of their rhizosphere microbial community (Costa *et al.*, 2006), and the host's susceptibility to pathogen invasion (Hein *et al.*, 2009). For example, rotation with ITC-containing rapeseed (*Brassica napus*) has been shown to cause different changes to microbial community composition than rotation with non-*Brassica* plants, enabling improved control of *Rhizoctonia* disease in potato (Larkin and Honeycutt, 2006). Moreover,

intercropping potato with cabbage (an ITC-containing *Brassica* plant) has been shown to decrease the density of the *Ralstonia solanacearum* bacterial plant pathogen by increasing the abundance of fluorescent pseudomonads, *Bacillus* and *Serratia* that are recruited by potato (Messiha *et al.*, 2019). Hence, biofumigation effects on rhizosphere microbiomes may be dependent on the specific plants used in crop rotation.

To identify the effects of ITC on microbial communities, we developed a model soil microcosm system where we tested the growth-inhibiting effects of allyl-ITC on *R. solanacearum* plant pathogenic bacterium growth in the rhizosphere microbiota of two crops, kale and potato. *R. solanacearum* is the causal agent of bacterial wilt and potato brown rot diseases and has a global distribution (Yabuuchi *et al.*, 1995; Elphinstone, 2005) and no effective control methods exist against this pathogen (Saddler, 2005). The two chosen plants, potato (*Solanum tuberosum*) and kale (*Brassica oleracea* var*. sabellica*), are commonly grown together as part of conventional agricultural crop rotation. These crop microbiomes were selected as kale belongs to the *Brassica* genera and likely has a similar rhizosphere microbiota to other *Brassica* biofumigant plants exposed to ITC during root growth (Schreiner and Koide, 1993). In contrast, potato is a natural host of the *R. solanacearum* pathogen and is likely to have a different microbiome to kale which is not naturally exposed to ITCs as these compounds are not produced by potato. Allyl-ITC was used to model biofumigation effects as it is the predominant antimicrobial allelochemical released during biofumigation with a common biofumigant crop, Indian mustard (*Brassica juncea*; Kirkegaard and Sarwar, 1998; Bending and Lincoln, 1999; Kirkegaard and Matthiessen, 2005; Mazzola, Hewavitharana and Strauss, 2015). We hypothesised that ITC effects on microbiota might be less pronounced with kale as its associated bacteria have experienced ITCs throughout their evolutionary history with this plant. In contrast, potato

microbiota could be more sensitive to ITCs as these compounds are not naturally released by this crop. We also expected that ITC could favour taxa that are potential antibiotic producers and hence enable cross-tolerance to ITC. To study this, we used natural microbiota derived from the rhizosphere of kale and potato at the end of harvest and exposed them to ITC and *R. solanacearum* in soil microcosms for 4 days. We used 16S rRNA gene sequencing to determine changes in the diversity and taxonomic composition of bacterial microbiota and quantified ITC effects on pathogen density. We found that *R. solanacearum* was equally sensitive to ITC in both rhizosphere microbiomes. Overall, ITC exposure had inhibitory effects on rare taxa and significantly changed the microbial community structure of both potato and kale microbiota by mainly enriching Actinobacteria and suppressing Firmicutes, Thaumarchaeota and Verrucomicrobia. Our results suggest that ITC release during biofumigation could cause large shifts in soil microbial community structure and drive certain rare species to extinction, resulting in potential negative effects on soil ecosystem functioning.

4.3. Materials and Methods

(a) Farming site and rhizosphere soil sampling

Potato and kale soil samples were collected in September 2019 from the Barworth Agriculture Ltd. field site located in Sleaford, Lincolnshire, UK. This site has no previous record of *R. solanacearum* infection. A 30 x 6 m plot was dedicated to sampling and was split by randomised block design into 6 x 2 m blocks per crop. Potato and kale crops were treated according to Standard Farming Practice, which included the application of fertiliser (100 kg nitrogen, 50 kg phosphorus and 50 kg potassium), irrigation as required, the application of organic insecticide (Pyrethrum 5EC) on two occasions, and mechanical weed

control on five occasions during the six-month sampling period. Seed potatoes were drilled and sown into the soil and kale was planted as plug plants. Prior to the establishment of the plot in April, the field was planted with spring barley. Soils at the site were typically sandy loam. Plots were sampled by taking a soil core from each of twenty sampling locations from each replicate plot (N=6) at the point of harvest. Cores were pooled into a single sample and soil washes were used to homogenise samples by mixing 10 g +/- 0.1 of soil from each replicate plot with 20 ml ddH_20. The solution was then vortexed thoroughly and allowed to settle for 30 minutes before 1 ml of soil wash from each sample was frozen at -80 °C in 30% glycerol. In the end, we had 6 independent samples from potato and kale crops at the point of harvest, which were used in further experiments in the lab.

(b) Pathogen strain and culture media

We used a virulent *R. solanacearum* strain (21415687) as the pathogenic bacterium in our experiment (phylotype II sequevar 1). The strain was originally isolated from the river Loddon in the UK (John Elphinstone, Fera Science, 2014), and is a representative of the endemic UK pathogen population which can inhabit Woody Nightshade secondary hosts leading to contamination of river water (Elphinstone, Stanford and Stead, 1998). The strain was cultured in CPG broth (1 g casamino acids, 10 g peptone and 5 g glucose per litre ddH_2O) for 48 hours at 28 °C with shaking (250 rpm) to create cryostocks (20% w/v glycerol) that were preserved at -80 °C.

(c) Soil microcosm model biofumigation experiment

To conduct a model biofumigation experiment, we used a soil system where *R. solanacearum* was inoculated in kale and potato rhizosphere communities and treated with synthetic ITC or sterile M9 buffer (negative control). Soil microcosms were set up using loosely lidded Universal bottles (30 ml) based on protocols described earlier (Hall *et al.*,

2016) to enable a near-natural environment with spatial structure and low-resource availability. To this end, 10 g of twice-autoclave sterilised John Innes #2 potting soil with 25% w/v water content were added to sterile Universal bottles and allowed to equilibrate for 1 hour before use. Bacterial densities of kale and potato microbiome liquid cultures were normalised to an optical density reading of 0.5 (OD 600 nm; Tecan Sunrise) and centrifuged (14,000 rpm) to form pellets. The supernatant was then removed, and the pellet was resuspended in 1 ml M9 buffer (128 g sodium phosphate dibasic, 30 g monopotassium phosphate, 5 g sodium chloride, 10 g ammonium chloride per litre ddH_2O; recipe for 10x stock concentration). The *R. solanacearum* inoculum was prepared similarly by normalising bacterial densities to 0.5 (OD 600 nm), equalling approximately 5 x 10^7 cells per ml and resuspended in M9 after centrifugation. The experiment was then started by inoculating 100 μl of the kale and potato rhizosphere suspensions to sterile soil microcosms, after 100 μl of *R. solanacearum* was immediately inoculated to pathogen treatments (100 μl M9 inoculated to no-pathogen treatments). Allyl-ITC was used as the model biofumigant as it is the predominant allelochemical released during biofumigation with Indian mustard (*Brassica juncea*), which is a common biofumigant crop (Kirkegaard and Sarwar, 1998; Bending and Lincoln, 1999; Kirkegaard and Matthiessen, 2005; Mazzola, Hewavitharana and Strauss, 2015). Additionally, we had already verified allyl-ITC as highly toxic to this strain of *R. solanacearum* in our previous work (Alderley, Greenrod and Friman, 2021). With all ITC-treated microcosms, allyl-ITC was mixed with sterile M9 buffer, resulting in final concentrations of 0.5 μmol g^{-1} soil. This concentration was chosen as it is relevant to concentrations achieved during field biofumigation (Borek *et al.*, 1995; Kirkegaard and Sarwar, 1998; Warton, Matthiessen and Shackleton, 2003b; Gimsing *et al.*, 2007; Rudolph *et al.*, 2015). In the case of the negative control treatment, equal volume of sterile M9 buffer

was added in the no-ITC treatment. All microcosms were then stored stationary at 28 °C for four days before the experiment was ended. The negative control treatments (only kale or potato microbiome) were replicated 3 times while other treatments were replicated 5 times (total number of microcosms=36).

All microcosms were destructively sampled after four days. Differences in total bacterial abundances between treatments were quantified using colony forming unit counts (CFU mL^{-1}). LB agar plates (lysogeny broth: 10 g tryptone, 5 g yeast, 10 g NaCl per litre of ddH_2O) were used for quantifying total bacterial abundances, while *R. solanacearum* densities were quantified using semi-selective SMSA agar plates containing specific nutrients, antibiotics and fungicide (Elphinstone, 2005). Before plating, samples were prepared by mixing 10 ml of sterile M9 buffer to each soil microcosm with 20 sterile glass beads and mixed thoroughly by vortexing to homogenise soil. Soil washes were then serially diluted and spotted onto agar plates which were incubated at 28 °C for 48 hours to determine CFUs. All soil washes were also cryopreserved at -80 °C in 30% glycerol for DNA extraction and Illumina MiSeq sequencing.

(d) DNA extraction and sample processing

Bacterial community composition of soil samples was investigated using Illumina MiSeq sequencing of 16S rRNA amplicon sequencing derived from the cryopreserved soil extracts. We also included pre-treatment samples derived from the 'at harvest' time point for both kale and potato rhizosphere samples (N=6 per crop, Total pre-treatment samples=12). DNA was purified from 0.25 g of soil wash using the Qiagen DNeasy PowerLyzer PowerSoil kit following the manufacturer's protocol. Bacterial 16S rDNA from soil extracts was PCR amplified using the oligonucleotide Illumina specific primers with Illumina adapters attached 515F-Y

(5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGYCAGCMGCCGCG GT AA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGACTACHVGGGTWTCTAAT-3') which encompasses the V4 hypervariable region (Parada, Needham and Fuhrman, 2016). PCRs were carried out using GoTaq Hot Start Polymerase (Promega Corporation). Reaction mixtures were set up according to the following protocol:

Component	Final concentration	PCR recipe for 50 μl reaction
Colourless GoTaq Flexi Buffer	1X	10 μl
$MgCl_2$ Solution, 25mM	1 mM	5 μl
dNTP	0.2 mM	1 μl
Forward primer	0.5 μM	0.5 μl
Reverse primer	0.5 μM	0.5 μl
GoTaq Hot Start Polymerase	5 U/μl	0.25 μl
Nuclease-Free Water		31.75 μl
Template DNA		1 μl

PCR amplification was then performed on a Biometra TProfessional Thermocycler (Biometra, Thermofisher), using the following PCR temperatures, times and cycles:

Temp (°C)	Time (min:s)	Cycles
95	2:00	1x
94	0:30	
53	0:45	30x

72	1:30	
72	5:00	1x

Agarose gel electrophoresis (1.5% agarose) was used to analyse the products of PCR reactions. Electrophoresis was then performed in a Bio-Rad horizontal gel electrophoresis tank, using the Bio-Rad PowerPac 300 power system (Bio-Rad) at 100 V for 55 minutes. Gels were imaged under UV illumination using a Bio-Rad ChemiDoc XRS+ imager.

Equal volumes of PCR products for each sample were pooled, cleaned (AMPure XP Beads, Beckman Coulter, USA) and indexed for sequencing (Nextera XT DNA Library Preperation Kit v2 set A, Illumina, USA). The DNA concentrations were determined using a Quant-iT dsDNA Broad-Range Assay kit (Thermo Scientific). All samples were quality checked by Bioanalyzer 2100 (Agilent, USA) before sequencing using Illumina MiSeq (2 x 300bp paired end reads) by the University of York Technology Facility.

Bioinformatics were performed using QIIME2 (Quantitative Insights into Microbial Ecology 1.8.0 (Bolyen *et al.*, 2019)). Raw sequence data were trimmed to remove PCR primer sequences using cutadapt followed by denoising with DADA2 (Callahan *et al.*, 2016). All amplicon sequence variants (ASVs) were aligned using mafft (Katoh *et al.*, 2002) and used to construct a phylogeny with fasttree (Price, Dehal and Arkin, 2010). Alpha-diversity metrics (richness, Shannon's diversity index, evenness and Chao1), beta diversity metrics (Bray-Curtis dissimilarity; Bray and Curtis, 1957) and weighted UniFrac Lozupone *et al.*, 2007) and principal coordinates analysis (PCoA) were estimated using q2-diversity after samples were rarefied (subsampled without replacement) to 60,000 sequences per sample. A total of 60,000 sequences per sample were chosen as our rarefaction depth to retain all paired samples and samples with fewer sequences were excluded from downstream analyses. However, in a subset of analyses where pre-treatment samples were also

considered, a rarefaction depth of 30,000 was used to account for differences in the number of sequences per sample. ASV taxonomy was assigned using the q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier (Bokulich *et al.*, 2018) against the SILVA 132 reference sequences at 99% similarity (Quast *et al.*, 2013).

(e) Statistical analysis

Two-way ANOVA was performed to analyse differences in CFU mL^{-1} between treatments and Tukey *post-hoc* tests were used to compare differences between subgroups ($p< 0.05$). To test for the effects of ITC exposure, microbiome origin and *R. solanacearum* presence on within sample diversity, four alpha-diversity metrics were used: Richness (total number of different ASVs), Shannon diversity (a quantitative measure of community richness, accounts for abundance and evenness of ASVs), Pielou's evenness (measures evenness of total number of different ASVs), and Chao1 (diversity based on abundance data weighing rare taxa). Significant differences between treatments were determined using Kruskal-Wallis non-parametric test. As for between sample beta diversity (community composition), principal coordinates analysis (PCoA) was used to determine the effect of different treatments (sampling time, ITC exposure, microbiome origin and *R. solanacearum* presence) on Bray-Curtis (differences in the presence, absence and abundance of ASVs) and weighted UniFrac (differences in the presence, absence, and abundance of ASVs while weighting differences in ASVs that are phylogenetically diverged) metrics. Components of variance were used to estimate the between sample vs within sample intraclass correlation coefficient for each microbiome measure. Cluster analysis was conducted using the 'stat_ellipse' function in R. To identify differentially abundant taxa, we used the LEfSe (Linear Discriminant Analysis Effect Size) approach via the Huttenhower Lab Galaxy Server (Goecks *et al.*, 2010). We tested for differentially abundant taxa at the phyla and genera

level under the different treatments (ITC exposure, microbiome origin and *R. solanacearum* presence). The LEfSe algorithm performs nonparametric statistical testing to determine whether individual taxa differed between treatments and ranks differentially abundant taxa by their linear discriminant analysis (LDA) log scores (Segata *et al.*, 2011). Differentially abundant taxa that were statistically significant based on alpha 0.05 and threshold on the logarithmic LDA score for discriminative features of at least +/- 2 were visually represented on bar plots.

To decipher the taxa that were shared by all microbiome samples, we took the genera level OTU tables produced by QIIME2 and used NetSets to produce Venn plots showing the overlap in genera between samples (Nagpal, Kuntal and Mande, 2021). We split samples by treatment variables (ITC exposure, microbiome origin and *R. solanacearum* presence). Genera present in all treatment samples were considered as the 'shared core microbiota'.

Microbial association networks were generated at the genera level based on significant Pearson correlations ($p < 0.05$), which were determined in R and compared in the presence and absence of ITC separately for the kale and potato microbiomes. Taxa classified as 'unknown microbe' or 'ambiguous microbe' by QIIME2 taxa determination were excluded from these analyses. Networks were visualised using CytoScape (Shannon *et al.*, 2003) and only significant associations unique to each treatment were visualised (associations present in one or more treatments were removed from plots to simplify the networks). The NetworkAnalyzer tool was used to calculate network topology parameters. Nodes represent genera and edges show statistically significant (Pearson correlation: $p < 0.05$) associations between nodes. Major hub nodes with many associations have a high betweenness centrality and this was used to determine node size in the network figure. Average path

length measures microbiome compactness, while the clustering coefficient describes the proportion of pairs of nodes connected to the same nodes.

To predict functional differences between microbiomes, Kyoto Encyclopaedia of Genes and Genomes (KEGG) Orthologs (KO), Enzyme Commission numbers (EC) and MetaCyc pathway abundance predictions were inferred using the PICRUSt2 (Langille *et al.*, 2013) plugin for QIIME2. Further information on the functions of differentially abundant pathways was collected using BioCyc. To compare the potential antimicrobial activity of microbiomes, we focused on genes commonly associated with antibiosis: non-ribosomal peptides (NRPs) and polyketides (Watanabe *et al.*, 2006; Ridley, Lee and Khosla, 2008). LEfSe was used to identify differentially abundant genes linked with antibiosis in the presence and absence of ITC and statistical significance was determined as described earlier (alpha 0.05 and threshold on the logarithmic LDA score for discriminative features of at least +/- 2). All statistical analyses and graphs were produced using R (R Foundation for Statistical Computing, R Studio Version (4.0.3). Packages: ggplot, tidyverse, rcompanion, Hmisc) and QIIME2 (1.8. 0).

4.4. Results

(a) *Ralstonia solanacearum* densities were reduced by ITC

To explore the effect of ITC, we quantified *R. solanacearum* densities before and after ITC application using selective plating and relative 16S rRNA sequence abundances. Based on colony counts on selective SMSA plates, ITC significantly reduced *R. solanacearum* densities ($F_{1, 24}$= 16.82, p< 0.001; Fig. 4.4.1A) and similar reduction was observed in the absence and presence of kale and potato microbiota ($F_{1, 24}$= 0.371, p> 0.05; Fig. 4.4.1). Furthermore, *R. solanacearum* densities did not differ in the kale or potato microbiomes ($F_{2, 20}$= 1.20, p=

0.32; Fig. 1A), and there were no synergistic effects of ITC exposure and microbiome presence on *R. solanacearum* density reduction (ITC*Microbiome: $F_{1, 22}$= 0.633, p> 0.05; Fig. 4.4.1A). We found similar suppressive ITC effects on relative *R. solanacearum* abundances based on 16S rRNA sequence counts ($F_{1, 18}$= 9.60, p< 0.01; Fig. 4.4.1B), and *R. solanacearum* density reduction did not differ in the potato or kale microbiomes ($F_{1, 18}$= 0.38, p> 0.05; Fig. 4.4.1B). Additionally, there were no synergistic effects of ITC exposure and the microbiota origin on *R. solanacearum* read count abundances (ITC*Microbiome origin: $F_{1, 16}$= 0.62, p> 0.05; Fig. 4.4.1B).

We also quantified CFU counts as a proxy of total cell abundances using LB agar plates, even though this is just an estimate as not all soil bacteria can grow on LB agar. We found that whilst ITC exposure had no effect on total bacterial abundance in the presence of *R. solanacearum* ($F_{1, 18}$= 0.83, p> 0.05; Fig. 4.4.1C), ITC application reduced total bacterial densities in the absence of *R. solanacearum* ($F_{1, 14}$= 5.48, p< 0.05; Fig. 4.4.1D). The presence of *R. solanacearum* pathogen had no effect on total bacterial abundance ($F_{1, 34}$= 0.78, p> 0.05; Fig. 4.4.1C-D). ITC was hence effective at reducing *R. solanacearum* densities in the presence and absence of other bacteria, while the densities of potato and kale microbiota were also negatively affected.

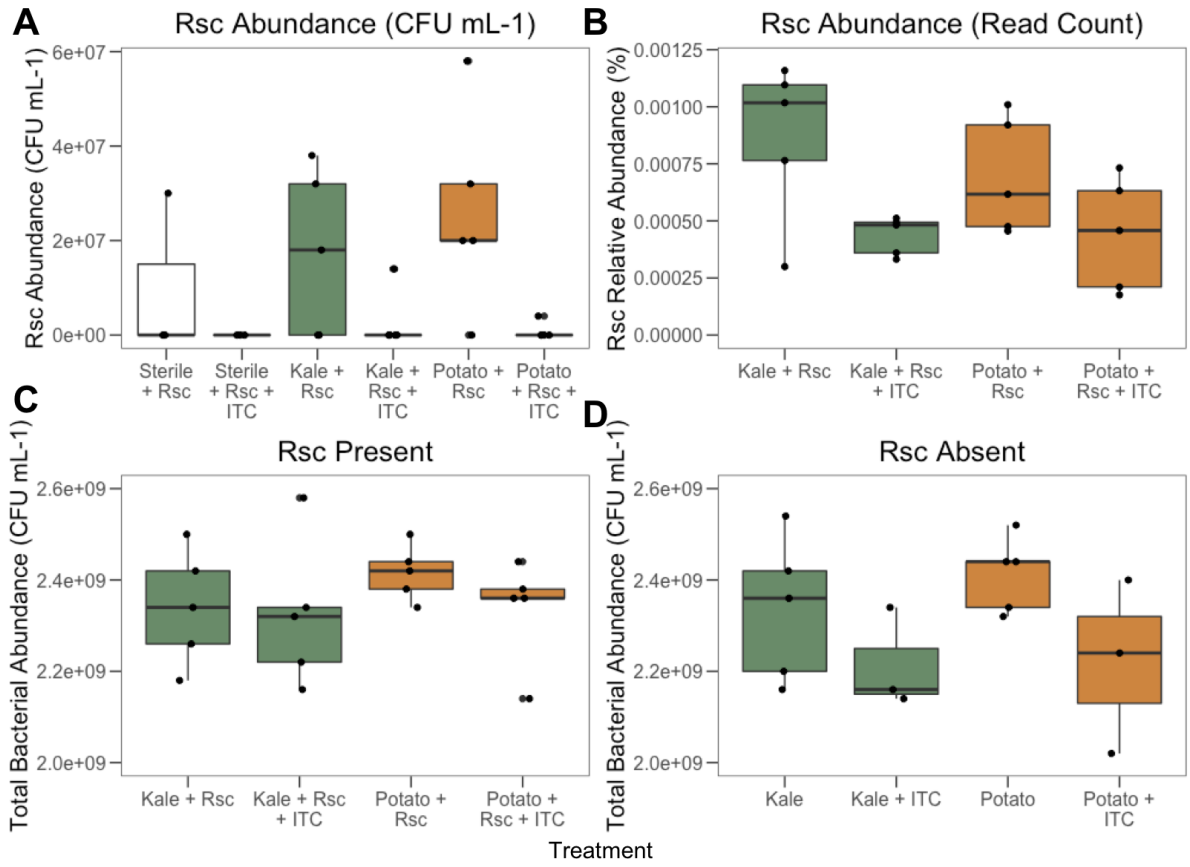


Figure 4.4.1. Effects of ITC exposure on *R. solanacearum* and kale and potato rhizosphere microbiota densities. Panels (A) and (B) show *R. solanacearum* (Rsc) densities in sterile, kale and potato microbiome soils in the absence and presence of ITCs as CFU mL^{-1} and relative read counts (%), respectively. Panels (C) and (D) show total bacterial abundances (CFU mL^{-1}) in the presence and absence of *R. solanacearum*, respectively. White boxplots denote for sterile soil, green for kale rhizosphere microbiota soil and orange for potato rhizosphere microbiota soil. All boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

(b) ITC exposure reduced overall microbial diversity and changed microbial community composition

We tested ITC effects on alpha diversity of potato and kale microbiomes using four metrics: species richness, Shannon diversity index, community evenness and Chao1 diversity index. ITC exposure reduced species richness and Chao1 diversity (highlighting the effects on rare species). However, no effect on Shannon diversity and a positive effect on evenness was observed (Richness: $H= 13.18$, $p< 0.001$; Chao1: $H= 13.64$, $p< 0.001$; Shannon diversity: $H= 0.146$, $p= 0.702$; Evenness: $H= 10.96$, $p< 0.001$, Fig. 4.4.2). None of the diversity metrics were affected by the microbiome origin or the presence of *R. solanacearum* (Richness: Microbiome origin: $H= 0.553$, $p= 0.457$; *R. solanacearum* presence: $H= 0.158$, $p> 0.05$; Evenness: Microbiome origin: $H= 0.001$, $p= 0.975$; *R. solanacearum* presence: $H= 0.123$, $p> 0.05$; Shannon diversity: Microbiome origin: $H= 0.53$, $p= 0.467$; *R. solanacearum* presence: $H= 0.259$, $p= 0.61$; Chao1: Microbiome origin: $H= 0.507$, $p= 0.476$; *R. solanacearum* presence: 0.134, $p= 0.714$; Fig. 4.4.2). Also, a significant interaction between *R. solanacearum* presence and ITC exposure on evenness was observed (ITC**R. solanacearum* presence: $F_{1, 32}= 5.33$, $p< 0.01$; Fig. 4.4.2B), and specifically, ITC exposure increased community evenness to a greater extent in the presence compared to the absence of *R. solanacearum* (Tukey: $p< 0.05$; Fig. 4.4.2B).

We next tested the effects of ITC on potato and kale microbiome community composition (beta-diversity) using two statistical approaches: non-parametric multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) using the weighted-UniFrac and Bray-Curtis beta-diversity metrics. ANOSIM tests whether distances between groups are greater than within groups whilst PERMANOVA tests whether distances differ between groups. As results were qualitatively similar between the two beta-diversity

metrics, we report here PERMANOVA results based on the Bray-Curtis metric while analyses based on ANOSIM test and Weighted-UniFrac metric are shown in Table 4.4.1.

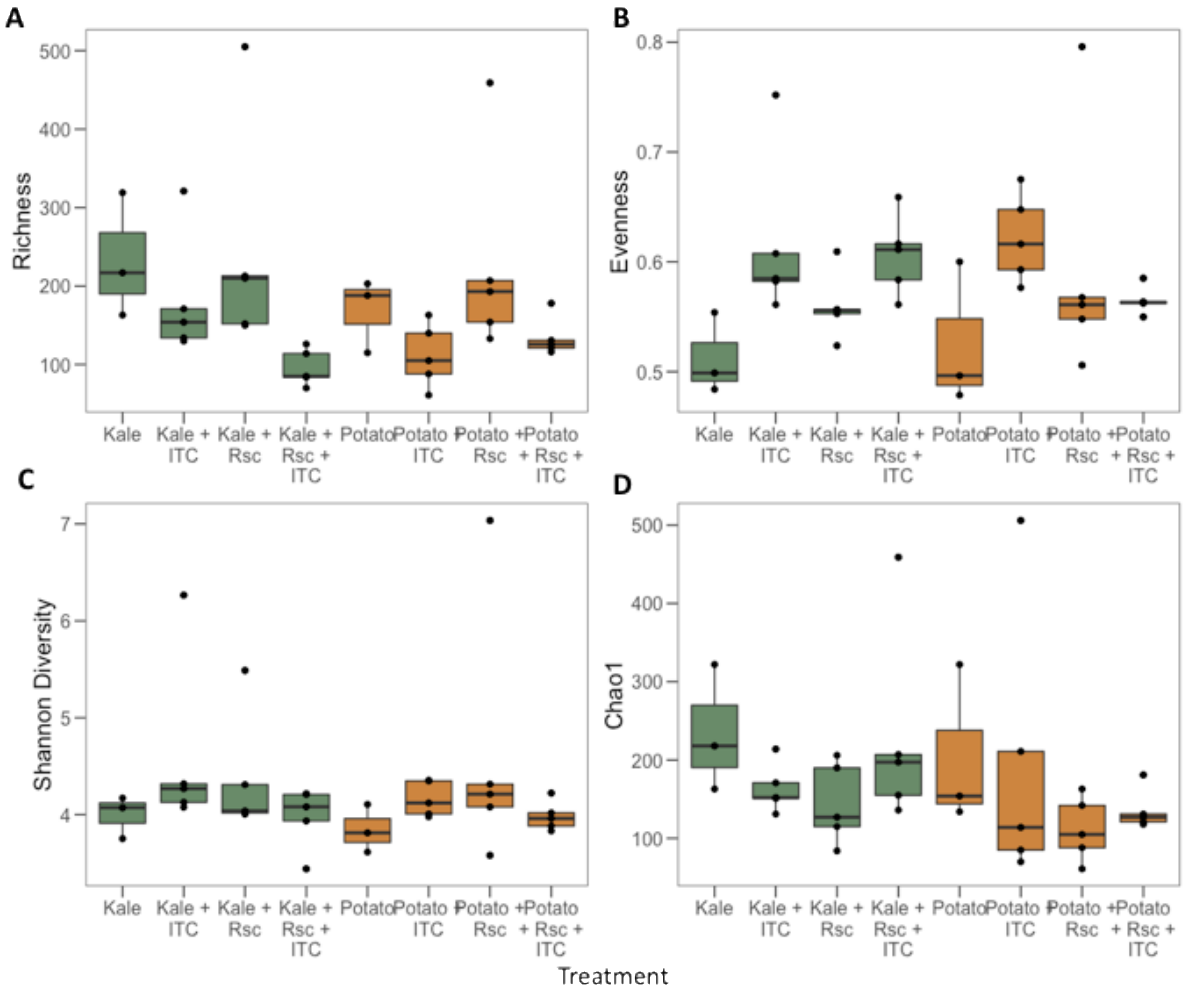


Figure 4.4.2. Effects of ITC exposure on potato and kale microbiota alpha diversity metrics in the absence and presence of the *R. solanacearum* pathogen. Panel (A) shows species richness, (B) shows community evenness, (C) shows Shannon diversity index and (D) shows Chao1 diversity index based on 16S rRNA amplicon sequence data sampled at the end of the experiment. Green boxplots denote for kale rhizosphere microbiota and orange for potato rhizosphere microbiota. Boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

Table 4.4.1. Effects of ITC exposure, microbiome origin and *R. solanacearum* presence on beta-diversity metric statistics, showing the effects on Bray-Curtis and Weighted-UniFrac beta-diversity metrics measured by ANOSIM and PERMANOVA and the test-statistic and p-value. An asterisk (*) after p-value indicates statistical significance (p< 0.05). Data show analyses of post-treatment samples only.

		Beta-diversity metric	
	Treatment	**Bray-Curtis**	**Weighted UniFrac**
ANOSIM	ITC	0.63, 0.001*	0.56, 0.001*
	Microbiome origin	0.21, 0.001*	0.12, 0.011*
	R. solanacearum presence	-0.01, 0.507	0.02, 0.255
PERMANOVA	ITC	14.95, 0.001*	10.75, 0.001*
	Microbiome origin	4.63, 0.002*	1.92, 0.096
	R. solanacearum presence	0.75, 0.591	0.62 0.682

We first compared the initial pre-treatment samples with the samples derived at the end of the biofumigation experiment and found that community composition changed drastically during the microcosm biofumigation experiment (PERMANOVA: Bray-Curtis: test statistic= 101.18, p= 0.001; Fig. 4.4.3A). Moreover, no difference in community composition of pre-treatment potato and kale microbiomes was found (PERMANOVA: Bray-Curtis: test statistic= 1.512, p= 0.196; Fig. 4.4.3A). We then compared the samples derived at the end of the model biofumigation experiment. ITC exposure (PERMANOVA: Bray-Curtis: test statistic= 14.95, p= 0.001; Appendix Figure B1A; Table 4.4.1) and microbiome origin (PERMANOVA: Bray-Curtis: test statistic= 4.63, p= 0.002; Appendix Figure B1A; Table 4.4.1) had strong effects on microbiome composition, while the effect of ITC exposure was relatively stronger than the effect of microbiome origin (Appendix Figure B1A). Moreover, the effect of microbiome origin was non-significant based on weighted-UniFrac metric (PERMANOVA: Weighted-UniFrac: test-statistic= 1.92, p= 0.096; Appendix Figure B1B; Table

4.4.1), which suggests that ITC exposure mainly affected the taxa abundances (Bray-Curtis significant), while closely related taxa were less affected (Weighted-UniFrac non-significant). In contrast, *R. solanacearum* presence had no effect on microbiota community composition (PERMANOVA: test-statistic= 0.753, p= 0.591; Appendix Figure B1A; Table 4.4.1) and this effect was similar for kale and potato microbiota (p> 0.05; Appendix Figure B1A).

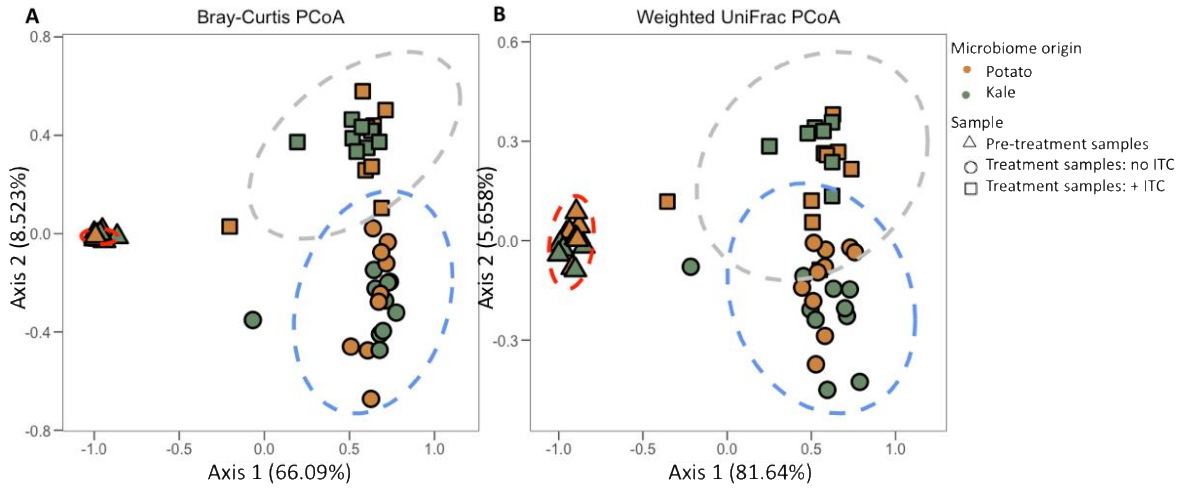


Figure 4.4.3. Effects of ITC exposure on potato and kale microbiota beta diversity metrics in the absence and presence of the *R. solanacearum* pathogen. Panel (A) shows Bray-Curtis (A) and panel (B) shows weighted UniFrac distance matrices. Confidence ellipsoids are clustered based on ITC exposure (red=pre-treatment samples, grey=no ITC and blue=ITC exposed samples). *R. solanacearum* presence had no significant effect on beta-diversity metrics and presented groups are averaged over pathogen presence treatment (N=6 for kale and potato microbiota pre-treatment samples, while in post-treatment samples N=3 for no-ITC and no *R. solanacearum* treatments, and N=5 for treatments with *R. solanacearum* and ITC).

To better understand differences and similarities between potato and kale microbiota, we quantified the core microbiota that were shared by post-treatment samples. The shared core microbiota predominantly consisted of *Pseudomonas*, Bacillales (order),

Enterobacteriaceae (family), *Rhodococcus, Stenotrophomonas, Rhodanobacter, Acinetobacter* and *Paenibacillus*, that were common for all treatments (Appendix Figure B2). We found significant overlap of 137 genera both in the presence and absence of ITC (Appendix Figure B2A). While genera including *Streptococcus*, *Dyella* and *Hyphomicrobium* were observed exclusively in the absence of ITC, *Parafilimonas*, *Agromyces* and *Domibacillus* were observed only in the presence of ITC. A total of 138 genera were present in both kale and potato microbiomes (Appendix Figure B2B), while a higher number of unique genera were observed in the potato compared to kale microbiome (38 and 27 genera, respectively). Finally, there was overlap of 137 genera both in the presence and absence of *R. solanacearum* (Appendix Figure B2C), and more unique genera were identified in the absence than in the presence of *R. solanacearum* (36 and 30 genera, respectively). Specifically, *Ochrobactrum* and *Holophaga* occurred exclusively in the absence of *R. solanacearum,* while *Terrimonas* and *Halomonas* were observed only in the presence of *R. solanacearum* (Appendix Figure B2C). Together, these results suggest that ITC effects were relatively subtle but associated with the enrichment and loss of certain taxa.

(c) ITC exposure enriched only Actinobacteria and reduced the abundance of several other phyla

To identify the effects of ITC exposure and *R. solanacearum* presence on different taxa in potato and kale microbiomes in more detail, we used LEfSe to determine differential abundance of the ten most abundant phyla. Of these, eight phyla were suppressed by ITC (Plactomycetes, Bacteroidetes, Gemmatimonadetes, Chloroflexi, Acidobacteria, Verrucomicrobia, Thaumarchaeota and Firmicutes; Fig. 4.4.4). In contrast, Actinobacteria was the only phylum that was enriched by ITC application ($F_{1, 34}$= 45.7, p< 0.001; Fig. 4.4.4), while Proteobacteria remained unaffected by ITC ($F_{1, 34}$= 0.102, p> 0.05; Fig. 4.4.4).

Moreover, some phyla were more affected by ITC depending on the rhizosphere microbiome origin. For example, Firmicutes were more sensitive to ITC in the kale rhizosphere microbiome (Microbiome*ITC: $F_{1, 32}$= 7.5, p< 0.01; Fig. 4.4.4). However, the abundances of the ten most abundant phyla were not affected by the presence of *R. solanacearum* in the kale or potato samples (p> 0.05; Fig. 4.4.4).

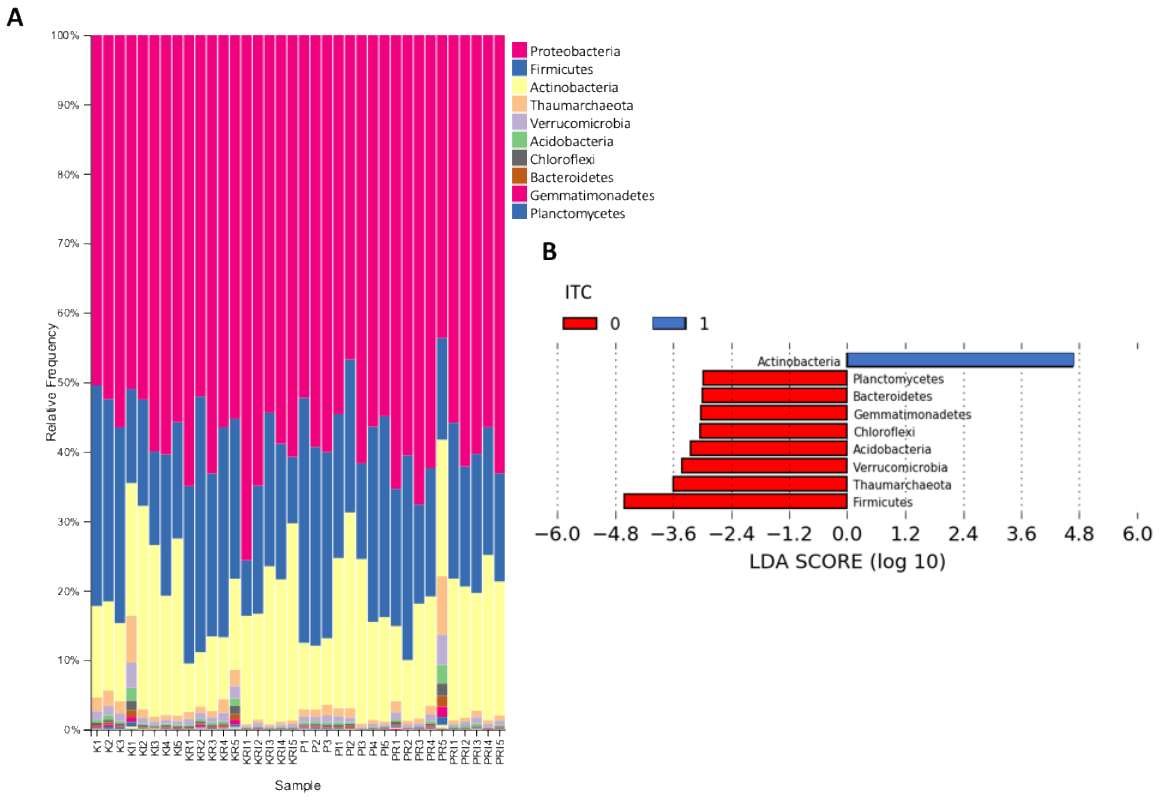


Figure 4.4.4. Effects of ITC exposure on potato and kale microbiota on phyla abundances (ten most abundant phyla) in the absence and presence of the *R. solanacearum* pathogen. Panel (A) shows relative frequency (%) of phyla abundances, while panel (B) shows differential phyla abundances (determined by LEfSe) when exposed to ITC. Data show post-treatment samples only. In panel (A), each bar indicates a replicate sample. X-axis labels indicate the sample number and treatment (K=kale microbiota, KI=kale microbiota and ITC, KR=kale microbiota and *R. solanacearum* presence, KRI=kale microbiota, *R. solanacearum* presence and ITC; similarly, P=potato microbiota). Bars are colour coded by the phylum (see

key). Panel (B) shows phyla that were significantly enriched or suppressed by ITC as blue or red bars, respectively. The length of the bar represents a log10 transformed LDA score (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

To further characterise taxa that benefitted or were sensitive to ITC, we used LEfSe to determine differentially abundant genera in potato and kale microbiomes. In total, 41 genera were found to be sensitive to ITC suppression. Of these, 11 genera (*Devosia, Gemmatimonas, Pseudolabrys, Mycobacterium, Candidatus Nitrosphaera, Candidatus Xiphinematobacter, Candidatus Nitrocosmicus, Pedomicrobium, Candidatus Udaeobacter, Fictibacillus* and *Paenibacillus*) were suppressed by ITC in both the kale and potato microbiomes (Fig. 4.4.5). Moreover, *Plantibacter* and *Cohnella* were exclusively enriched by ITC in the kale and potato microbiomes, respectively (Fig. 4.4.5A-B). Some genera changes were also unique to microbiome origin and *R. solanacearum* presence. For example, *Plantibacter, Ochrobactrum, Achromobacter* and *Acinetobacter* were enriched in the kale microbiome overall, while *Stenotrophomonas, Burkholderia-Caballeronia-Paraburkholderia* and *Marmoricola* were enriched in the potato microbiome (Appendix Figure B3A). Furthermore, *Novosphingobium* were enriched in the presence of *R. solanacearum*, while *Brevundimonas* and *Cohnella* were suppressed (Appendix Figure B3B). Together these results suggest that ITC had negative effects on the abundance of several bacterial genera across multiple phyla, while only Actinobacteria benefitted from ITC exposure.

(d) ITC exposure reduced bacterial co-occurrence network connectivity

Bacterial co-occurrence networks were constructed based on significant taxa correlations at the genera level (Pearson: $p < 0.05$) within kale and potato microbiota treatments in the absence and presence of ITC (4 networks in total). Overall, ITC exposure reduced the

number of nodes, edges and path length and decreased the average number of neighbours in co-occurrence networks (Fig. 4.4.6; Table 4.4.2). These effects were seen in the kale (125 nodes, 1160 edges, 1.62 path length, 18.56 neighbours in kale in the absence of ITC, compared to 102 nodes, 778 edges, 1.766 path length and 15.26 neighbours in kale samples in the presence of ITC; Fig. 6A-B; Table 2) and potato microbiota (136 nodes, 2544 edges, 1.507 path length and 37.41 neighbours in potato in the absence of ITC, compared to 78 nodes, 193 edges, 1.686 path length and 4.95 neighbours in potato in the presence of ITC; Fig. 4.4.6C-D; Table 4.4.2). Some genera also formed different associations in the presence and absence of ITC. For example, in the kale microbiome, the number of negative associations between *Rhodococcus* and other genera increased in the presence of ITC from one to 32 (no change in positive associations; Fig. 4.4.6A-B). In contrast, the number of negative associations between *Paenibacillus* and other genera were considerably reduced from 47 to none in the presence of ITC in the potato microbiome (Fig. 4.4.6C-D). This suggests that ITC exposure reduced co-occurrence network connectivity leading to fewer unique network associations, potentially due to the loss of certain taxa.

Table 4.4.2. Effects of ITC application and microbiome origin on genera level co-occurrence network statistics. Network metrics were determined using CytoScape in samples of different microbiome origin (kale or potato) and in the presence or absence of ITC exposure.

	Kale	Kale + ITC	Potato	Potato + ITC
Number of nodes	125	102	136	78
Number of edges	1160	778	2544	193
Average number of neighbours	18.56	15.26	37.41	4.95
Network diameter	5	5	4	4
Network radius	1	1	1	1
Characteristic path length	1.623	1.766	1.507	1.686
Clustering coefficient	0.332	0.333	0.375	0.238
Network density	0.075	0.076	0.139	0.032
Connected components	3	2	5	5

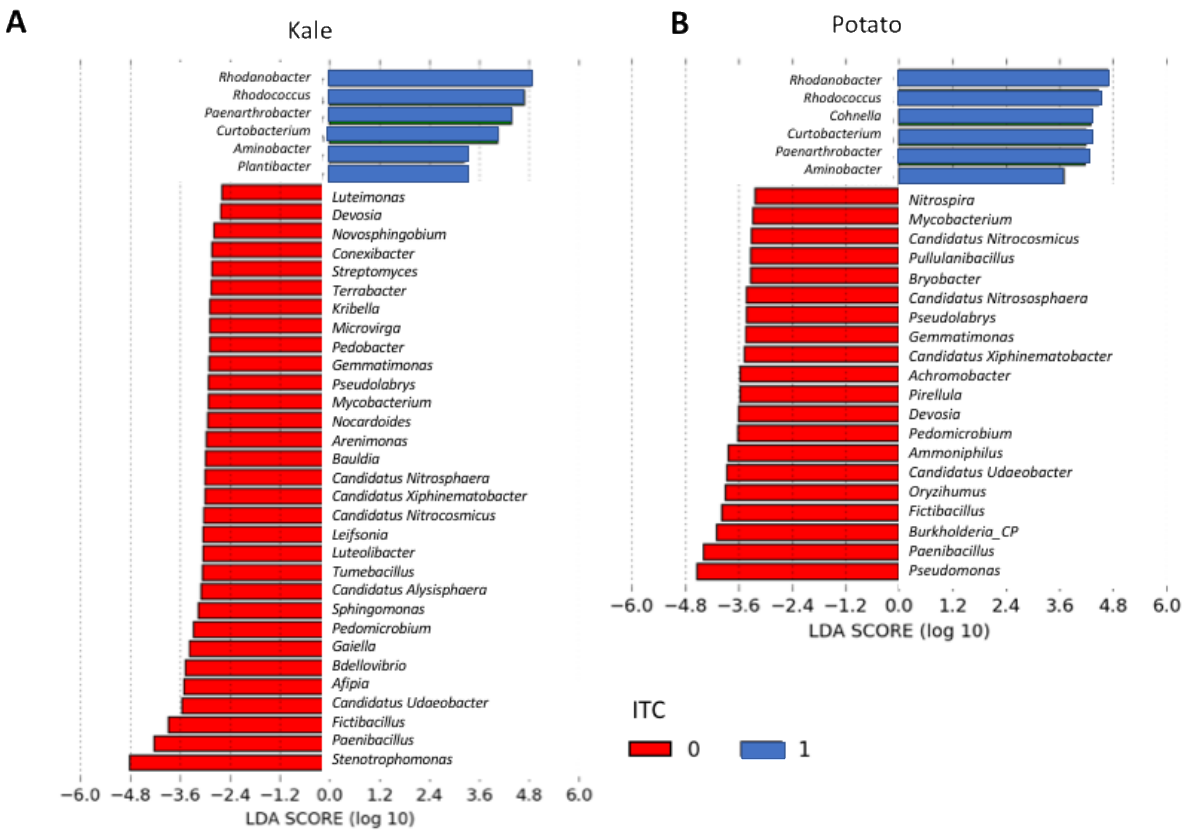


Figure 4.4.5. Effects of ITC exposure on potato and kale microbiome genera abundances in the absence and presence of the *R. solanacearum* pathogen. Panels show genera that were significantly enriched (blue) by ITC or enriched in the absence of ITC exposure (red) based on LEfSe output in the kale (A) and potato (B) rhizosphere microbiome communities (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC). The length of the bar represents a log10 transformed LDA score. Data show post-treatment samples only.

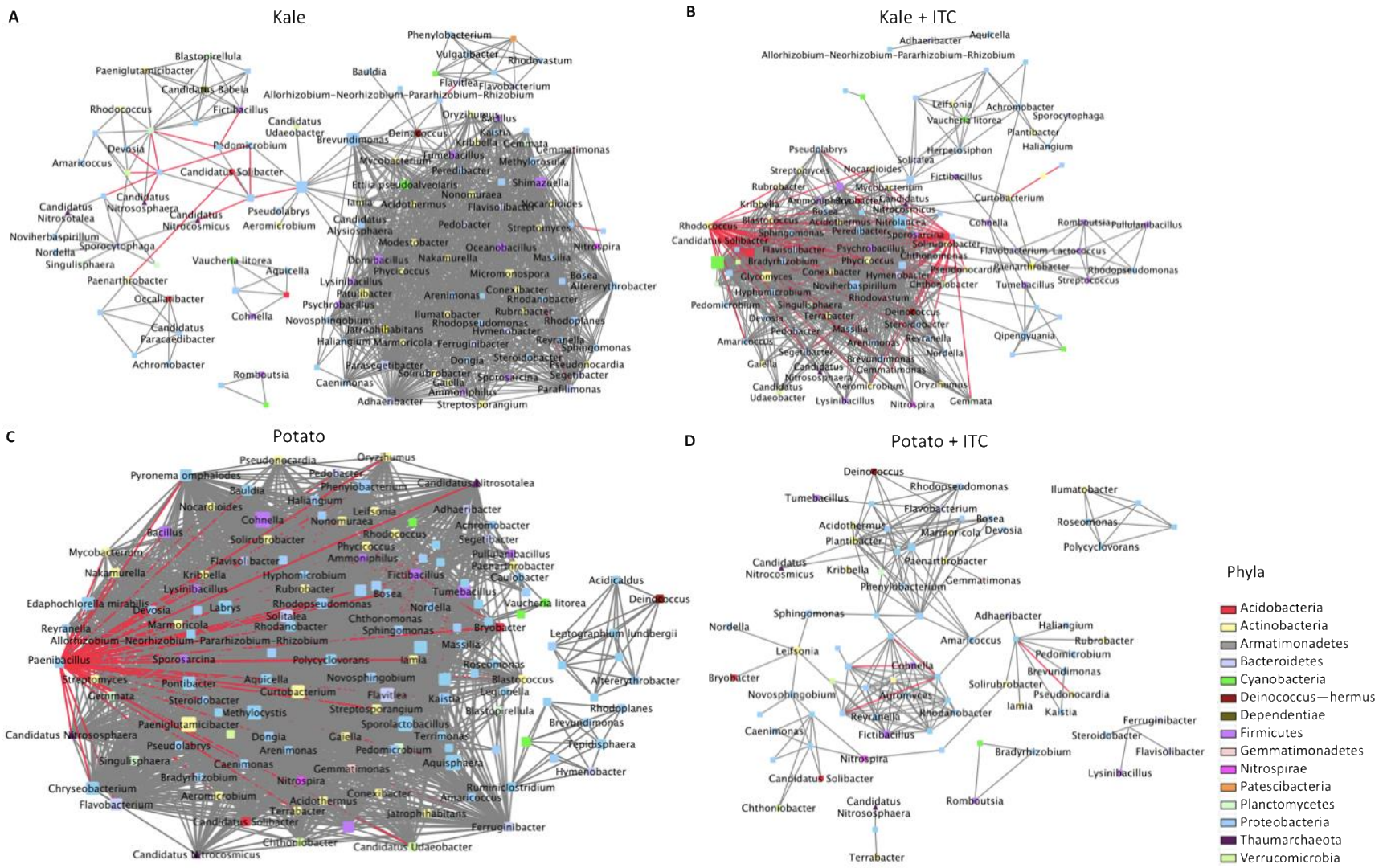

A
Kale
B
Kale + ITC
C
Potato
D
Potato + ITC
Phyla
Acidobacteria
Actinobacteria
Armatimonadetes
Bacteroidetes
Cyanobacteria
Deinococcus—hermus
Dependentiae
Firmicutes
Gemmatimonadetes
Nitrospirae
Patescibacteria
Planctomycetes
Proteobacteria
Thaumarchaeota
Verrucomicrobia

Figure 4.4.6. Effects of ITC on genera level co-occurrence networks in kale and potato microbiomes. Panels A and B show genera from the kale rhizosphere in the absence and presence of ITC, respectively. Panels C and D show genera from the potato rhizosphere in the absence and presence of ITC, respectively. Networks include samples in both the presence and absence of *R. solanacearum* pathogen. Genera with average abundance > 0.1%, and $p < 0.05$ (Pearson correlation) are included in the networks. Nodes are coloured based on the phyla classification of the genera (see key). Square nodes indicate bacteria whereas triangles represent archaea. Grey links indicate positive associations between genera whilst red links show negative associations. Genera are only shown if they were unique to the treatment. Node size is correlated with betweenness centrality (large node= high betweenness centrality value). Genera names are only displayed if they formed a highly significant ($p< 0.001$) association with other genera (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

(e) Predicted genes linked with antibiosis are differentially abundant in the absence and presence of ITC

To compare whether species that responded positively to ITC exposure were associated with antibiosis-related genes (NRPs and polyketides), we used PICRUSt predictions. We identified 11 KEGGs linked with NRPs and polyketides that were associated with antibiotic synthesis (actVII, aveA1, dhbF, entE, irp1, mbtE, mxcG, pchF, ppsB, sgcE and srfAA; Appendix Figure B4; B5). Five of these KEGGs were enriched and three suppressed by ITC exposure (Appendix Figure B4; B5). Moreover, while pchF was enriched in potato microbiome, irp1 was enriched in the presence of *R. solanacearum*. This indicates that certain antibiosis related genes might respond to ITC exposure, potentially because antibiotic production is also coupled with resistance, that could provide cross-tolerance to the biocidal activity of ITC.

4.5 Discussion

Here we studied the effects of *Brassica*-derived allyl-ITC allelochemical on *R. solanacearum* pathogen and the rhizosphere microbiota of two crop plants commonly grown in rotation: kale and potato. We found that *R. solanacearum* densities were reduced by ITC both in terms of CFU mL^{-1} and read counts. Moreover, we observed a reduction in microbial community diversity and clear taxa-specific changes in microbiota composition following ITC exposure. ITC effects on the surrounding microbiota were taxa specific. Only the Actinobacteria phylum responded positively to ITC exposure, whilst many other phyla, including Firmicutes, Thaumarchaeota and Verrucomicrobia were suppressed. Network analyses further revealed ITC exposure reduced the number of unique associations in both crop microbiomes likely due to the loss of certain taxa. Together, our results suggest that

allyl-ITC derived from Indian mustard biofumigant is effective at suppressing the growth of the *R. solanacearum* pathogen in soil microcosms. However, ITC exposure also influenced the surrounding microbial community, which may have detrimental effects on soil fertility and ecosystem functioning.

ITC application had a negative effect on *R. solanacearum* densities in our model biofumigation experiment. Moreover, the level of *R. solanacearum* density reduction in response to ITC did not differ when introduced to the kale or potato rhizosphere microbiome. This has positive implications for the potential for biofumigation to control against bacterial wilt disease and corroborates other studies which document the sensitivity of *R. solanacearum* to ITC allelochemicals (Arthy *et al.*, 2005; Ren *et al.*, 2018). However, we also found that ITC had suppressive effects on non-target microbiota in potato and kale microbiomes. Specifically, ITC exposure reduced taxa richness. This may have several negative effects on the soil microbiome functioning, including reduced disease suppressiveness as decreased community richness may increase the likelihood of pathogen invasion through reduced resource (Wei *et al.*, 2015) or interference competition (Hu *et al.*, 2017). In contrast to our finding, previous research has linked biofumigation with increased bacterial community richness (Wang *et al.*, 2014). We propose these contrasting results may be explained by the application of pure ITC in our study, whereas in the study by Wang *et al.* (2014) biofumigation was induced using rapeseed (*Brassica napus*) meal, thus preventing the separation of the effects of organic matter incorporation from direct biocidal effects of ITC. Moreover, our experiment took place in the absence of plants and therefore no carbon input, and had a relatively much shorter timescale, which could explain the discrepancy between these studies. We also found that ITC exposure reduced the abundance of rare taxa specifically, as indicated by relatively lower Chao1 diversity index. Rare species in

microbial communities have been correlated with improved plant health (Wei *et al.*, 2019) and biogeochemical cycle functioning (Jousset *et al.*, 2017) and their loss could be detrimental for soil ecosystem functioning. In contrast, we observed an increase in community evenness following ITC application, which could correlate with increased microbial community stability, plant productivity and soil health (Wittebolle *et al.*, 2009; Crowder *et al.*, 2010). Further research is hence required to link these diversity effects with soil ecosystem functioning and could involve analyses of changes to rates of nitrogen and carbon cycling following biofumigation, for instance.

ITC exposure also significantly changed the soil microbial community structure, in keeping with other studies (Omirou *et al.*, 2011b; Hu *et al.*, 2015). Overall, we found that most significantly different taxa showed sensitivity to ITC and only Actinobacteria increased in relative abundance after ITC application. ITC exposure also significantly reduced co-occurrence network connectivity, which is likely due to the reduced density or loss of certain taxa. This result is concerning as highly connected microbial networks are often associated with improved resistance to pathogen invasion (Wei, Yang, Friman, *et al.*, 2015; Xiong *et al.*, 2017). In regards to biofumigation, it has been shown that ITCs released by *B. napus* reduced microbial diversity but enriched ITC-tolerant microorganisms, many of which have plant growth promoting activities (Siebers *et al.*, 2018). Similarly, a previous study found simplification of microbial associations after conventional fumigation to control for *Fusarium* wilt (Ge *et al.*, 2021). Thus, ITC application may reduce overall microbial connectivity, but enrich the relative abundance of plant growth-promoting rhizobacterial taxa. A reduction in Actinobacteria and Firmicutes densities have previously been associated with *R. solanacearum* infections (Lee *et al.*, 2021), and it is thus possible that ITC-induced changes in microbiome composition could have consequences for natural disease

suppressiveness of soils. In contrast to our study, it has been reported that ITC application led to a transient increase in the proportion of Firmicutes (Hu *et al.*, 2015). Again, these contradictory findings may be due to differences in the initial soil community structure (Wei *et al.*, 2019) or the method of biofumigation, as in our study we applied the allyl-ITC compound directly, while others integrate the biofumigant plant that also contains nutrients to soil (Omirou *et al.*, 2011b; Baldrian *et al.*, 2012; Hollister *et al.*, 2013a). Previous studies have linked changes in soil microbiome structure with incorporation of organic matter, rather than biocidal effects of ITC release (Ochiai *et al.*, 2002), which often increases the abundance of ammonia oxidising bacteria and nitrification rates (Bending and Lincoln, 1999; Omirou *et al.*, 2011b). Moreover, our experiment was also considerably shorter and it is possible that we did not yet observe transient changes, which could be identified only after 7 days of biofumigation in the previous study (Hu *et al.*, 2015).

Interestingly, Actinobacteria were the only phylum that responded positively to ITC exposure, including *Rhodococcus*, *Paenarthrobacter*, *Microbacterium*, *Plantibacter* and *Curtobacterium* genera. Actinobacteria are well-known for their secondary metabolism and capability to produce several, highly potent antibiotics (Franco-Correa *et al.*, 2010; Palaniyandi *et al.*, 2013). As a result, they are often also highly resistant to antimicrobials and could have thus benefitted from competitive release and reduction in the abundance of ITC-sensitive species. For example, the biocontrol activity of *Curtobacterium* has been attributed to antibiotic production, which makes it highly antagonistic against crop diseases such as watermelon seedling blight and fruit blotch disease (Horuz and Aysan, 2018). Similarly, the enrichment of Actinobacteria has previously been linked to disease suppressive soils (Trivedi *et al.*, 2017), and specifically the suppression of *R. solanacearum* (Upreti and Thomas, 2015). Actinobacteria could also have indirect effects on the pathogen

via interactions with plants. For example, Cohen *et al.* (2005) attributed the control of *R. solani* following *B. napus* seed meal amendment to an increase in *Streptomyces* spp. abundance and nitric oxide production which triggered plant systemic resistance. The beneficial effects of biofumigation could thus also be explained by changes in the abundance of pathogen suppressing microbes or induction of systemic resistance in host plants (Ochiai *et al.*, 2002; Cohen, Yamasaki and Mazzola, 2005; Omirou *et al.*, 2011b).

To explore the link between ITC exposure and potential antibiotic resistance of kale and potato microbiota, we explored changes in predicted antibiosis genes using PICRUSt and LEfSe. We were able to identify 5 KEGGs that were enriched and 3 KEGGs that were suppressed by ITC exposure associated with antibiosis (NRPs and polyketides). These genes are often linked with antibiotic synthesis (Minowa, Araki and Kanehisa, 2007), and may be selected for under ITC exposure. Specifically, the KEGGs actVII, sgcE, mxcG, dhbF and aveA1 were found to be significantly more abundant in ITC exposed samples, which have been linked to electron transport inhibition (Silakowski *et al.*, 2001) and siderophore synthesis (Hofemeister *et al.*, 2004). The selective advantage of microbes possessing antibiosis-related genes may explain the previously documented increase in pathogen suppression following biofumigation (Omirou *et al.*, 2011b; Weerakoon *et al.*, 2012; Wang *et al.*, 2014). However, it is important to note that our analyses were based on functional gene predictions and should be validated using metagenomic or direct culture approaches in the future.

In conclusion, our findings demonstrate that ITC could potentially be used to suppress the growth of *R. solanacearum* plant pathogen in microbial communities. However, ITC exposure also caused collateral damage to non-target taxa. Crucially, more work is required to determine if ITC-induced rearrangement of the soil microbial community structure, *e.g.* suppression of Firmicutes and enrichment of Actinobacteria could strengthen

or constrain natural soil disease suppressiveness. Future work is also required to understand the consequences of changes in community composition for microbiome ecosystem functioning and to better understand how our results might differ if ITC application is combined with organic matter incorporation as during conventional field biofumigation.

Chapter 5. Collateral effects of model biofumigation on the crop and non-target soil microbiota

5.1. Abstract

Biofumigation is a biocontrol technique that involves the mulching of *Brassica* plant tissues into soil to release isothiocyanates (ITCs). While the biocidal effects of ITCs against plant pathogens are well documented, effects on the surrounding microbiome and the associated crop are still relatively understudied. Here we used an *in vivo* tomato model system to evaluate the efficacy of allyl-ITC against *Ralstonia solanacearum* plant pathogenic bacterium, tomato growth and the surrounding microbiota. We found that ITC was efficient at suppressing *R. solanacearum* growth in the rhizosphere but only in the presence of microbiota. Despite this, ITC application reduced wilt disease symptoms both in the absence and presence of microbiota. Interestingly, ITC application was associated with the reduction in tomato dry weight, even in the absence of pathogen and microbiota, indicative of potential direct negative effects of ITC on the crop. Moreover, ITC exposure reduced soil microbiota richness and eliminated rare taxa but increased the connectedness of bacterial co-occurrence networks. We also found evidence for potential ITC tolerance by *R. solanacearum* in the single and weekly ITC application treatments. Together, our results suggest that while ITC application can successfully inhibit *R. solanacearum* growth and reduce disease symptom severity, there were also potential negative collateral effects on the plant and the surrounding microbiota. Further research is hence required to identify types of ITCs that would be more specific to the pathogen.

5.2. Introduction

Crop disease accounts for the loss of almost a third of global food production annually (Strange and Scott, 2005; Raaijmakers *et al.*, 2009). Conventional agrochemical pathogen control methods often have poor pathogen specificity, harm non-target microbiota and have damaging effects on the environment (Sederholm *et al.*, 2018). Biocontrol, the use of natural microbial antagonists of the pathogen, may offer environmentally sustainable alternatives to broad-spectrum agrochemicals.

Biofumigation is a biocontrol technique that uses *Brassica* plant allelochemicals to eliminate pathogens. *Brassica* plants like oilseed rape (*B. napus*), cabbage (*B. oleracea*) and Indian mustard (*B. juncea*) are elevated in glucosinolates (GSLs), and myrosinase enzymes that react upon contact to release pathogen suppressive by-products, notably isothiocyanates (ITCs) (Sarwar *et al.*, 1998). To apply biofumigation, *Brassica* are generally incorporated as green manure or seed meal as an integrated part of conventional crop rotation (Kirkegaard *et al.*, 2000). The biocidal effects of ITCs against bacteria are attributed to destruction of the outer cell membrane, causing leakage of cell metabolites (Hanschen *et al.*, 2015a), or disruption of sulfhydryl groups and disulfide bonds and enzyme breakdown (Brown, 1997). The biocidal effects of ITC are often short-lived due to their high volatility, which varies based on ITC chemical structure (Matthiessen and Shackleton, 2005). ITCs thus typically have short half-lives and persist in the soil for less than 48 hours before escaping into the atmosphere (Borek *et al.*, 1995; Gimsing and Kirkegaard, 2006). Due to the relatively short exposure time, it is hence unlikely that bacterial pathogens could evolve resistance or tolerance to ITC in contrast to antimicrobial exposure in pathogens in clinical settings (Levin-Reisman *et al.*, 2017). However, it has recently been shown that ITC

tolerance can evolve rapidly in liquid microcosms (Alderley, Greenrod and Friman, 2021); Chapter 2), although the potential for ITC tolerance to evolve in the plant rhizosphere has not been experimentally evaluated.

Despite the documented effects of biofumigation on pathogen suppression (Matthiessen and Kirkegaard, 2006; Larkin and Griffin, 2007), its effects on the surrounding microbiome have been researched much less. The rhizosphere microbiome often also includes plant growth promoting rhizobacteria (PGPR) that can stimulate plant growth and reduce levels of crop disease through pathogen antibiosis, resource competition and induction of systemic resistance (Lugtenberg and Kamilova, 2009). As ITCs are often found to have broad spectrum activity, they have been reported to cause collateral damage to beneficial bacteria, potentially undermining the benefits of biofumigation on crop yields (Bending and Lincoln, 1999; Rumberger and Marschner, 2003). In other cases, biofumigation has been shown to selectively enrich PGPRs (Cohen, Yamasaki and Mazzola, 2005; Larkin and Honeycutt, 2006; Friberg *et al.*, 2009b; Hollister *et al.*, 2013b). One explanation for this is that PGPRs often produce antibiotics themselves and could hence be cross-tolerant to the biocidal activity of ITCs. Alternatively, some species might be better at competing for nutrients following loss of ITC sensitive species and niche liberation as biofumigation also increases the levels of soil carbon and nitrogen as a side-effect of *Brassica* organic matter incorporation (Mazzola and Gu, 2002; Wang *et al.*, 2014). As a result, ITCs could either stimulate or suppress non-target microorganisms.

ITCs may also have phytotoxic effects on the crop plants. It has been long-established that *Brassica* have lost the ability to form arbuscular mycorrhizas (Poveda *et al.*, 2019) and make poor companion plants (Chew, 1988), probably due to the release of ITC allelochemicals from the roots whilst cell integrity is compromised during its growth

(Choesin and Boerner, 1991; Rumberger and Marschner, 2003). For instance, one field experiment found inhibition of lettuce germination and growth following biofumigation using *Brassica* seed meal (Intanon *et al.*, 2014), while another study found high-GSL seed meals had the most severe phytotoxic effects on plants, which could undermine the disease suppressive effects of biofumigation and reduce crop yield. In order to optimise the benefits of biofumigation, it is hence important to identify compounds that are more harmful to the pathogen relative to the soil microbiota and crop plant.

To study the effects of ITCs on microbial communities, we developed a model *in vivo* system where we tested the growth-inhibiting effects of allyl-ITC on *R. solanacearum* plant pathogenic bacterium in the absence or presence of rhizosphere microbiota with tomato. *R. solanacearum* causes bacterial wilt disease (BWD) globally and can infect many crops, including important solanaceous crop plants such as tomato and potato (Hayward, 1991). Crucially, no effective control method against this pathogen exists (Saddler, 2005). Allyl-ITC was chosen as a model biofumigant compound due to its high biocidal activity against *R. solanacearum.* ITC application was stimulated by exposing tomato and rhizosphere bacteria to allyl-ITC once at the beginning of the experiment, or repeatedly once every week for a total of four weeks. Changes in bacterial densities and community composition were quantified using microbiological methods and 16S rRNA amplicon sequencing and effects on the tomato plant growth and health were monitored throughout the experiment. *R. solanacearum* was found to be sensitive to ITC suppression but only in the presence of microbiota. However, the pathogen density reduction did not directly translate into reduced disease severity and a clear reduction in BWD symptoms by ITC was observed also in the absence of microbiota. Collateral effects of ITC included reduced microbiota diversity and tomato dry weight, indicative of phytotoxic effects of biofumigation on the crop.

Furthermore, we found evidence for the evolution of ITC tolerance in *R. solanacearum.* Together, these results suggest that the non-specificity of allyl-ITC could potentially reduce the benefits of biofumigation for *R. solanacearum* pathogen biocontrol.

5.3. Materials and Methods

(a) Pathogen strain and culture media

We used a virulent *R. solanacearum* strain (21415687) as the pathogenic bacterium in our experiment (phylotype II sequevar 1). The strain was originally isolated from the river Loddon in the UK (John Elphinstone, Fera Science, 2014), and is a representative of the endemic UK pathogen population which can inhabit Woody Nightshade secondary hosts leading to contamination of river water (Elphinstone, Stanford and Stead, 1998). The strain was cultured in CPG broth (1 g casamino acids, 10 g peptone and 5 g glucose per litre ddH_2O) for 48 hours at 28 °C with shaking (250 rpm) to create cryostocks (20% w/v glycerol) that were preserved at -80 °C.

(b) Farming site and rhizosphere soil sampling

We focused on the effects of ITC on potato microbiota because it is a major host crop for *R. solanacearum* in the UK. Moreover, biofumigation has shown to be effective in protecting against potato plant parasitic nematodes (Ploeg, 2008; Ntalli and Caboni, 2017). Potatoes (*Solanum tuberosum*) were harvested in September 2019 from the Barworth Agriculture Ltd. field site located in Sleaford, Lincolnshire, UK. This site has no previous record of *R. solanacearum* infection. A 30 x 6 m plot was dedicated to sampling and was split by randomised block design into 6 x 2 m blocks. Potatoes were drilled and sown into the soil and grown under Standard Farming Practice, which included the application of fertiliser (100 kg nitrogen, 50 kg phosphorus and 50 kg potassium) and organic insecticide (Pyrethrum

5EC) on two occasions, irrigation as required, and mechanical weed control on five occasions during the six-month growth period. Soils at the site were typically sandy loam. Soil samples were taken at the point of harvest using a soil corer from each of twenty sampling locations from each replicate plot (N=6). Cores were pooled into a single sample and soil washes were made to enable a homogenised sample to be used in later experiments. Washes were made by taking 10 g +/- 0.1 soil from each replicate plot and mixing with 20 ml ddH_20 by vortex. The solution was then allowed to settle for 30 minutes before 1 ml of soil wash from each sample was frozen at -80 °C in 30% glycerol for later experiments. In the end, we had 6 independent rhizosphere samples from potato crops at the point of harvest, which were used in further experiments in the lab. Due to difficulty in growth and infection of the potato plant model, we used tomato which is from the same genus as tomato and has a very similar microbiota (Goswami, Kashyap and Awasthi, 2019).

(c) Plant growth chamber model biofumigation experiment

To construct a model biofumigation experiment, *R. solanacearum* was inoculated in sterile soil in the absence or presence of rhizosphere microbiota with tomato and treated with synthetic ITC, which was applied once or 4 times at weekly intervals throughout the experiment (sterile M9 buffer was used as negative control). Seeds of the cultivar Micro Tom tomato (*Solanum lycopersicum*) were sown in 30 g twice-autoclave sterilised John Innes #2 potting soil (Sterile soil), or in the same soil inoculated with the potato rhizosphere microbiota (Microbiome soil). The sterile soil acted as a control in comparisons of microbiome effects on crop health and growth. Microbiome inoculum was prepared from potato rhizosphere soil wash cryostocks which were thawed and homogenised by vortex-mixing before inoculation to CPG media and growth at 28 °C for 48 hours. Bacterial densities of the potato microbiome liquid cultures were then normalised to an optical density reading

of 0.5 (OD 600 nm; Tecan Sunrise) and spun by centrifugation (14,000 rpm) to form a pellet. The supernatant was removed, and the pellet was resuspended in 6 ml M9 buffer (128 g sodium phosphate dibasic, 30 g monopotassium phosphate, 5 g sodium chloride, 10 g ammonium chloride per litre ddH_2O; recipe for 10x stock concentration). 300 μl of the microbiome suspension was then added to the microbiome treatment soil and mixed before sowing tomato seeds. After 25 days of growth, the roots of all tomato seedlings were cut using a sterile scalpel to mimic pathogen entry sites in natural conditions and to increase the consistency of infection between replicates. 1 ml of *R. solanacearum* suspension was inoculated to plants in the pathogen treatments after normalising bacterial densities to 0.4 (OD 600 nm; equalling approximately 10^9 CFU bacteria g^{-1} soil) and washing the bacteria from nutrients as described earlier. Every treatment was replicated 7 times (microbiota; microbiota + *R. solanacearum*; microbiota + ITC (applied once); microbiota + *R. solanacearum* + ITC (applied once); microbiota + ITC (applied weekly); microbiota + *R. solanacearum* + ITC (applied weekly)) and also the same treatments in sterile conditions, resulting in a total of 84 tomato mesocosms. All mesocosms were watered as required using sterile water, and plants were kept in growth chambers with 12-hour dark/light cycles at 28 °C during the day and 20 °C at night. Mesocosms were rearranged randomly within the plant growth chamber every two days.

Allyl-ITC was used to model biofumigation effects as it is the predominant antimicrobial allelochemical released during biofumigation with Indian mustard (*Brassica juncea*), a common biofumigant crop (Kirkegaard and Sarwar, 1999; Ngala *et al.*, 2015). Additionally, we had already verified allyl-ITC as highly toxic to this strain of *R. solanacearum* in our previous work (Alderley, Greenrod and Friman, 2021). ITC was applied at two different frequencies: on a single occasion two days after pathogen inoculation, and once

per week after pathogen inoculation for a total of 4 weeks. ITC application frequency manipulation enabled comparisons of biofumigation effects based on quick and slower ITC release which is affected by the extent of bruising and pulverisation of *Brassica* tissues, soil type and climatic conditions (Sarwar *et al.*, 1998). Before application, ITC was mixed with M9 buffer, resulting in final concentrations of 0.5 μmol g^{-1} soil, and 1 ml of this solution was added to the soil surface. This concentration was chosen as it is relevant to concentrations achieved during field biofumigation (Borek *et al.*, 1995; Kirkegaard and Sarwar, 1998; Warton, Matthiessen and Shackleton, 2003a; Gimsing *et al.*, 2007; Rudolph *et al.*, 2015). For the negative control treatment, equal volume of sterile M9 buffer was added. To prevent cross-volatilisation of ITC, the seedlings that were exposed to ITC were kept in a separate growth chamber. Following *R. solanacearum* inoculation, bacterial wilt disease symptoms were monitored based on visual observations every 2 days using an index of 0-4 according to Roberts *et al.* (1988), with a score of 0 meaning no leaves wilted, 1 meaning 25% of leaves wilted, 2 meaning 26-50% of leaves wilted, 3 meaning 51-75% of leaves wilted and 4 meaning 76-100% of leaves wilted.

At the end of the experiment (37 days after *R. solanacearum* infection), plants were cut at the base and the aboveground section of the plant was dried in a drying oven at 70 °C for 4 days before measuring dry weight. Tomato rhizosphere soil samples were collected from all replicate plant mesocosms by washing the soil surrounding the roots in 20% w/v glycerol. Soil suspensions were allowed to settle for 30 minutes before 1.25 ml from each sample was frozen at -80 °C in 50% glycerol for later experiments. To quantify *R. solanacearum* abundances, semi-selective SMSA agar plates containing specific nutrients, antibiotics and fungicide (Elphinstone, 2005) were used. *R. solanacearum* treatment soil washes were then serially diluted and spotted onto agar plates which were incubated at 28

°C for 48 hours to determine CFUs. All soil washes were also used for DNA extraction and Illumina MiSeq sequencing as described in the following section.

(d) DNA extraction and sample processing

Bacterial community composition of soil samples was determined using Illumina MiSeq sequencing of 16S rDNA amplicon sequencing, focusing on microbiota-containing treatments at the end of the model biofumigation experiment (total of 42 rhizosphere samples). DNA was extracted for PCR amplification and purified from 0.25 g of soil wash using the Qiagen DNeasy PowerLyzer PowerSoil kit following the manufacturer's protocol. Bacterial 16S rDNA was PCR amplified using the oligonucleotide Illumina specific primers with Illumina adapters attached 515F-Y (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGANNNHNNNWNNNHGTGYCAGCMGCCGCG GT AA-3') and 806R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGTGGACTACHVGGGTWTCTAAT-3') which encompasses the V4 hypervariable region (Parada, Needham and Fuhrman, 2016). PCRs were carried out using GoTaq Hot Start Polymerase (Promega Corporation). Reaction mixtures were set up according to the following protocol:

Component	Final concentration	PCR recipe for 50 μl reaction
Colourless GoTaq Flexi Buffer	1X	10 μl
$MgCl_2$ Solution, 25mM	1 mM	5 μl
dNTP	0.2 mM	1 μl
Forward primer	0.5 μM	0.5 μl
Reverse primer	0.5 μM	0.5 μl

GoTaq Hot Start Polymerase	5 U/μl	0.25 μl
Nuclease-Free Water		31.75 μl
Template DNA		1 μl

PCR amplification was then performed on a Biometra TProfessional Thermocycler (Biometra, Thermofisher), using the following PCR temperatures, times and cycles:

Temp (°C)	Time (min:s)	Cycles
95	2:00	1x
94	0:30	
53	0:45	30x
72	1:30	
72	5:00	1x

Agarose gel electrophoresis (1.5% agarose) was used to analyse the products of PCR reactions. Electrophoresis was then performed in a Bio-Rad horizontal gel electrophoresis tank, using the Bio-Rad PowerPac 300 power system (Bio-Rad) performed at 100 V for 55 minutes. Gels were imaged under UV illumination using a Bio-Rad ChemiDoc XRS+ imager.

Equal volumes of PCR products for each sample were pooled, cleaned (AMPure XP Beads, Beckman Coulter, USA) and indexed for sequencing (Nextera XT DNA Library Preperation Kit v2 set A, Illumina, USA). The DNA concentrations were determined using a Quant-iT dsDNA Broad-Range Assay kit (Thermo Scientific). All samples were further quality checked by Bioanalyzer 2100 (Agilent, USA) before sequencing using Illumina MiSeq (2 x 300bp paired end reads) by the University of York Technology Facility.

Bioinformatics were performed using QIIME2 (Quantitative Insights into Microbial Ecology) 1.8. 0 (Bolyen *et al.*, 2019)). Raw sequence data were trimmed to remove PCR

primer sequences using cutadapt followed by denoising with DADA2 (Callahan *et al.*, 2016). All amplicon sequence variants (ASVs) were aligned with mafft (Katoh *et al.*, 2002) and used to construct a phylogeny with fasttree (Price, Dehal and Arkin, 2010). Alpha-diversity metrics (richness, evenness, Shannon's diversity and Chao1), beta diversity metrics (Bray-Curtis dissimilarity (Bray and Curtis, 1957) and weighted UniFrac (Lozupone *et al.*, 2007) and principal coordinates analysis (PCoA) were estimated using q2-diversity after samples were rarefied (subsampled without replacement) to 45,000 sequences per sample to retain all paired samples. ASV taxonomy was assigned using the q2-feature-classifier classify-sklearn naïve Bayes taxonomy classifier (Bokulich *et al.*, 2018) against the SILVA 132 reference sequences at 99% similarity (Quast *et al.*, 2013).

(e) Determining evolutionary changes in *R. solanacearum* tolerance to ITC

To investigate the potential evolution of *R. solanacearum* ITC tolerance, we used fitness assays that compared the densities of ancestral and evolved control (non-ITC exposed) and ITC-exposed *R. solanacearum* populations when re-exposed to ITC in lab conditions, using the final time point samples (37 days post pathogen inoculation). Eight ancestral and three evolved *R. solanacearum* colonies per each seven replicate plants within each pathogen-containing treatment were isolated from SMSA agar plates using sterile loops, and frozen in 30% glycerol at -80 °C. All clones were inoculated into 200 μl of CPG media in 96-well microtiter plates (Total of 126 evolved clones and 8 ancestral clones). After 24 hours of growth at 28 °C, bacterial densities were normalised to an OD reading of 0.1 (600 nm) and 10 μl of each replicate colony was inoculated in new liquid microcosms in 96-well microplates containing 200 μl CPG or CPG media with 500 μM allyl-ITC concentration similar to ITC exposure during the tomato mesocosm experiment. Bacterial density measurements were recorded at 24-hour intervals for a period of 72 hours based on OD readings (600 nm).

After taking the mean bacterial density of each clone, relative growth inhibition by ITC was determined by comparing growth in the absence of ITC (CPG only) with growth in the presence of ITC. Therefore, a value of 0 indicates the strain grows equally well in the absence and presence of ITC, while values below 0 indicate the suppressive effect of ITC. We hypothesised that single or weekly ITC exposure may influence the capacity for *R. solanacearum* to develop tolerance to biofumigant hydrolysis products by affecting the selective pressure.

(f) Statistical analyses

Two-way ANOVA was performed to analyse differences in CFU g^{-1} and tomato dry weight between treatments and Tukey *post-hoc* tests were used to compare differences between subgroups ($p< 0.05$). Chi-squared test was used to determine differences in the severity of disease symptoms (index 0-4) between treatments. Non-parametric Kruskal-Wallis test was used to analyse differences in alpha-diversity metrics and other data that did not fit the assumptions of a normal distribution. To test for the effects of ITC exposure and *R. solanacearum* presence on within sample diversity, four alpha-diversity metrics were used: Richness (total number of different ASVs), Shannon diversity (a quantitative measure of community richness, accounts for abundance and evenness of ASVs), Pielou's evenness (measures evenness of total number of different ASVs), and Chao1 (diversity based on abundance data weighing rare taxa). As for between sample beta diversity (community composition), principal coordinate analysis (PCoA) was used to determine the effect of different treatments (ITC exposure and *R. solanacearum* presence) on the Bray-Curtis (differences in the presence, absence and abundance of ASVs) metric. Components of variance were used to estimate the between sample vs within sample intraclass correlation coefficient for each microbiome measure. Cluster analysis was conducted using the

'stat_ellipse' function in R. To identify differentially abundant taxa at the phyla and genera level, we used the LEfSe (Linear Discriminant Analysis Effect Size) approach via the Huttenhower Lab Galaxy Server (Goecks *et al.*, 2010). The LEfSe algorithm performs nonparametric statistical testing to determine whether individual taxa differed between treatments and ranks differentially abundant taxa by their linear discriminant analysis (LDA) log scores (Segata *et al.*, 2011). Differentially abundant taxa (based on ITC exposure and *R. solanacearum* presence) that were statistically significant based on alpha 0.05 and threshold on the logarithmic LDA score for discriminative features of at least +/- 2 were visually represented on bar plots.

To decipher the taxa that was shared by all microbiome samples, we took the genera level OTU tables produced by QIIME2 and used NetSets to produce Venn plots showing the overlap in genera between samples (Nagpal, Kuntal and Mande, 2021). We split samples by treatment variables (ITC exposure and *R. solanacearum* presence). Genera present in all treatment samples were considered as the 'shared core microbiota'.

Microbial association networks (at the genera level) were generated based on significant Pearson correlations ($p < 0.05$), which were determined using R. We compared microbial association networks in the presence and absence of ITC separately. Taxa classified as 'unknown microbe' or 'ambiguous microbe' by QIIME2 taxa determination were excluded from analyses. These networks were visualised using CytoScape (Shannon *et al.*, 2003) and only significant associations unique to each treatment were visualised (associations present in all treatments were removed from plots to simplify networks). The NetworkAnalyzer tool was used to calculate network topology parameters. Nodes represent genera and edges show statistically significant (Pearson correlation: $p < 0.05$) associations between nodes. Major hub nodes with many connections have a high betweenness

centrality and this was used to determine node size in the network figure. Average path length measures microbiome compactness, while the clustering coefficient describes the proportion of pairs of nodes connected to the same nodes. All statistical analyses and graphs were produced using R (R Foundation for Statistical Computing, R Studio Version (4.0.3). Packages: ggplot, tidyverse, rcompanion, Hmisc) and QIIME2 (1.8. 0).

5.4. Results

(a) *Ralstonia solanacearum* densities were reduced by ITC in microbiota soil treatments

To explore the effect of ITC, we quantified *R. solanacearum* densities in the presence and absence of ITC using selective plating. Based on colony counts on selective SMSA plates, ITC had no effect overall on *R. solanacearum* abundances ($F_{2, 36}$= 0.08, p> 0.05; Fig. 5.4.1A). However, the presence of a microbiota significantly reduced *R. solanacearum* densities ($F_{1, 36}$= 56.15, p< 0.001; Fig. 5.4.1A), and ITC exposure (single or weekly application) had negative effects on *R. solanacearum* in the presence of microbiota (ITC*Microbiome: $F_{2, 36}$= 14.89, p< 0.001; Fig. 5.4.1A). Despite the lack of *R. solanacearum* density reduction in the absence of microbiota, ITC exposure led to clear reduction in disease symptoms (X^2= 2.83, df= 2, p< 0.05; Fig. 5.4.1B), while the presence of microbiota reduced disease symptoms even in the absence of ITC (X^2= 0.16, df= 1, p< 0.05; Fig. 5.4.1B), an effect which was not further magnified in the presence of ITC (X^2= 0.38, df= 2, p= 0.83; Fig. 5.4.1B). *R. solanacearum* presence reduced tomato plant aboveground dry weight ($F_{1, 82}$= 19.3, p< 0.001; Fig. 5.4.1C), and while the microbiota alleviated the effects of *R. solanacearum* infection in the absence of ITC ($F_{1, 26}$= 6.17, p< 0.05; Fig. 5.4.1C), no effect was observed when ITC was applied ($F_{1, 26}$= 0.028, p> 0.05, Fig. 5.4.1C). Similar to disease symptom data,

ITC application did not alleviate the negative effects of *R. solanacearum* infection ($F_{2, 39}$= 0.82, p> 0.05; Fig. 5.4.1C). Instead, weekly ITC applications significantly reduced tomato dry weight in the absence of *R. solanacearum* ($F_{2, 39}$= 10.96, p< 0.001; Tukey: p< 0.05; Fig. 5.4.1C). Together, these results suggest that ITC application led to reduced disease symptoms but also lowered tomato biomass.

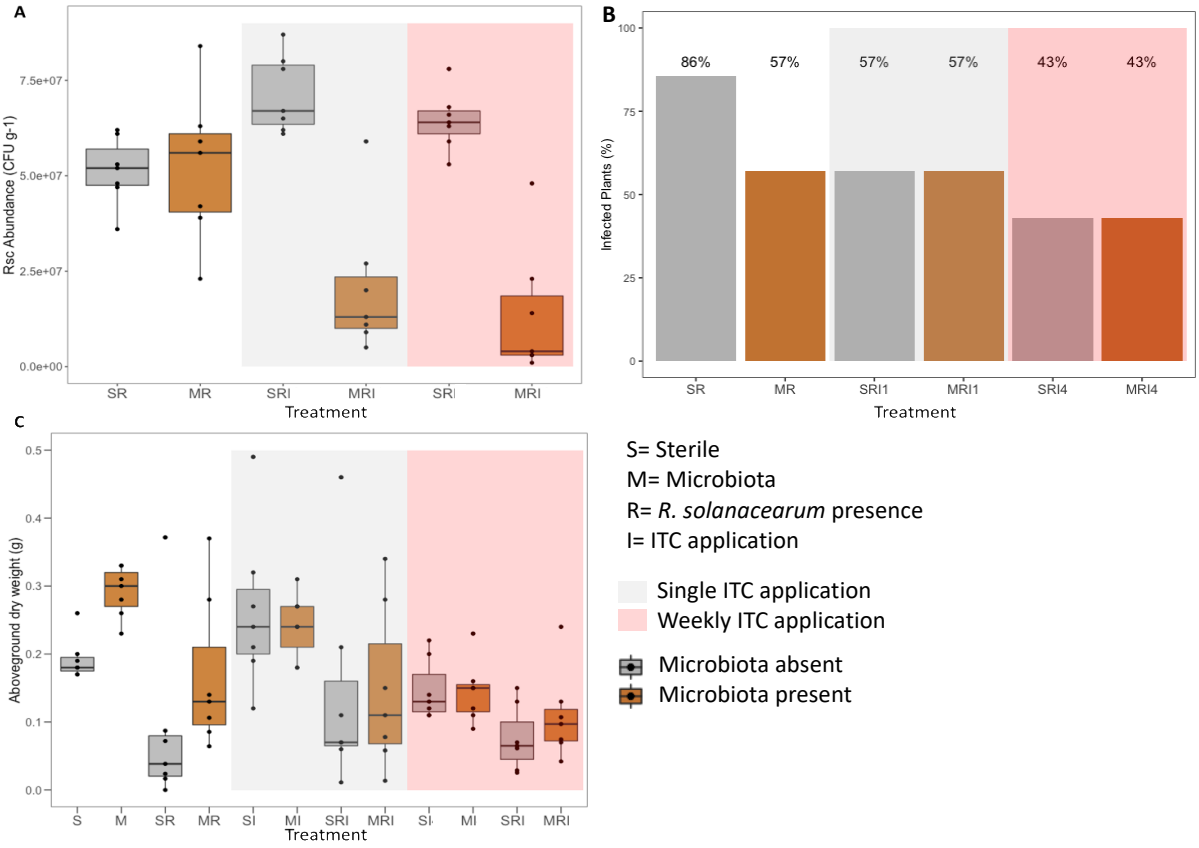


Figure 5.4.1. Effects of ITC exposure (single or weekly) and microbiome presence on *R. solanacearum* abundances, bacterial wilt disease symptoms and tomato dry weight. Panel (A) shows *R. solanacearum* (Rsc) abundances (CFU g^{-1}) in the absence (grey bars) and presence (orange bars) of microbiota when ITC was not applied (white background), applied once (grey background) or applied repeatedly once per week for 4 weeks (red background). Panels (B) shows the percentage of wilted plants and panel (C) tomato plant dry weight in the same treatments in addition to sterile soil (S) and microbiota-only (M) control

treatments. All boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=4 for all control treatments (no bacteria inoculated) and N=7 for all other treatments).

(b) Weekly ITC exposure had collateral effects on microbial diversity and changed microbial community composition

ITC effects on alpha diversity of microbiomes were tested using four metrics: species richness, Shannon diversity index, community evenness and Chao1 diversity (to determine the effect on rare species). ITC application had no effect on community evenness ($H= 2.81$, $p= 0.25$, Fig. 5.4.2B), and none of the diversity metrics were affected by the presence of *R. solanacearum* (Richness: $H= 0.93$; $p> 0.05$; Evenness: $H= 1.14$, $p= 0.287$; Shannon diversity: $H= 2.27$, $p> 0.05$; Chao1: $H= 1.01$, $p> 0.05$; Fig. 5.4.2). However, single and weekly ITC application reduced taxa richness and had a relatively larger negative effect on rare species, as indicated by reduced Chao1 diversity (Richness: $H= 14.21$, $p< 0.001$; Chao1: $H= 14.26$, $p< 0.001$; Fig. 5.4.2A, D). Moreover, weekly ITC exposure also reduced Shannon diversity ($H= 8.15$, $p< 0.05$; Fig. 5.4.2C), while single ITC exposure had no effect (Tukey: $p> 0.05$). We also found that the presence of *R. solanacearum* had positive effects on richness and Chao1 when ITC was applied weekly (ITC**R. solanacearum* interaction for Richness: $F_{2, 32}= 4.16$, $p< 0.05$; Chao1 diversity: $F_{2, 32}= 4.12$, $p< 0.05$; Fig. 5.4.2A; D).

We next compared the effects of ITC on microbiome community composition (beta-diversity) using two statistical approaches: non-parametric multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM) using the Bray-Curtis and weighted-UniFrac beta-diversity metrics. ANOSIM tests whether distances between groups are greater than within groups whilst PERMANOVA tests whether distance differs between groups. As results were qualitatively similar between the two beta-diversity metrics and statistical

tests, we report here PERMANOVA tests based on the Bray-Curtis metric while analyses based on ANOSIM test and Weighted-UniFrac metric are shown in Table 5.4.1.

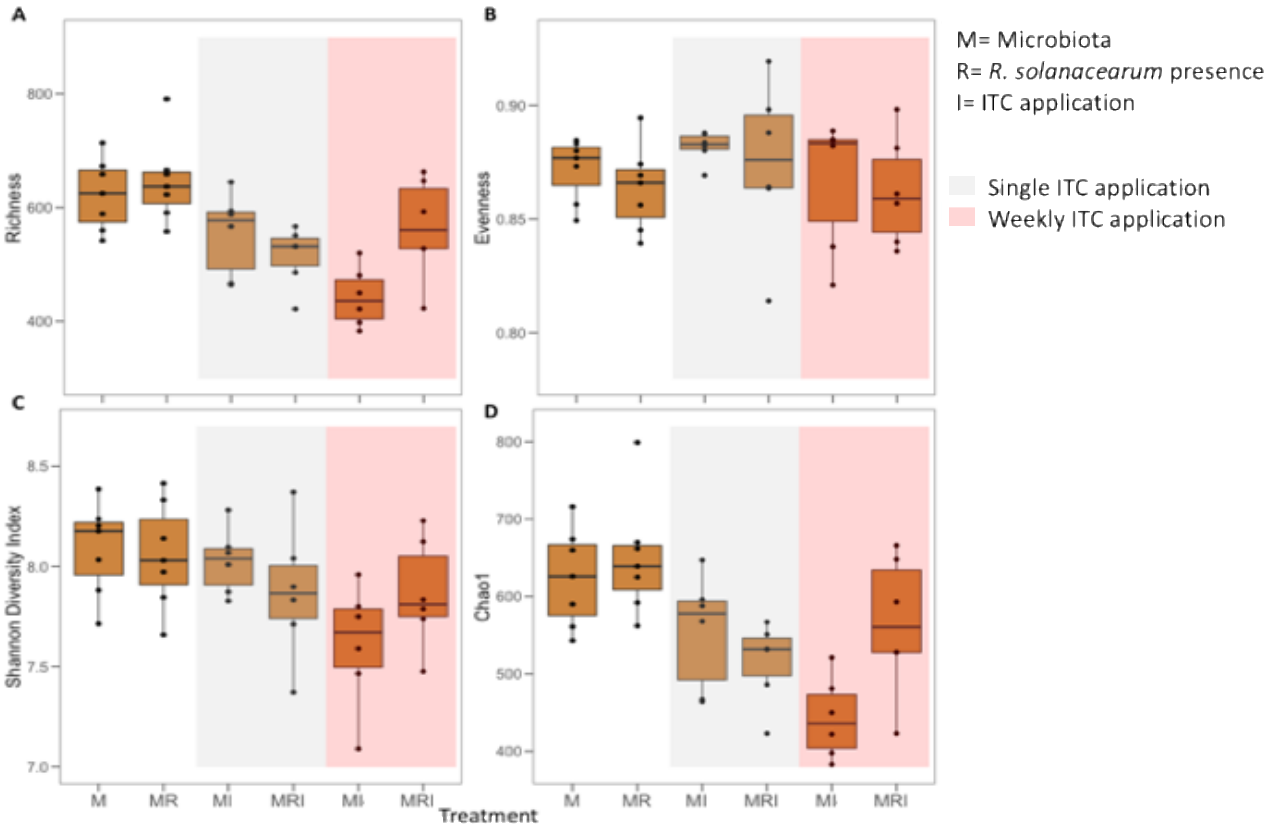


Figure 5.4.2. Effects of ITC exposure (single or weekly) on microbiota alpha diversity metrics in the absence and presence of the *R. solanacearum* pathogen. Panel (A) shows species richness, (B) shows community evenness, (C) shows Shannon diversity index and (D) shows Chao1 diversity index based on 16S rRNA amplicon sequence data sampled at the end of the mesocosm experiment. White, grey and red background shading indicate no, single and weekly ITC application, respectively. All boxplots show the minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=7 for all treatments).

Table 5.4.1. Effects of ITC exposure and *R. solanacearum* presence on microbiota beta-diversity metric statistics, showing the effects on Bray-Curtis and Weighted-UniFrac beta-diversity metrics measured by ANOSIM and PERMANOVA and the test-statistic and p-value. An asterisk (*) after p-value indicates statistical significance (p< 0.05).

	Bray-Curtis		**Weighted-UniFrac**	
	ANOSIM	**PERMANOVA**	**ANOSIM**	**PERMANOVA**
ITC application	H= 0.114 p= 0.02 *	H= 1.71 p= 0.019 *	H= 0.0145 p= 0.001 *	H= 1.99 p= 0.014 *
***R. solanacearum* presence**	H= 0.314 p= 0.001 *	H= 4.336 p= 0.001 *	H= 0.271 p= 0.001 *	H= 6.175 p= 0.001 *

We found that ITC exposure and *R. solanacearum* presence both affected microbiome composition (ITC: 1.71, p= 0.019; *R. solanacearum* presence: 4.336, p= 0.001; Fig. 5.4.3; Table 5.4.1), and weekly ITC application had relatively larger effects that single ITC application treatments (Tukey: p< 0.05; Fig. 5.4.3). To better understand similarities and differences between no-ITC and ITC-exposed microbiota, we quantified the core microbiota that were shared by all treatment samples. The shared core microbiota common to all treatments predominantly consisted of *Parasegetibacter*, Microscillaceae (family), *Bacillus, Dyella, Pirellula, Sphingomonas* and *Flavisolibacter.* Many more unique taxa were observed in the no-ITC compared to either single or weekly ITC application treatments (53 genera in no-ITC compared to 19 genera in single instance ITC and 9 genera in weekly ITC application treatments; Appendix Fig. B1A). While genera including *Lysinibacillus*, *Marmoricola* and *Gaiella* were observed exclusively in the absence of ITC, *Solirubrobacteriales* were observed only in the presence of ITC. Finally, there was overlap of 234 genera both in the presence and absence of *R. solanacearum* (Appendix Fig. B1B), and four more unique genera were identified in the presence of *R. solanacearum* (42 versus 38, respectively). Specifically, *Acinetobacter, Pseudaminobacter* and *Leifsonia* occurred exclusively in the absence of *R.*

solanacearum, while *Roseimicrobium, Mycobacterium* and *Undibacterium* were observed only in the presence of *R. solanacearum* (Appendix Fig. B1B). Together, these results suggest that similar taxa were found to be present in all treatments despite the application of ITC and *R. solanacearum* presence.

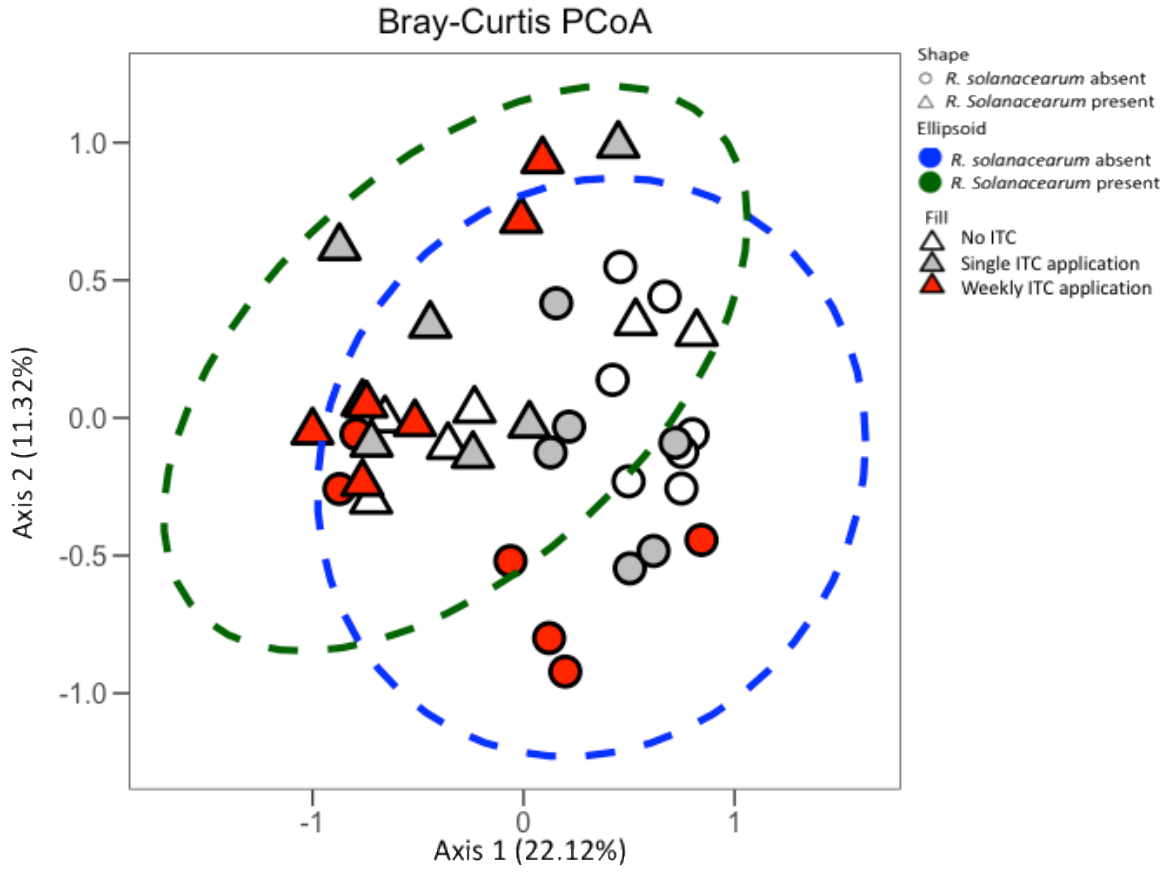


Figure 5.4.3. Effects of ITC exposure (single or weekly) and *R. solanacearum* presence on microbiota composition. Figure shows community differences based on the Bray-Curtis distance matrix. Confidence ellipsoids are clustered based on *R. solanacearum* presence (blue; triangles) or absence (green; circles), while white, grey and red colours denote for ITC application treatments (N=7 for all treatments).

(c) ITC exposure and *R. solanacearum* presence had differential effects on surrounding microbiome taxa abundances

LEfSe was used to explore the effects of ITC application and *R. solanacearum* presence on taxa abundances of the ten most abundant phyla and differential bacterial genera in more detail. At the phyla level, only Planctomycetes were suppressed by ITC, while no phyla were enriched by ITC (Fig. 5.4.4A). Moreover, Firmicutes, Proteobacteria, Gemmatimonadetes and Chloroflexi were enriched in *R. solanacearum* presence, while Bacteroidetes and Planctomycetes were suppressed in the presence of *R. solanacearum* (Fig. 5.4.4B). We also characterised taxa differences at the genus level. In total, 39 genera were found to be sensitive to ITC suppression, including *Pirellula, Dyella* and *Flavisolibacter* (Fig. 5.4.5A), while 20 genera were enriched by ITC, including *Chitinophaga, Rhodococcus* and *Pseudomonas* (Fig. 5.4.5A). Moreover, 37 genera were enriched (e.g. Bacillales (order), *Hyphomicrobium* and *Chitinophaga*) while 33 genera (e.g. *Pirellula, Dyella* and *Bacillus*) were suppressed in the presence of *R. solanacearum* (Fig. 5.4.5B). Together these results suggest that ITC had negative effects on the abundance of several bacterial genera across multiple phyla.

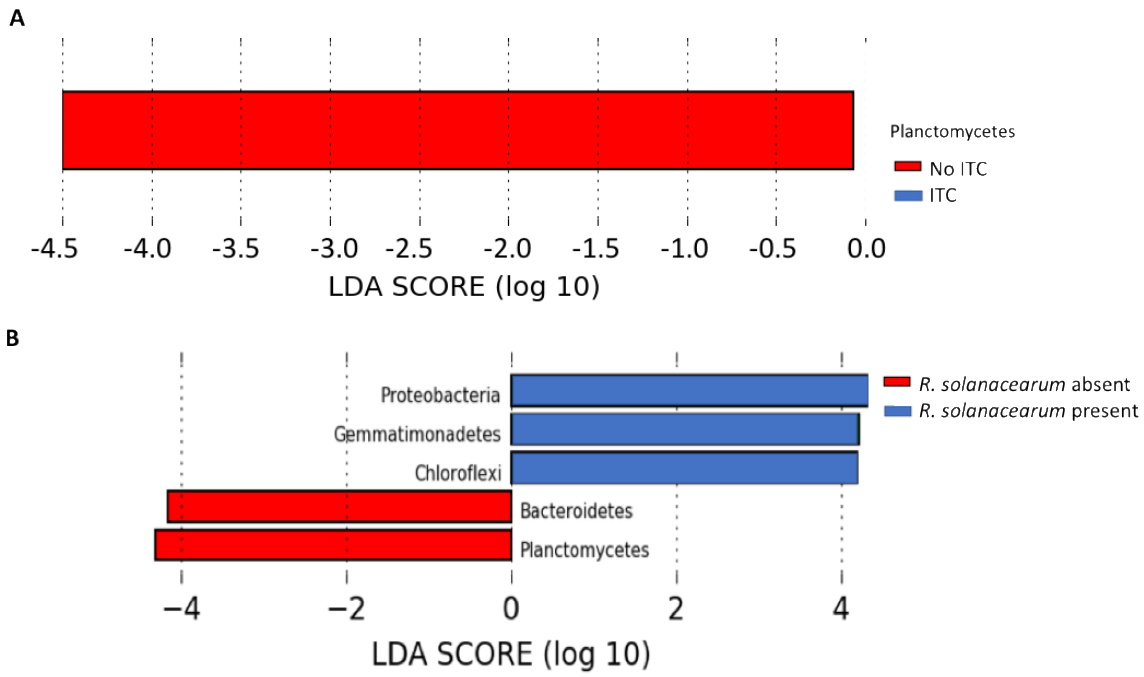


Figure 5.4.4. Effects of ITC exposure and *R. solanacearum* presence on microbiota phyla (ten most abundant phyla) abundances. Panels show differential phyla abundances (determined by LEfSe) when exposed to ITC (A) and in the presence of *R. solanacearum* (B). Bar colours show phyla that were enriched (blue) or suppressed (red). The lengths of the bars represent log10 transformed LDA scores (N=7 for all treatments).

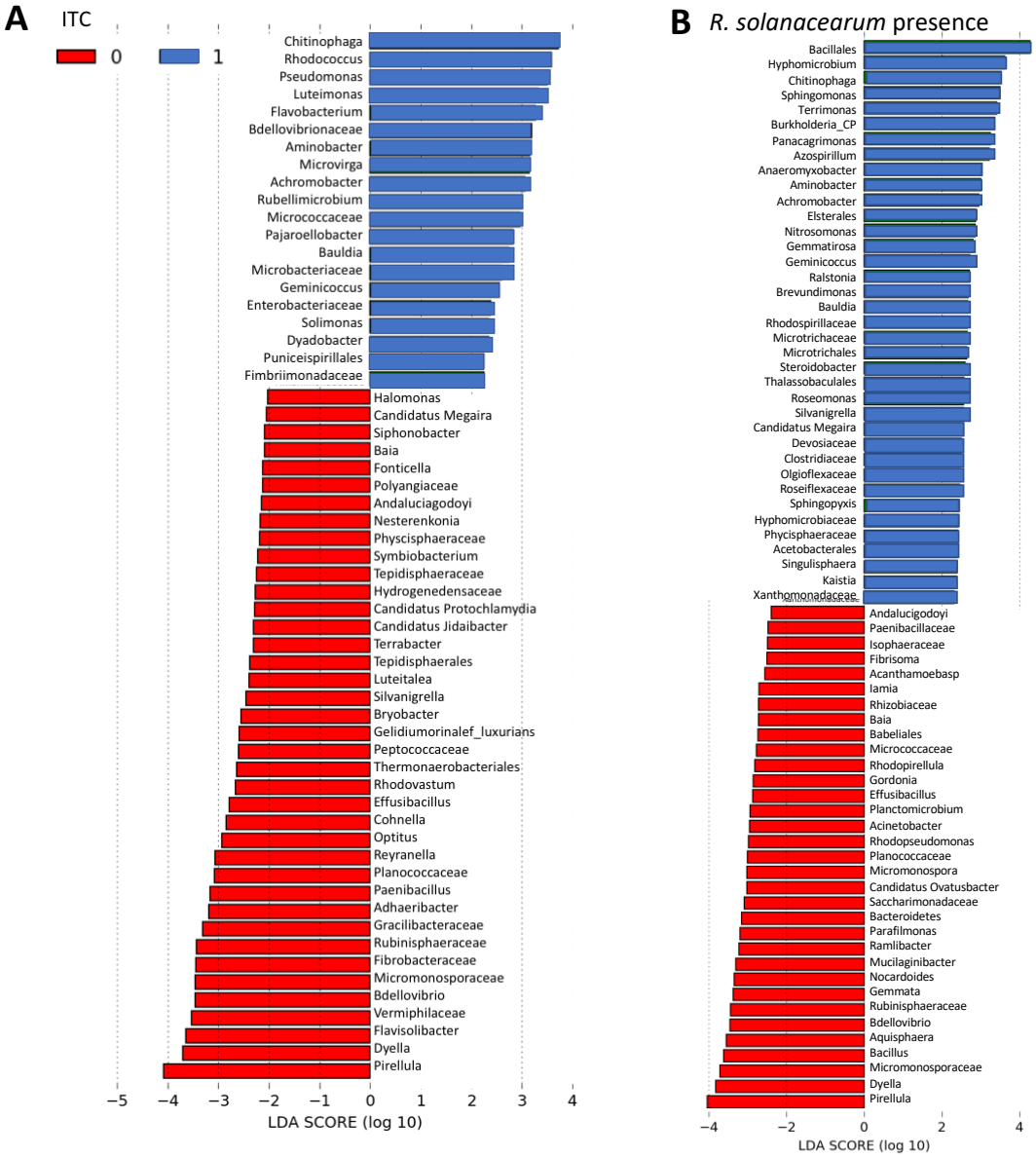

A
ITC
0
1
Chitinophaga
Rhodococcus
Pseudomonas
Luteimonas
Flavobacterium
Bdellovibrionaceae
Aminobacter
Microvirga
Achromobacter
Rubellimicrobium
Micrococcaceae
Pajaroellobacter
Bauldia
Microbacteriaceae
Geminicoccus
Enterobacteriaceae
Solimonas
Dyadobacter
Puniceispirillales
Fimbriimonadaceae
Halomonas
Candidatus Megaira
Siphonobacter
Baia
Fonticella
Polyangiaceae
Andaluciagodoyi
Nesterenkonia
Physcisphaeraceae
Symbiobacterium
Tepidisphaeraceae
Hydrogenedensaceae
Candidatus Protochlamydia
Candidatus Jidaibacter
Terrabacter
Tepidisphaerales
Luteitalea
Silvanigrella
Bryobacter
Gelidiumorinalef_luxurians
Peptococcaceae
Thermonaerobacteriales
Rhodovastum
Effusibacillus
Cohnella
Optitus
Reyranella
Planococcaceae
Paenibacillus
Adhaeribacter
Gracilibacteraceae
Rubinisphaeraceae
Fibrobacteraceae
Micromonosporaceae
Bdellovibrio
Vermiphilaceae
Flavisolibacter
Dyella
Pirellula
−5 −4 −3 −2 −1 0 1 2 3 4
LDA SCORE (log 10)
B
R. solanacearum presence
Bacillales
Hyphomicrobium
Chitinophaga
Sphingomonas
Terrimonas
Burkholderia_CP
Panacagrimonas
Azospirillum
Anaeromyxobacter
Aminobacter
Achromobacter
Elsterales
Nitrosomonas
Gemmatirosa
Geminicoccus
Ralstonia
Brevundimonas
Bauldia
Rhodospirillaceae
Microtrichaceae
Microtrichales
Steroidobacter
Thalassobaculales
Roseomonas
Silvanigrella
Candidatus Megaira
Devosiaceae
Clostridiaceae
Olgioflexaceae
Roseiflexaceae
Sphingopyxis
Hyphomicrobiaceae
Phycisphaeraceae
Acetobacterales
Singulisphaera
Kaistia
Xanthomonadaceae
Andalucigodoyi
Paenibacillaceae
Isophaeraceae
Fibrisoma
Acanthamoebasp
Iamia
Rhizobiaceae
Baia
Babeliales
Micrococcaceae
Rhodopirellula
Gordonia
Effusibacillus
Planctomicrobium
Acinetobacter
Rhodopseudomonas
Planococcaceae
Micromonospora
Candidatus Ovatusbacter
Saccharimonadaceae
Bacteroidetes
Parafilmonas
Ramlibacter
Mucilaginibacter
Nocardoides
Gemmata
Rubinisphaeraceae
Bdellovibrio
Aquisphaera
Bacillus
Micromonosporaceae
Dyella
Pirellula
−4 −2 0 2 4
LDA SCORE (log 10)

Figure 5.4.5. Effects of ITC exposure and *R. solanacearum* presence on microbiota genera abundances. Panels show differential genera abundances (determined by LEfSe) when exposed to ITC (A) and in the presence of *R. solanacearum* (B). Bar colours show genera that were enriched (blue) or suppressed (red). The length of the bars represents log10 transformed LDA scores (N=7 for all treatments).

(d) ITC effect on bacterial co-occurrence networks

Bacterial co-occurrence networks were constructed based on significant taxa correlations at the genera level (Pearson: $p< 0.05$) within microbiome treatments in the absence and presence of ITC (single and weekly ITC application treatments pooled together both networks pooled over *R. solanacearum* presence). While the number of nodes was similar between the treatments (No-ITC: 231 nodes, ITC applied: 272 nodes), ITC exposure increased the number of edges (No-ITC: 1983 edges, ITC applied: 6467 edges; Fig. 5.4.6; Table 5.4.2). Additionally, there were fewer negative taxa associations in the presence of ITC (Fig. 5.4.6; Table 5.4.2). Despite this, other factors including path length and clustering coefficient remained similar between treatments (Fig. 5.4.6; Table 5.4.2). Together, these results suggest that ITC application mainly increased co-occurrence network connectivity.

Table 5.4.2. Effects of ITC exposure and *R. solanacearum* presence on microbiota genera level co-occurrence network statistics. Network metrics were determined using CytoScape in samples in the presence or absence of ITC.

	No ITC	**ITC**
Number of nodes	231	272
Number of edges	1983	6467
Average number of neighbours	17.17	17.75
Network diameter	8	8
Network radius	1	1
Characteristic path length	2.69	2.74
Clustering coefficient	0.204	0.174
Network density	0.037	0.033
Connected components	1	2

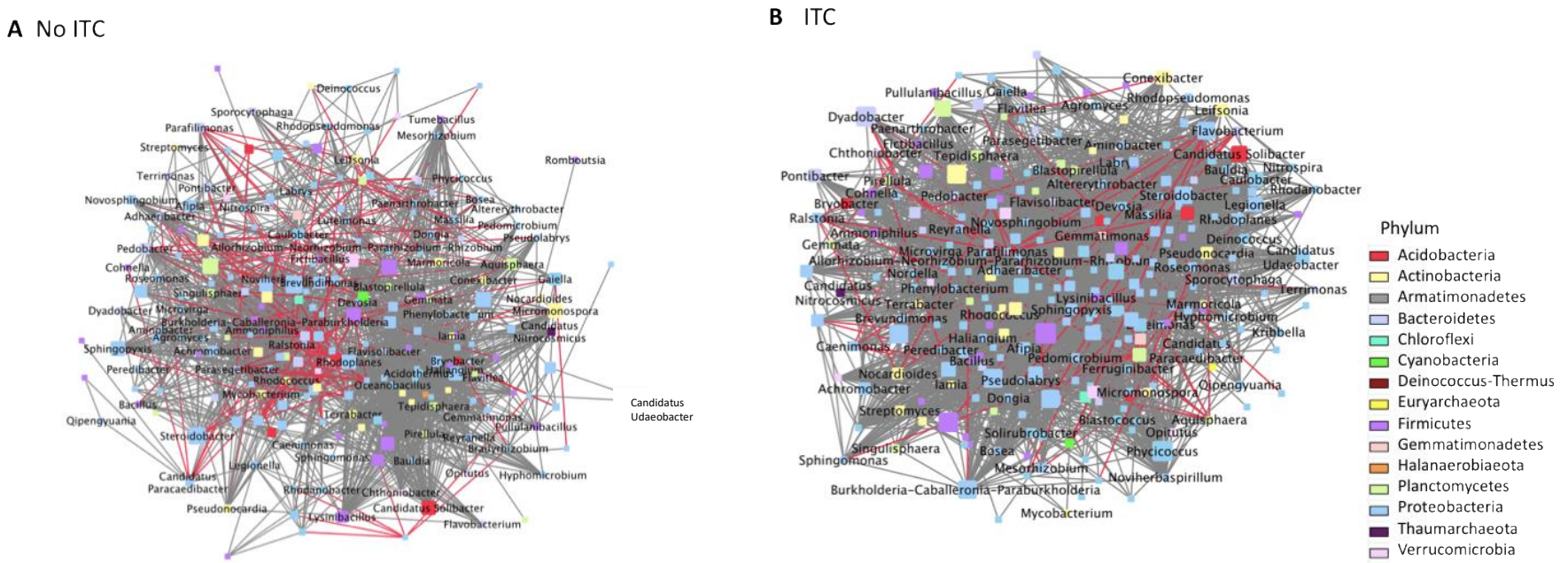


Figure 5.4.6. Effects of ITC application on genera level co-occurrence networks. Panels (A) and (B) show genera in the absence and presence of ITC, respectively. Networks include samples both in the presence and absence of *R. solanacearum* pathogen, and ITC network is pooled over single and weekly ITC application treatments. Only genera with average abundances > 0.1%, and $p < 0.05$ (Pearson correlation) are included in the networks. Nodes are coloured based on the phyla classification of the genera (see key). Square nodes indicate bacteria whereas triangles represent archaea. Grey links indicate positive associations between genera whilst red links show negative associations. Genera are only shown if they were unique to the treatment. Node size is correlated with betweenness centrality (large node= high betweenness centrality value). Genera names are only displayed if they formed a highly significant ($p< 0.001$) association with other genera (N=7 for all treatments).

(e) Quantifying *R. solanacearum* tolerance evolution to ITC

To test for potential ITC tolerance evolution under ITC exposure, *R. solanacearum* isolates that had evolved in the presence and absence of the microbiota were re-exposed to ITC in a separate liquid microcosm experiment. Previous ITC exposure improved ITC tolerance compared to the ancestral and clones not exposed to ITC ($F_{3, 46} = 7.69$; $p<0.001$; Tukey: $p< 0.05$; Fig. 5.4.7). Furthermore, *R. solanacearum* clones previously grown in the presence of a microbiome community reached significantly lower bacterial densities ($F_{1,40} = 6.51$, $p< 0.05$; Fig. 5.4.7), while *R. solanacearum* clones showed improved ITC tolerance when derived from sterile soil and exposed to ITC weekly (ITC*Microbiome: $F_{2, 43} = 16.02$, $p< 0.001$; Fig. 5.4.7). Together, these results suggest that *R. solanacearum* showed signs of ITC tolerance evolution in the tomato rhizosphere and these effects were particularly pronounced with clones exposed to ITC weekly.

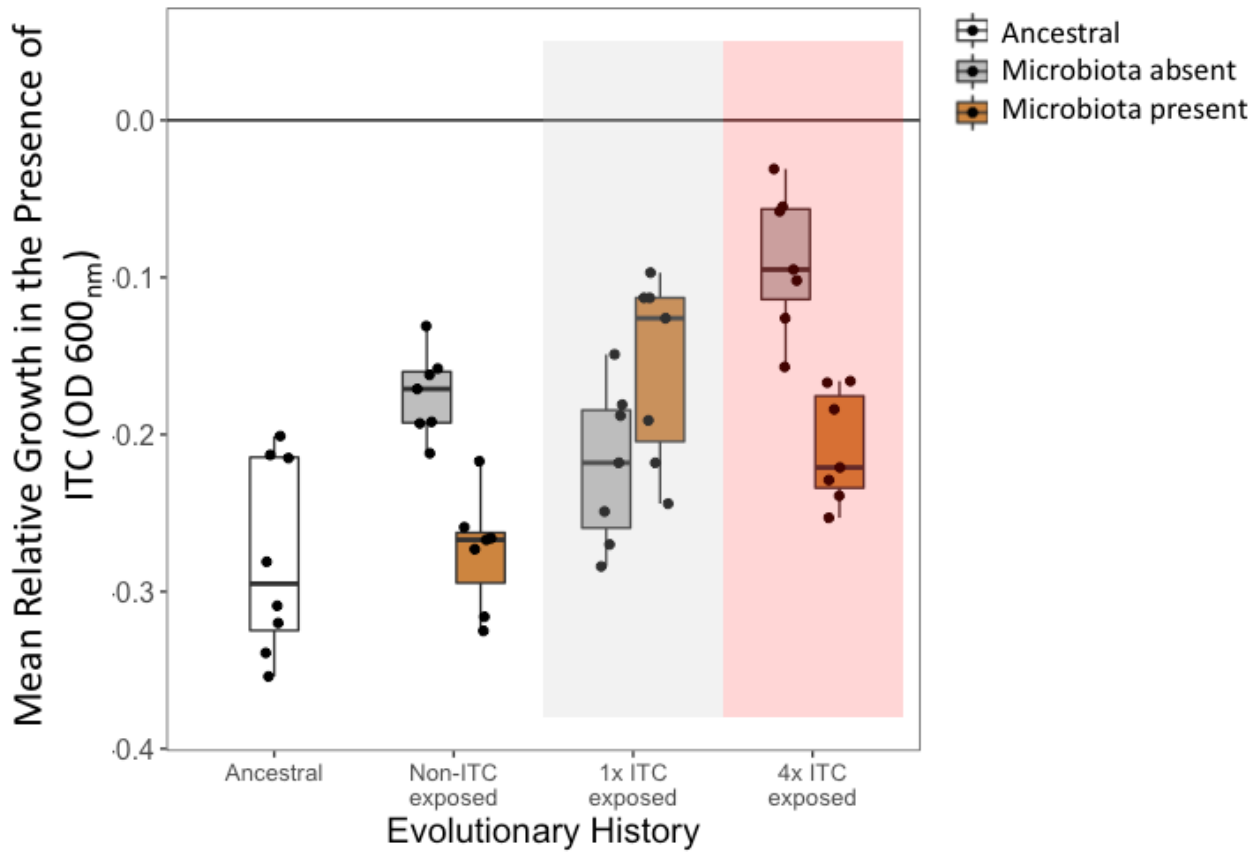


Figure 5.4.7. *R. solanacearum* ITC tolerance measured at the end of the tomato mesocosm experiment. ITC tolerance was measured as the relative growth of bacteria in the presence versus absence of ITC for the ancestral clone (white) and evolved clones from the ITC exposed and non-ITC exposed control *R. solanacearum* isolates. The black horizontal line indicates no effect of ITC on bacterial growth, while observations below the line denote for negative effects of ITC on *R. solanacearum* growth. White, grey and red background shading indicate treatment histories with no, single and weekly ITC application, respectively. The boxplots show the minimum, maximum, interquartile range and the median (black line). Individual data points show bacterial densities for each biological replicate (N=8 for the ancestral and N=7 for all evolved clones).

5.5. Discussion

Here we studied the effects of *Brassica*-derived allyl-ITC allelochemical on *R. solanacearum* and the rhizosphere microbiota in tomato mesocosms. We found that while ITC suppressed *R. solanacearum* densities only in the presence of microbiota, reduction in disease severity by ITC was observed also in the absence of microbiota. Our evidence indicated that ITC also had collateral effects on the microbiota by causing a reduction in microbial community diversity and clear taxa-specific changes in microbiota composition. ITC effects on the surrounding microbiota were taxa-specific, and while no phyla were enriched by ITC, Planctomycetes were suppressed. At the genera level, a relatively higher number of genera were suppressed by ITC than were enriched and network analyses further revealed that ITC exposure increased the number of unique associations, potentially due to enrichment of certain genera. Of concern, ITC exposure reduced tomato dry weight also in the absence of microbes, indicative of phytotoxic effects. Furthermore, we observed signs of ITC tolerance evolution. Together, our results suggest that allyl-ITC derived from Indian mustard biofumigant is effective at suppressing the growth of *R. solanacearum* in the tomato rhizosphere. However, ITC exposure influenced the surrounding microbial community and tomato growth, which could limit the benefits of this biocontrol method.

ITC application had a negative effect on *R. solanacearum* densities in our model biofumigation experiment only when grown in the presence of microbiota. This suggests ITC exposure may be effective against *R. solanacearum* in natural soil microbial communities, and corroborates other studies that show *R. solanacearum* sensitivity to ITC (Smith and Kirkegaard, 2002; Arthy *et al.*, 2005; Olivier *et al.*, 2006). However, we observed disease reduction by ITC even in the absence of microbiota despite not finding any effects on

pathogen densities. One explanation for this is that ITC exposure affected gene expression of *R. solanacearum*, leading to reduced virulence. In support for this, it has been shown previously that different plant derived compounds, such as coumarins, can *suppress R. solanacearum* virulence gene expression (Yang *et al.*, 2017; Han *et al.*, 2021). It is thus possible that ITC effects could have also altered pathogen gene expression in addition to biocidal effects.

We also found that especially weekly ITC exposure reduced tomato dry weight in the absence of *R. solanacearum*, which may have implications for plant reproductive output and crop yields. In line with our finding, previous studies have identified phytotoxic effects of biofumigation on host plants (Intanon *et al.*, 2014), and the severity of these effects has been shown to correlate with biofumigant GSL levels and ITC concentrations (Handiseni *et al.*, 2013). The ITC concentration applied in the current study (0.5 μmol g^{-1} soil) may be higher than those achieved during field biofumigation and could explain the phytotoxic effects. Furthermore, while recommended biofumigation practice suggests allowing at least two weeks following biofumigation before sowing the following crop (Cohen and Mazzola, 2006b), we applied ITC directly to cut crop roots which likely exacerbated ITC phytotoxicity and is not reflective of field biofumigation. Our findings support those identified in our previous chapter (Chapter 3), where ITC application reduced tomato dry weight in sterile soil. Importantly, since this finding was only observed under repeated ITC exposures, there may be the potential to reduce the frequency of ITC release during biofumigation to minimise phytotoxic effects. For instance, thorough mulching of *Brassica* tissue into soil may maximise the likelihood of high ITC release in a single instance with little harmful effects on the crop plant (Sarwar *et al.*, 1998).

ITC exposure also reduced microbial diversity and changed microbial community composition with collateral effects on non-target microbiota. Genera including *Pirellula, Dyella* and *Flavisolibacter* were particularly vulnerable to ITC suppression. Reduced microbial diversity may increase the likelihood of future pathogen invasion as microbiome diversity has been associated with reduced pathogen invasion success (Wang *et al.*, 2014; Hu *et al.*, 2016). However, one study showed that whilst ITC reduced microbial diversity, ITC-tolerant microorganisms were enriched and many of these microbes have known PGPR properties (Siebers *et al.*, 2018), which may override the effects of reduced diversity to enhance microbiome pathogen invasion resistance. In line with this, we observed enrichment of *Pseudomonas,* a known PGPR genus (Hakim *et al.*, 2021). Furthermore, whilst *R. solanacearum* invasion had no effect on microbial diversity, the presence of *R. solanacearum* alleviated the negative effects of ITC in the weekly ITC application treatment. While it is not clear why this occurred, this result suggests that pathogens may have a buffering effect on microbiome stability when exposed to ITC. We must also note that the microbiota used originated from a potato field, which likely affected the growth of tomato, associated microbiome assembly and potential interactions with ITC and *R. solanacearum.* ITC exposure was also associated with higher network connectivity, which has been found to correlate with reduced pathogen invasion success in the tomato rhizosphere (Wei *et al.*, 2015). Interestingly, this is in contrast with the results observed in the previous chapter and the most likely explanation for this is that the current experiment was much longer and affected by the presence of the plant. Further research is thus needed to determine ITC effects in more realistic field settings, which are likely to be more dynamic and variable.

The evolution of ITC tolerance was observed in *R. solanacearum* clones previously exposed to ITC at single and weekly intervals. Allyl-ITC tolerance evolution has previously

been documented to occur in *in vitro* liquid microcosm experiments under ITC exposure at three-day intervals, and the genetic mechanisms underlying this have been linked to insertion sequence movement, particularly in the megaplasmid (Alderley, Greenrod and Friman, 2021); Chapter 2). Whilst our finding is concerning for the long-term efficiency of biofumigation, there may be ways to mitigate tolerance evolution potential. For example, using a combination of *Brassica* species with different GSL profiles and hence, ITC release mixtures, such as *Brassica juncea* and *Brassica napus* during biofumigation have been shown to maximise pathogen suppression (Mazzola *et al.*, 2007b), and may limit the potential for tolerance evolution by applying multiple selective pressures concurrently.

In conclusion, our findings demonstrate that ITC could potentially be used to suppress the growth of *R. solanacearum* plant pathogen. However, ITC exposure had poor pathogen specificity and also resulted in the suppression of non-target taxa and reduced tomato biomass. We also identified the potential for *R. solanacearum* ITC tolerance evolution which may compromise the long-term success of biofumigation. Future work should validate the effects of allyl-ITC exposure during real field biofumigation.

Chapter 6. General Discussion

6.1. Overview

Food security is threatened by crop losses due to disease and hence, environmentally friendly alternatives to agrochemicals, like biofumigation, are required. A review of the published literature shows variable effects of biofumigation in plant pathogen control. The overall purpose of this PhD project was to determine the long-term efficiency of ITCs released during biofumigation against *R. solanacearum* bacterial plant pathogen, to evaluate the effects of ITCs on non-target microorganisms and how this affects the health and yield of the host crop using a combination of *in vitro* liquid and soil lab experiments and *in vivo* growth chamber experiments. This chapter provides an overview and synthesis of the results of this thesis and discusses them in the context of the three central aims, and their significance and contribution to broader knowledge in the biofumigation research field (Fig. 6.1.1).

This research has progressed the understanding of biofumigation as a biocontrol method and suggests that ITC offers an effective mechanism in controlling the *R. solanacearum* bacterial plant pathogen. Allyl-ITC gave the most effective control of *R. solanacearum* compared to sec-butyl and 2-phenylethyl ITCs, but tolerance developed when serially transferred at three-day intervals in *in vitro* settings. Mechanistically, the genetic changes underlying this were unclear but may have been partly due to insertion mutations in genes linked with antibiotic resistance (dehydrogenase-like protein) and transmembrane protein movement (Tat pathway signal protein) and insertion sequence movement at four positions in genes associated with stress responses, antibiotic production, calcium sequestration and iron storage. The effects of ITC on non-target bacteria were tested,

focusing initially on two species of plant growth promoting *Pseudomonas* in model communities in *in vitro* liquid and soil microcosms, and then upscaling research to test ITC effects in natural rhizosphere communities in the presence of a tomato plant *in vivo*. It was found that *Pseudomonas* were less susceptible to ITC suppression than *R. solanacearum* and there were synergistic suppressive effects of the presence of *Pseudomonas* and ITC on *R. solanacearum* growth. Similarly, when validated in natural rhizosphere communities, *R. solanacearum* was suppressed by ITC and this was clearer in the presence of microbiota. Furthermore, evidence for ITC tolerance evolution was also observed in *in vivo* settings where *R. solanacearum* had previously been exposed to ITC once and four times at weekly intervals, particularly when *R. solanacearum* was isolated from sterile soil. However, ITC tolerance was less obvious across all *in vivo* experiments than *in vitro*, potentially due to a greater range of selective pressures acting simultaneously compared to the less complex *in vitro* settings. Furthermore, the potential costs of tolerance could have been greater in the soil. Moreover, suppressive effects of ITC were observed on overall microbial diversity in rhizosphere communities and certain rare taxa were driven to extinction. While phyla like Firmicutes and Planctomycetes were particularly susceptible to ITC exposure, other phyla including Actinobacteria benefitted from ITC exposure. Interestingly, despite the suppressive effects of ITC exposure on *R. solanacearum* densities, this did not consistently correlate with reduced wilt disease symptoms in tomato hosts, and instead, reduced tomato dry weight and flowering in the presence of *Pseudomonas* PGPR. Further research is required to determine whether the shifts to microbiome structure induced by ITC have positive or negative effects on soil ecosystem functioning, including future pathogen invasion.

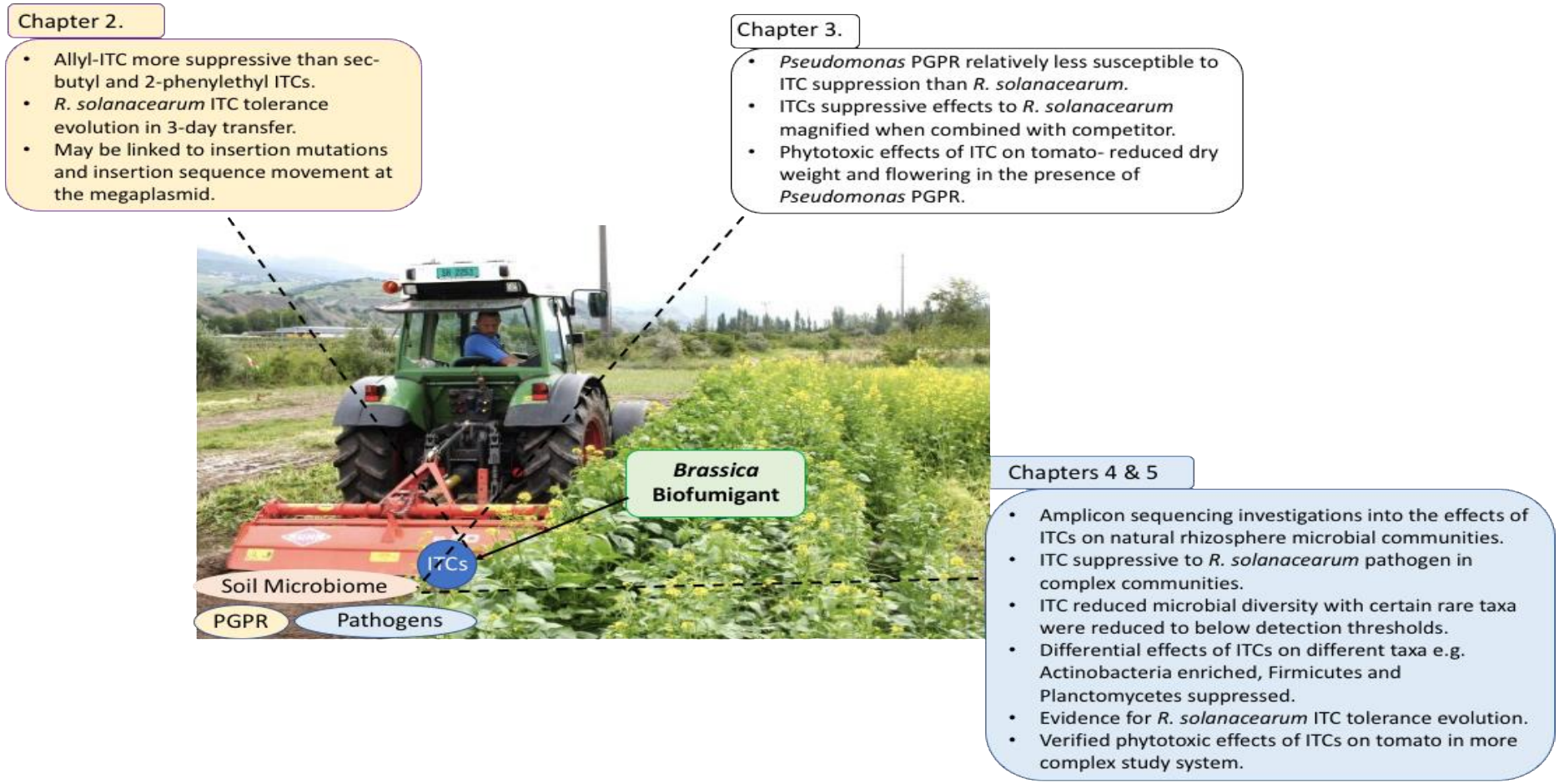


Figure 6.1.1. Thesis Data Chapter summary. The main findings of each chapter and how this progresses the knowledge of biofumigation and its wider microbiome effects.

6.2. Evolution of tolerance to ITC in *R. solanacearum*

To ensure the long-term efficiency of biofumigation, it is important to verify that pathogens do not evolve tolerance or resistance to biocidal ITCs under repeated exposure. This was modelled in simplified lab experiments *in vitro* and it was found that *R. solanacearum* developed tolerance to allyl-ITC when exposed at three-day intervals. Interestingly, this tolerance was also observed in control samples that had not previously been exposed to ITC. In this case, ITC tolerance is likely a result of media adaptation as when tested in a new media, ITC tolerance was lost in these samples. At the genetic level, ITC tolerance was linked with insertion mutations in the dehydrogenase-like uncharacterised protein and Tat pathway signal protein, although these mutations did not occur consistently across all ITC tolerant control clones. To investigate this further, we also analysed the presence and absence of intermediate indels (≥50 bp), prophages and insertion sequence movement. Similarly for the intermediate indels and prophages, we did not identify any clear changes between the ITC tolerant and non-tolerant or ancestral strains. However, insertion sequence movement at four locations, especially in the megaplasmid (three positions), was highly specific to ITC tolerant strains. The movement of these insertion sequences could have enabled ITC tolerance by knocking out costly genes, deleting genes following homologous recombination, or causing gene and gene cluster duplications (Lee *et al.*, 2016; Hawkey *et al.*, 2020; Sentausa *et al.*, 2020; Arashida *et al.*, 2021). Despite this, we did not identify any genetic changes exclusive to ITC-exposed clones compared to no-ITC clones. This may suggest that ITC tolerance was the result of a combination of epistatic genetic changes and as such is difficult to identify using conventional approaches. Overall, the regularity of serial transfers had a relatively larger effect on *de novo* mutations and insertion

sequence movement than ITC exposure. Since ITC tolerance was only observed when serial transfers occurred at low frequency, there may be the potential to control for pathogen ITC tolerance evolution during field biofumigation by limiting repeated exposures. This may be achieved by ensuring high ITC incorporation levels to maximise pathogen elimination and thorough mulching of high-GSL producing biofumigant material into soil followed by irrigation (Kirkegaard and Matthiessen, 2005). This should reduce the extent of later or residual ITC release during *Brassica* degradation and seal ITCs into soils.

Similarly, when tested in more complex rhizosphere microbial communities, evidence for ITC tolerance evolution was also observed (Chapter 5) both when exposed at a single instance or repeatedly every week. Enhanced biodegradation has previously been documented after single ITC exposure (Warton, Matthiessen and Shackleton, 2003a). In the presence of a rhizosphere microbiome, *R. solanacearum* tolerance was particularly clear when exposed to ITC at weekly intervals, potentially due to repeated exposures increasing the selective pressure to develop ITC tolerance. Although concerning for the long-term efficiency of biofumigation, ITC tolerance evolvability was reduced in more near-natural complex rhizosphere communities compared to *in vitro* settings. To constrain ITC tolerance in the field, growers should aim to maximise ITC release from biofumigants as a single release to reduce the selection for *de novo* mutants during repeated ITC exposure.

Despite ITC tolerance evolution in *in vitro* liquid microcosms (Chapter 2) and *in vivo* tomato rhizosphere experiments (Chapter 5), when *R. solanacearum* ITC tolerance was tested in *in vitro* soil microcosms when grown alone or in co-culture with a *Pseudomonas* species under a single instance of ITC exposure with no tomato host plant (Chapter 3), there was no evidence for *R. solanacearum* ITC tolerance evolution. This may be due to the very

short experiment, lasting only 4 days, which may not have enabled sufficient time for the resistant mutants to emerge and increase in frequency.

The use of different study systems allowed the determination of *R. solanacearum* ITC tolerance evolution in *in vitro* and *in vivo* settings. This research could act as a platform to identify ITC tolerance evolution in more realistic settings for biofumigation. Future work should determine ITC tolerance evolution in the field by applying allyl-ITC as in the present studies directly to plant roots at different frequencies (single ITC exposure and weekly ITC exposure) in *R. solanacearum* infected fields and isolating *R. solanacearum* colonies following ITC fitness assays in the lab. This would enable validation of ITC tolerance studies presented here in natural rhizosphere communities in field conditions. It will also be important to identify ways to mitigate *R. solanacearum* tolerance evolution by maximising ITC release and preventing multiple ITC exposures.

6.3. ITC effects on non-target microorganisms

Optimal biofumigation should have highly target-specific pathogen suppressive effects with minimal effects on non-target microbiota. Previous research has shown that even minor changes to individual microbes can have broader ecosystem effects, as is well-established in the gut microbiome (Hacquard *et al.*, 2015; Shin, Whon and Bae, 2015). In the case of biofumigation, effects on rhizosphere microbiome composition have been shown to correlate with ITC concentration (Rumberger and Marschner, 2003). Using a series of experiments, starting initially with simple *in vitro* liquid and soil microcosms and increasing complexity to *in vivo* tomato mesocosm experiments with near-natural rhizosphere communities, the effects of allyl-ITC on non-target microorganisms were tested. In Chapter 4, *R. solanacearum* was introduced to microbial communities derived from the kale and

potato rhizosphere in soil microcosms and exposed to ITC for a short period of time. In Chapter 5, *R. solanacearum* invasability was tested in microbial communities derived from potato in tomato mesocosms when exposed to ITC at different rates for a longer period. This enabled comparison of ITC effects in different crop rhizosphere microbiomes both in the presence and absence of a host crop which likely recruits its own rhizosphere also.

In model communities tested *in vitro*, there were suppressive effects of ITC to *Pseudomonas* PGPR, though pathogenic *R. solanacearum* was suppressed to a relatively greater extent by ITC. There were also synergistic effects of ITC exposure and the presence of a competitor on *R. solanacearum* densities, suggesting that in the highly complex soil microbiome, ITC may have stronger suppressive effects on the pathogen. Meanwhile, in rhizosphere microbial communities, whilst *R. solanacearum* was still suppressed by ITC in all study systems, there were also collateral effects of ITC on soil microbiome composition and diversity and certain rare taxa were eliminated. Specifically, in Chapter 4, Actinobacteria were enriched by ITC, while many phyla, including Firmicutes were suppressed, while in Chapter 5, Planctomycetes were suppressed by ITC and no phyla were enriched. Rearrangement of microbial community structure may have wider effects on nutrient cycling and the sensitivity of the microbiome to future pathogen invasion (Hu *et al.*, 2020). However, effects on co-occurrence networks differed between study systems. Specifically, in soil microcosms, ITC exposure led to reduced network connectivity (Chapter 4), while network connectivity was increased by ITC in *in vivo* tomato mesocosm experiments (Chapter 5). Network connectivity has been associated with microbiome susceptibility for pathogen invasion (Thébault and Fontaine, 2010; Wei, Yang, Friman, *et al.*, 2015b; Wei *et al.*, 2018), and these results could have implications for levels of soil disease suppressiveness following ITC exposure. Potentially, the presence of the tomato host plant

in Chapter 5 but absence in Chapter 4, as well as the differences in the length of the experiments (Chapter 5= 37 days following *R. solanacearum* infection, Chapter 4= 4 days following infection) resulted in the differences observed between the two experiments. Additionally, in both rhizosphere microbiome chapters (4 and 5), we found that ITC exposure reduced Chao1 index which suggests larger reductive effects on rare taxa (Chao, 1984). Whilst rare taxa (and high Chao1 index values) are often associated with disease resistance (Hol *et al.*, 2015; Jousset *et al.*, 2017; Wei *et al.*, 2019), in terms of *R. solanacearum* invasion specifically, diseased plants have been associated with higher Chao1 indexes in their rhizosphere than healthy plants (Hu *et al.*, 2020). This suggests that the ITC associated reduction in rare taxa abundance identified in this work may increase tolerance to *R. solanacearum* pathogen invasion. However, since the consensus generally appears to be that rare taxa reduce microbiome pathogen invasion (Hol *et al.*, 2015; Jousset *et al.*, 2017; Wei *et al.*, 2019), ITC could also increase susceptibility to microbiome invasion by other pathogens. This work has revealed the broad-spectrum action of ITC and the rearrangement of communities following ITC exposure. The long-term effects of this on levels of soil disease suppression, microbial ecosystem functioning, and whether the microbiome restores to its pre-ITC exposed state following ITC loss by degradation and volatilisation remains to be elucidated.

6.4. ITC effects on the host crop

The overarching aim of biofumigation and ITC application is to improve crop yields by limiting disease. This thesis investigates the effects of ITC in controlling against *R. solanacearum* growth and infectivity in the tomato host plant. This was tested in sterile soil inoculated with model co-cultures (Chapter 3) and in soil inoculated with the potato

rhizosphere microbiome (Chapter 5) to investigate the direct effects of ITC on the plant in the absence of microbial interference and the indirect effects of ITC on soil microbes and how this affects host crop health.

In both tomato experiments (Chapter 3 and 5), ITC exposure reduced *R. solanacearum* growth and abundance and this resulted in less severe BWD symptoms. However, ITC also reduced tomato dry weight in both experiments, and in Chapter 3, reduced tomato flowering where *Pseudomonas* were inoculated, potentially due to the relatively high ITC concentration used. Whilst higher ITC concentrations have been shown to confer higher levels of pathogen suppression (Bending and Lincoln, 1999; Morra and Kirkegaard, 2002), and crop yield boosting effects (Angus *et al.*, 1994; Triky-Dotan *et al.*, 2007), there may also be phytotoxic side-effects on host crop health (Morales-Rodriguez and Wanner, 2015), particularly when ITCs are incorporated at high concentrations (Handiseni *et al.*, 2013; Mohamed *et al.*, 2013; Intanon *et al.*, 2014). The ITC concentrations achievable during biofumigation are much lower than those used for soil sterilisation (Kirkegaard, Smith and Morra, 2001). A wide range of soil ITC concentrations have been documented following biofumigation, from as little as 0 and 12119 pmol g^{-1} (Rumberger and Marschner, 2003), to as high as 8 µmol g^{-1} (Mattner *et al.*, 2008), and can be maximised by ensuring complete maceration and irrigation immediately following *Brassica* incorporation (Matthiessen *et al*., 2004). However, ultimately, the concentration of ITCs produced depends on many factors including soil texture, temperature, soil moisture, microbial community and pH (Ploeg, 2008). The ITC concentrations used in the experiments in this thesis (0.5 µmol g^{-1} soil) are at the higher end of the established range achievable with biofumigation. The application of liquid allyl-ITC directly to tomato root wounds in this thesis may have increased the phytotoxic effects of ITC on tomato.

6.5. Application of findings in the agricultural context

The work presented in this thesis has progressed our understanding of biofumigation and have implications for biofumigation in the field. Whilst biofumigation has previously been achieved through *Brassica* incorporation as green manure, seed meal and even partial biofumigation (*Brassica* growth without incorporation), the work of this thesis demonstrates the potential to apply pure allyl-ITC, extracted from *Brassica* to control a soilborne pathogenic bacterium. Whilst conventional biofumigation may be more convenient and environmentally sustainable than pure ITCs, *Brassica* ITC release is often low and other, less toxic release products are exuded as well (Bending and Lincoln, 1999; Gardiner *et al.*, 1999; Morra and Kirkegaard, 2002; Gimsing and Kirkegaard, 2006). Furthermore, combinations of different ITCs are secreted from *Brassica* tissues which may be less toxic than single ITC release (Alderley, Greenrod and Friman, 2021); Chapter 2) and growers would need to incorporate large amounts of *Brassica* material into the soil (approximately 20 kg ha^{-1} (Doheny-Adams *et al.,* 2018) to achieve sufficient ITC concentrations to enable pest control. Additionally, *Brassica* reach maximum biomass when grown in spring and harvested in the autumn (Matthiessen and Kirkegaard, 2006), taking out a whole growth season with large economic consequences. Moreover, some studies have found no difference in pathogen densities following *Brassica* amendment (Mazzola, Agostini and Cohen, 2017). In this thesis we demonstrate the lethality of allyl-ITC to *R. solanacearum.* This may indicate the potential to control against bacterial wilt infection of crops by applying allyl-ITC to soil, potentially via drip injection or using tractor-mounted shank injection.

Application of plant extract ITCs like allyl-ITC may have less environmentally damaging effects than synthetic fumigants (Caboni and Ntalli, 2014) and have short half-

lives (Borek *et al.*, 1995), meaning their persistence in the environment is very short and the likelihood of leaching into water systems is low. However, ITCs released during biofumigation have been shown to be just as toxic or even more toxic than synthetic fumigants (Gimsing and Kirkegaard, 2009a). Allyl-ITC has been used in crop production as a biopesticide since the early 1960's and can be used in organic farming when derived from natural sources including essential oil of mustard which can be liberated from dry distilled seeds (Lazzeri and Malaguti, 2004). Alternatively, allyl-ITC is available commercially as part of a formula called Dazitol and Bugitol and has shown promising results for controlling parasitic nematodes in tomato (Hajji-Hedfi *et al.*, 2018) and potato (Turner *et al*., 2007). However, the concentration of allyl-ITC in these formulations is very low (< 3.7%) (Hajji-Hedfi *et al.*, 2018). In the US, a synthetic biofumigant called Dominus has been developed which is composed of 96.3% allyl-ITC as the active ingredient (Janis, 2016).

Aside from plant-derived ITCs, fumigation using the pesticide Dazomet, which converts to methyl-ITC during decomposition, is commonly used as an alternative to methyl bromide. However, fumigated soils suffer long-term reductions in bacterial and fungal abundance and diversity (Ridge and Theodorou, 1972), and nitrogen fixing bacteria are particularly vulnerable (Fang *et al.*, 2018). Additionally, methyl ITC fumigation has environmentally damaging effects, resulting in increased emissions of the greenhouse gas nitrous oxide for 48 days following fumigation (Spokas, Wang and Venterea, 2005).

The success of pure ITCs in pathogen control have previously been demonstrated against fungi (Kurt, Güneş and Soylu, 2011; Ren *et al.*, 2018), nematodes (Dahlin and Hallmann, 2020; Lazzeri *et al.*, 2004; Hajji-Hedfi *et al.*, 2018) and bacteria (Dussault, Vu and Lacroix, 2014; Ren *et al.*, 2018). To date, allyl-ITC is registered in over 30 states in the US (Dominus, Isagro USA Inc., Morrisville, NC, USA) and is most often applied by shank or drip-

injection which provides similar levels of weed and plant parasitic nematode control to agrochemical fumigants (Devkota and Norsworthy, 2014; Ntalli and Caboni, 2017; Ren *et al.*, 2018) and uses machinery generally already available to farmers. The recommended dosage of allyl-ITC is 30-50 g/m^2 (Zhu *et al.*, 2020). Shank injection involves the integration of liquid formulations below the soil surface by a specialised tractor implement. However, due to the volatility of allyl-ITC this method may be less effective in the long-term (Ren *et al.*, 2018). Alternatively, allyl-ITC could be added to irrigation water and integrated with pre-existing fumigant irrigation systems. This may be a more economical and environmentally friendly method (Ajwa *et al.*, 2007). One study compared different allyl-ITC application methods, including single or dual-port shanks and drip injection through one or two tapes, along with different formulations of allyl-ITC to control against a fungal pathogen and observed most effective control when applied via shank injection with a dual port rig (Baggio *et al.*, 2018). The effectiveness of shank injection is supported by other studies (Schneider *et al.*, 2009). Thus, growers should be able to incorporate liquid allyl-ITC cheaply and easily to their fields.

More efficient biocontrol could be achieved by combining allyl-ITC application with other biocontrol techniques as part of an integrated pest management scheme. This may also reduce the likelihood of pathogen resistance evolution by enforcing multiple selective pressures simultaneously. For instance, combining biofumigation using *B. juncea* with allyl-ITC application maximised pathogen control and improved soil structure (Dahlin and Hallmann, 2020). Additionally, combining allyl-ITC with soil solarisation, a soil thermal disinfection method, was shown to be more effective than single biocontrol measures (Hajji-Hedfi *et al.*, 2018). However, other studies have found combining soil solarisation with biofumigation decreased disease control levels, potentially due to increased nitrogen levels which reduced plant growth (Oz, Coskan and Atilgan, 2016; Stevens, David and Storkey,

2018). Furthermore, integrated pest management could benefit from application of PGPR microbes, trap crops and regular crop rotations alongside ITC application (Kirkegaard and Matthiessen, 2005). Previously, bacteriophage have shown promise in controlling against *R. solanacearum* (Fujiwara *et al.*, 2011a; Ramírez, Neuman and Ramírez, 2020) and benefit from being highly specific, with limited effects on non-target microbes, and low risk of phage resistance evolution (Buttimer *et al.*, 2017). Combining phage with other biocontrol methods like PGPR supplementation has shown promise in controlling *R. solanacearum* (Wang *et al.*, 2019). Whilst combining phage with ITC application is yet to be tested directly, this may offer a more effective integrated biocontrol solution. However, careful selection of bacteriophage is important as some phage can increase *R. solanacearum* virulence (Bae *et al.*, 2012; Yamada, 2013). Hence, allyl-ITC biocontrol may function best as part of an integrated pest management scheme.

6.6. Future developments and limitations of biocontrol

While this thesis documents the potential for allyl-ITC to reduce *R. solanacearum* densities in *in vitro* liquid and soil microcosms and *in vivo* tomato growth chamber experiments, there were several concerning findings that may affect allyl-ITC's viability as biocontrol. Of particular concern is the potential for *R. solanacearum* to develop allyl-ITC tolerance. Integration of allyl-ITC application alongside other biocontrol methods may inhibit tolerance evolution by having a broader spectrum of activity which may further reduce pathogen densities and provide concurrently acting, potentially conflicting selective pressures. Additionally, there were phytotoxic effects of allyl-ITC on tomato dry weight and inhibition of flowering where *Pseudomonas* PGPR were inoculated. This could potentially be prevented by applying lower concentrations of allyl-ITC, though this may reduce the

efficiency of pathogen control. Phytotoxic effects may have been augmented by the cutting of tomato roots and application of allyl-ITC directly to wounds, which would not occur during allyl-ITC application in the field. Furthermore, there were collateral effects of allyl-ITC application on non-target microbes and overall microbial diversity was reduced. Our findings corroborate other studies that show that despite a reduction in bacterial diversity following biofumigation, the relative abundance of PGPR in the phylum Actinobacteria were increased (Zhang *et al.*, 2020), which may overall reduce disease susceptibility. Future work should investigate what this means for long-term pathogen invasion potential directly.

Despite the vast amount of existing work on the subject, there are other aspects of biofumigation that need investigation, including the effects of allyl-ITC induced changes to microbial community structure on pathogen invasion, the potential for allyl-ITC to be combined with other biocontrol methods and identifying the most efficient and environmentally friendly ways to extract and apply allyl-ITC. Future research could use the upscaling experimental approach documented in this thesis: beginning with *in vitro* liquid experiments to increasing complexity in soil microcosms, to *in vivo* crop growth chamber experiments and ultimately, to field experiments. This thesis focuses on the potential for allyl-ITC application to control against a single bacterial plant pathogen species, *R. solanacearum*. Other research documents the success of allyl-ITC against *R. solani* (Kirkegaard and Sarwar, 1998; Chung *et al.*, 2010), *V. dahlia* (Olivier *et al.*, 1999), *F. oxysporum* (Smolinska and Horbowicz, 1999), *P. aphanidermatum* and *P. capsica* (Chung *et al.*, 2010) pathogens. However, there is little work published on the effects of ITCs on bacterial plant pathogens. Future work should investigate the potential for allyl-ITC to control against other important bacterial pathogens like *Pseudomonas syringae* (Mansfield *et al.*, 2012).

Application of allyl-ITC may have unprecedented effects on soil fertility by altering nutrient levels, for example. Whilst biofumigation by *Brassica* green manuring has been proven to enhance soil fertility through the incorporation of organic matter and nutrients to soil (Thorup-Kristensen, Magid and Jensen, 2003; Zhang *et al.*, 2020), there has been little research into pure allyl-ITC effects on soil fertility. One study found that allyl-ITC fumigation promoted the growth of subsequent plants by having a 'fertiliser effect' (Ren *et al.*, 2018). This may be due to the enrichment of beneficial microorganisms in the soil as well as increases in the abundance of fungi involved in organic matter decomposition through cellulase and hemicellulose production (Basotra *et al.*, 2016; Ren *et al.*, 2018). However, it has also been shown that low doses of allyl-ITC can inhibit the growth of soil bacteria like *Humicola* that function in nutrient cycling through cellulase, hemicellulose and ligninase secretion (Yang *et al.*, 2006; Ren *et al.*, 2018). Thus, the effects of allyl-ITC on soil fertility are unclear and require further investigation.

6.7. Concluding remarks

This thesis has given an in-depth investigation into allyl-ITC as biocontrol against *R. solanacearum* bacterial plant pathogen. It has considered the specificity of allyl-ITC pathogen control and long-term effects both *in vitro* and *in vivo* and revealed ITC tolerance evolution may threaten long-term biocontrol efficiency.

Allyl-ITC may offer an effective biocontrol against *R. solanacearum* with likely minimal environmental effects due to its short persistence and derivation from natural sources. However, we identify several worrying findings. Firstly, *R. solanacearum* pathogen tolerance evolution threatens the long-term efficiency of allyl-ITC biocontrol. Secondly, there were collateral effects of ITC on non-target microbes which may increase or decrease

future pathogen invasion potential and may have broader ecosystem effects. Thirdly, we observed phytotoxic effects of ITC on tomato that may indicate reduced yield in terms of reduced dry weight and inhibition of flowering where *Pseudomonas* were inoculated. Future work should aim to test these findings in controlled experiments in the field.

Appendices

Appendix A: Chapter 2

Appendix Table A.1. The mean density reduction (%) of *Ralstonia solanacearum* bacterium when exposed to 500 or 1000 µM allyl, sec-butyl and 2-phenylethyl ITCs in CPG growth media after 24, 48 or 72 hours relative to when grown in the absence of ITCs. This table is based on the same data presented in Appendix Fig. A.2.

ITC Type and Concentration (µM)	Time (h)	Bacterial density reduction (%) compared to control
Allyl-ITC, 500	24	66
	48	54
	72	27
Allyl-ITC, 1000	24	66
	48	47
	72	41
Sec-Butyl ITC, 500	24	33
	48	26
	72	9
Sec-Butyl ITC, 1000	24	30
	48	27
	72	8
2-Phenylethyl ITC, 500	24	39
	48	13
	72	10
2-Phenylethyl ITC, 1000	24	38
	48	18
	72	13

Appendix Table A.2. Estimation of ancestral *R. solanacearum* clone doubling time in the presence and absence of ITC during three growth periods of the transfer frequency cycles (0-24h, 24-48h and 48-72h). This information was used to estimate the total number of generations per transfer frequency cycle and the 16-day long selection experiment. The doubling time was calculated based on the ancestral *R. solanacearum* clone cell densities (CFU per mL) at 0h, 24h, 48h and 72h sampling time points in CPG media with and without allyl-ITC (500 μM).

	Doubling time (h)			**Estimate total number of generations per transfer frequency cycle**			**Estimated number of generations experienced during the 16-day selection experiment**	
Growth period	**No ITC**	**ITC**	**Transfer frequency**	**No ITC**	**ITC**	**Transfer frequency**	**No ITC**	**ITC**
0-24h	1.4	1.77	High	17.1	13.5	High	274.2	216.9
24-48h	9.5	14.1	Intermediate	19.6	15.2	Intermediate	157.3	122
48-72h	26.5	17.2	Low	20.5	16.6	Low	120	96.8

Appendix Table A.3. Prophage information of ancestral and experimental isolate assemblies as determined using flanking regions mapped to UY031. Replicates are named by treatments, IntNoITC= Intermediate transfer frequency, no ITC; LowNoITC= Low transfer frequency, no ITC; LowITC= Low transfer frequency, ITC.

Clone	Prophage	Left flank UY031 position	Right flank UY031 position	Length (kb)	GC content (%)	Total proteins #
UY031	Unclassified A	-	-	37	62.76	44
	RS551	-	-	13.4	61.24	16
	PHAGE_Vibrio_VHML_NC_004456	-	-	18.5	64.64	29
Ancestor	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
IntNoITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17

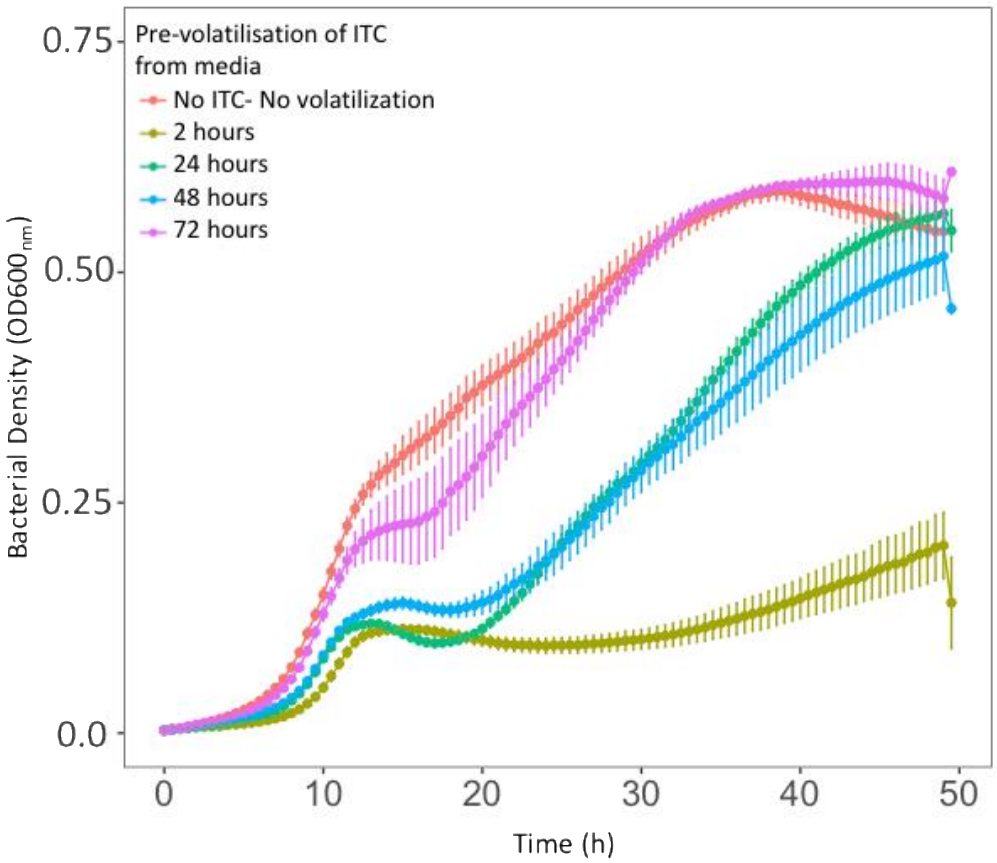
IntNoITC2	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
IntNoITC3	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
IntNoITC4	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC5	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC6	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
IntNoITC7	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	43

	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1218233-1223232	13.1	58.58	17
IntNoITC8	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
LowNoITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17
LowNoITC2	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC3	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC4	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218233-1223232	NZ_CP012687.1:1236385-1237640	13.1	58.58	17

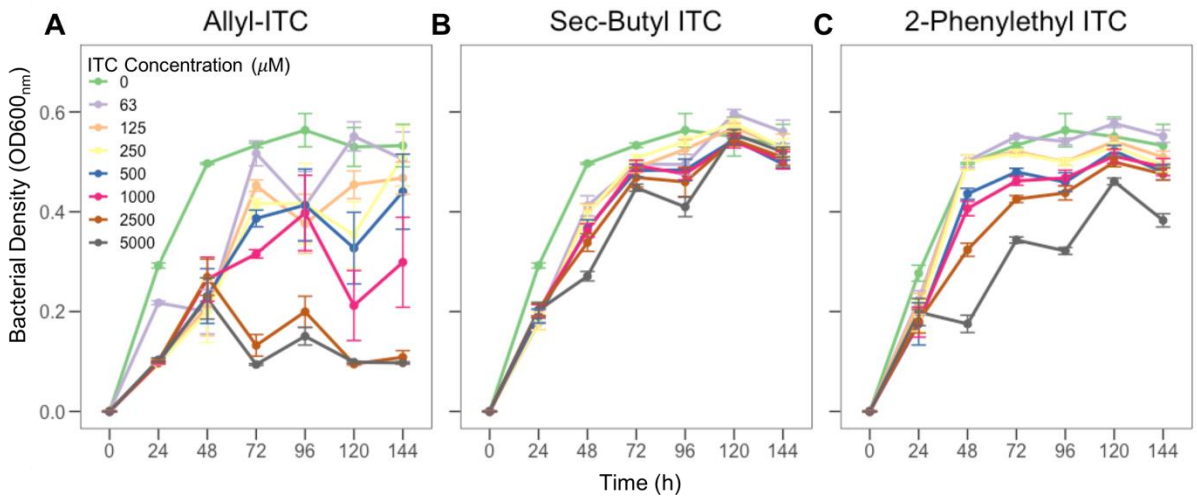
LowNoITC 5	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236385-1237640	13.1	58.58	18
LowNoITC 6	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	18
LowNoITC 7	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	18
LowNoITC 8	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	17
LowITC1	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	43
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	17
LowITC2	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	43

	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	17
LowITC3	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237640	13.1	58.58	18
LowITC4	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
LowITC5	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18
LowITC6	Unclassified A	NZ_CP012687.1:1121824-1126823	NZ_CP012687.1:1162291-1162775	35.4	62.85	42
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	17
LowITC7	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18

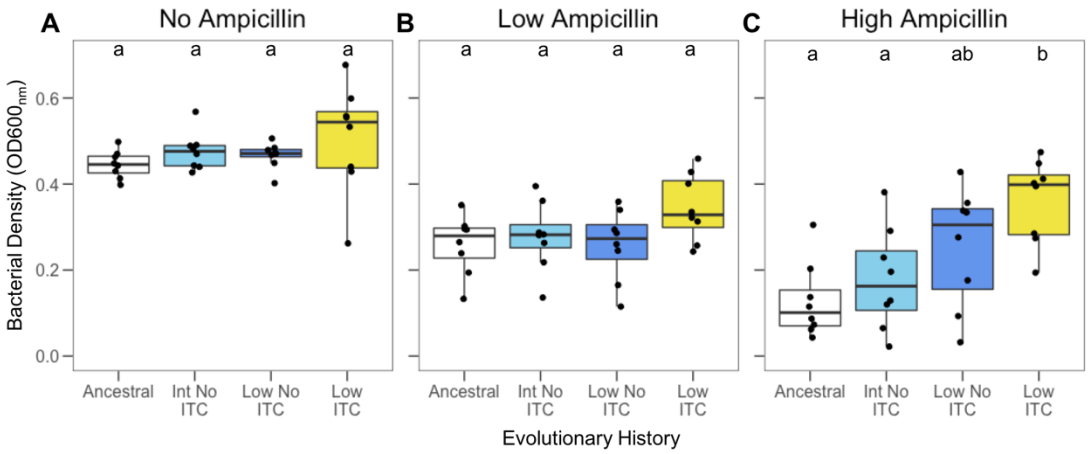
LowITC8	Unclassified A	NZ_CP012687.1:1121823-1126822	NZ_CP012687.1:1162290-1162775	35.4	62.85	41
	RS551	NZ_CP012687.1:1218220-1223219	NZ_CP012687.1:1236384-1237639	13.1	58.58	18



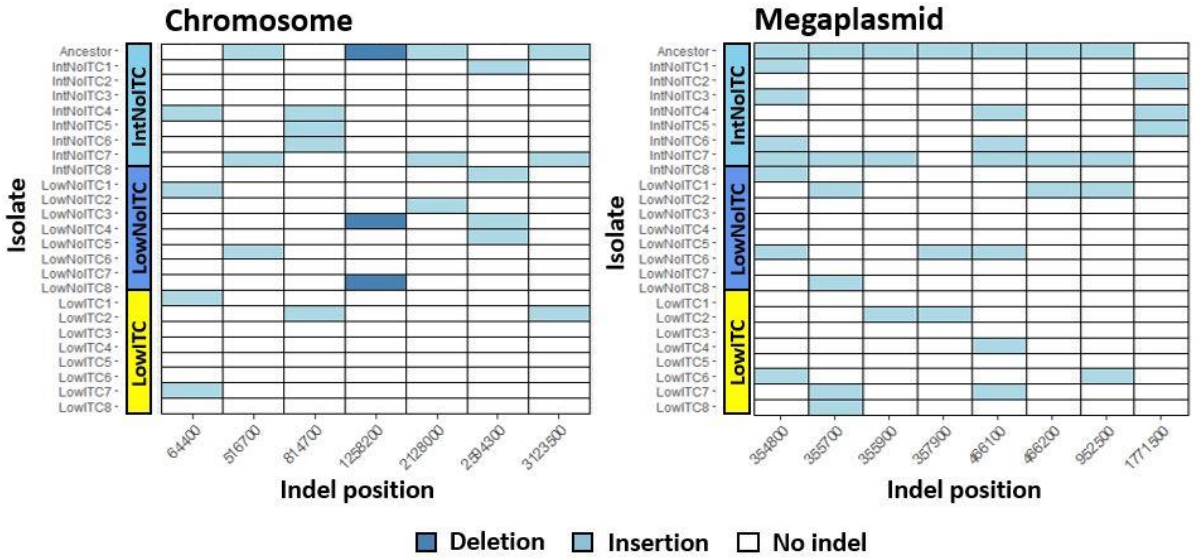
Appendix Figure A1. The effect of allyl-ITC pre-volatilisation for antibacterial activity against *Ralstonia solanacearum*. *R. solanacearum* bacterial growth was measured in CPG media supplemented with 0 (No allyl-ITC) or 500 μM of allyl-ITC that had been allowed to volatilise for 2, 24, 48 or 72 hours (see key). All data points show the mean of eight technical replicates and bars show ±1 standard error of the mean (SEM).



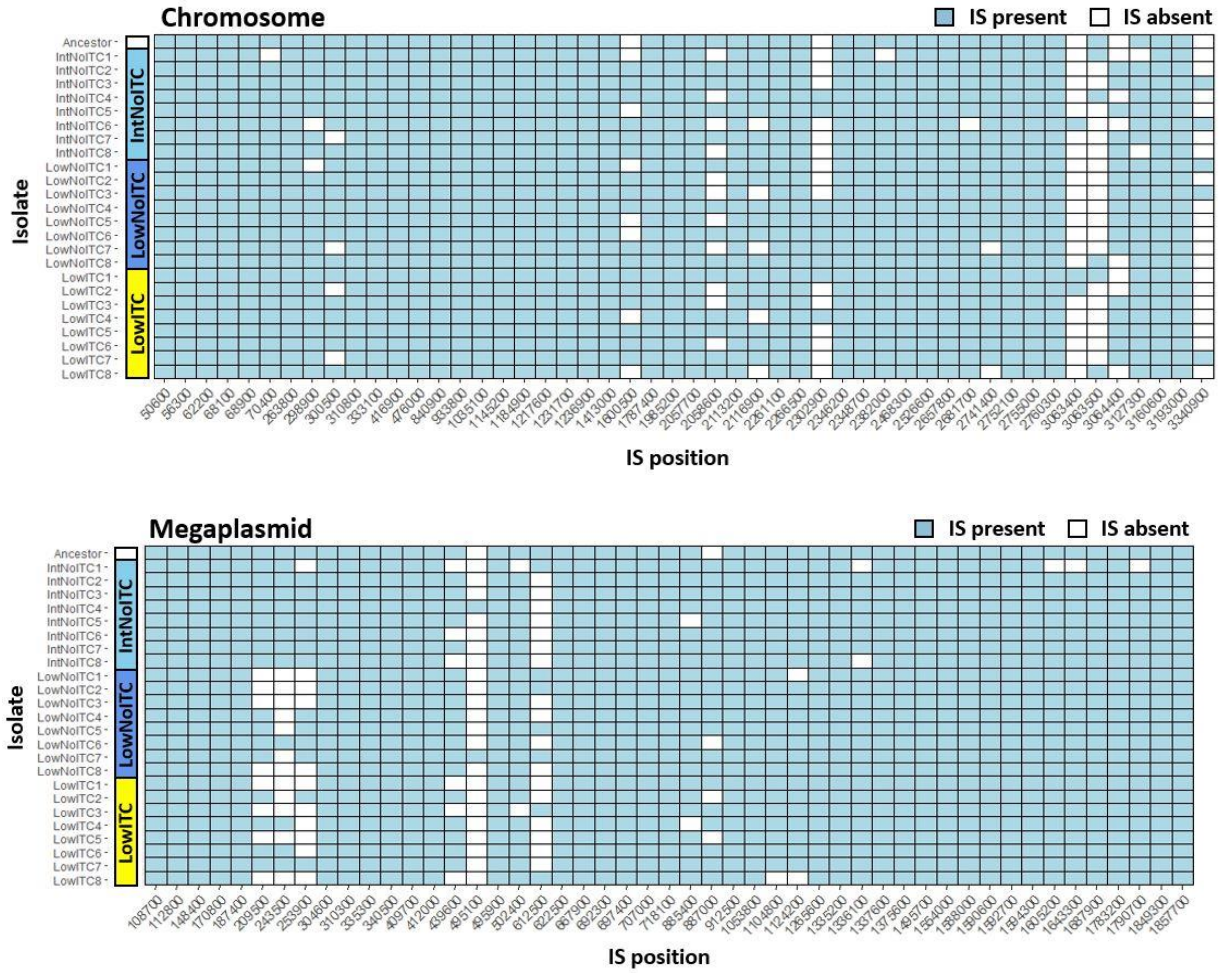
Appendix Figure A2. Effects of allyl, sec-butyl and 2-phenylethyl ITCs on *Ralstonia solanacearum* growth at different ITC concentrations. In all panels, *R. solanacearum* bacterial densities are shown on the Y-axis as optical density ($OD600_{nm}$), measured at 24-hour intervals (X-axis). In all panels, different line colours refer to different ITC concentrations (see key in A). All data points show the mean of eight technical replicates and bars show ±1 standard error of the mean (SEM).



Appendix Figure A3. *Ralstonia solanacearum* tolerance to ampicillin beta-lactam antibiotic. Ampicillin tolerance was measured as the growth of ancestral and evolved *R. solanacearum* clones isolated from intermediate (Int) and low transfer frequency (Low) control treatments (no-ITC) and ITC-exposed low transfer frequency treatment in the absence (A) and presence (B-C) of ampicillin (15 and 30 µg/ml concentrations). Boxplots show the minimum, maximum, interquartile range and the median (black line) after 48 hours. Individual data points show bacterial densities for each biological replicate clone (N= 8). Different small case letters above boxplots indicate significant pairwise differences (Tukey: $p < 0.05$) between treatments within each panel.

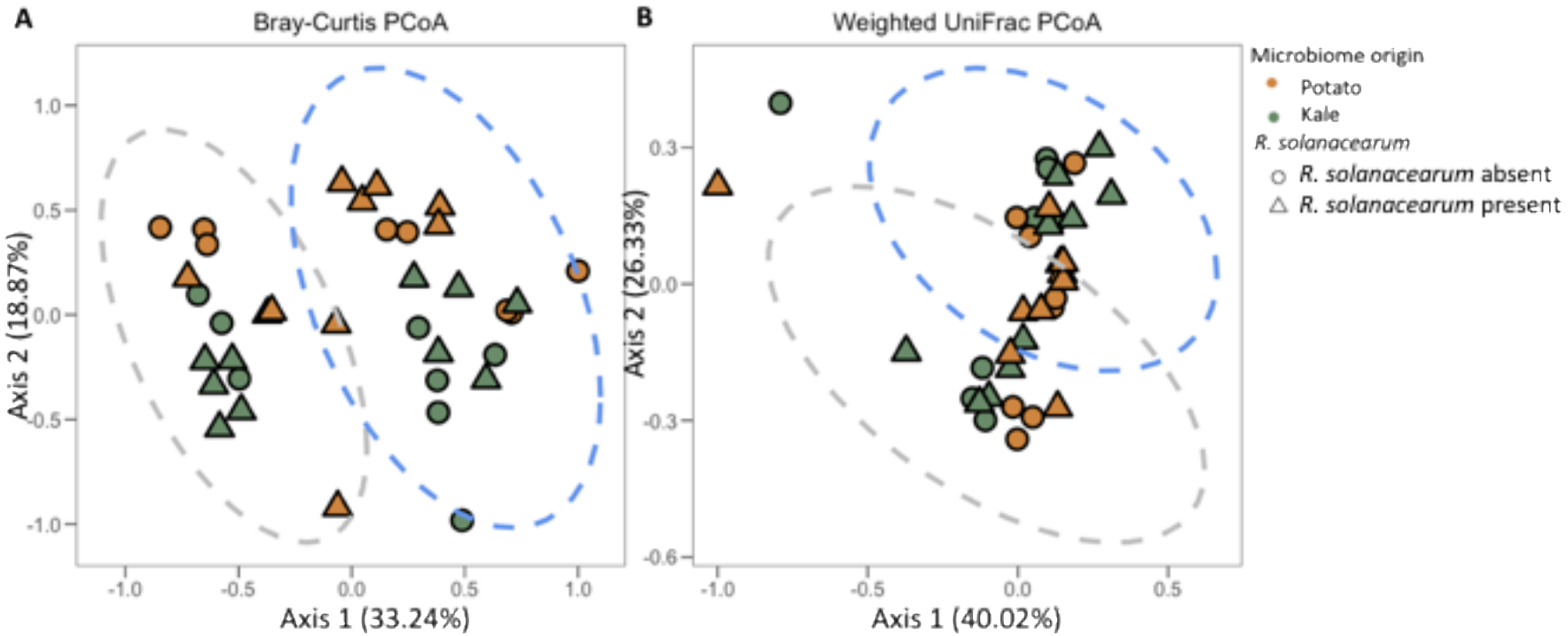


Appendix Figure A4. Presence and absence of intermediate indels found in more than two isolates in the chromosome and megaplasmid. The X-axis shows the indel position rounded to the nearest 100bp. The Y-axis shows isolates grouped as shown in Figure 5.



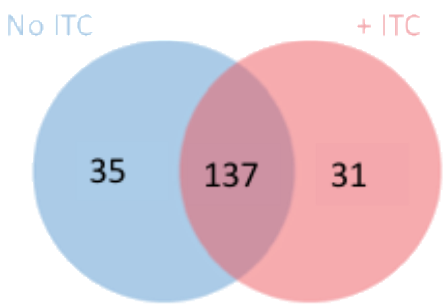
Appendix Figure A5. Presence and absence of insertion sequences in the chromosome and megaplasmid. The X and Y-axes show the insertion sequence position and experimental isolate, respectively, as outlined in Figure 2.4.5.

Appendix B: Chapter 4

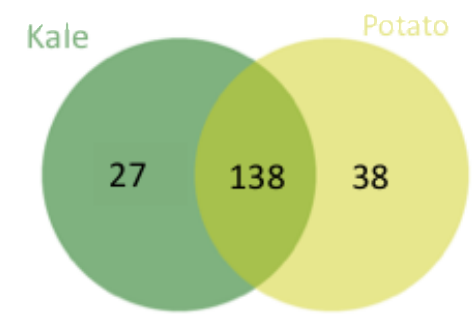


Appendix Figure B1. Effects of microbiome origin and ITC exposure on beta-diversity metrics. Panel (A) shows Bray-Curtis (A) and panel (B) shows weighted UniFrac distance matrices. Confidence ellipsoids are clustered based on ITC exposure (grey=no ITC, blue=ITC-exposed samples). *R. solanacearum* presence had no significant effect on beta-diversity metrics and presented groups are averaged over pathogen presence treatment (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

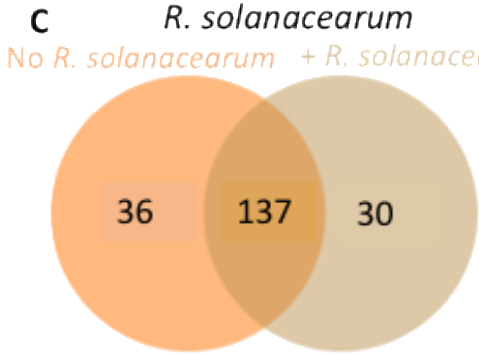
A



B



C



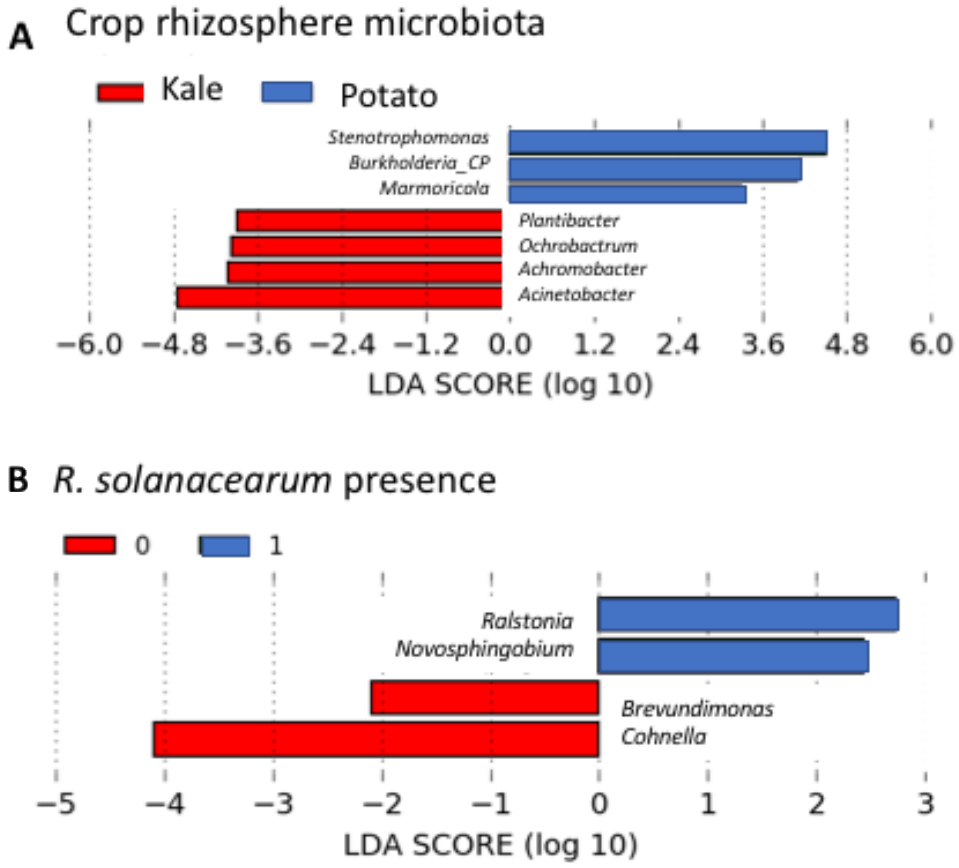
D

Unique to Kale	Unique to Kale+Rsc	Unique to Kale+ITC	Unique to Kale+Rsc+ITC
Zavarzinella		*Methylorosula*	*Terrimonas*
Isosphaeraceae_1		*Oceanobacillus*	*Tepidisphaera*
Candidatus Babela		*Parafilimonas*	*Legionella*
Fimbriimonadaceae_1		*Parasegetibacter*	*Halomonas*
		Dokdonella	*Anaeromyxobacter*
		Micromonospora	
		Rhodobacteraceae_1	
		Domibacillus	
		Modestobacter	
		Occallatibacter	
		Shimazuella	
		Vulgatibacter	
		Verrucomicrobium	

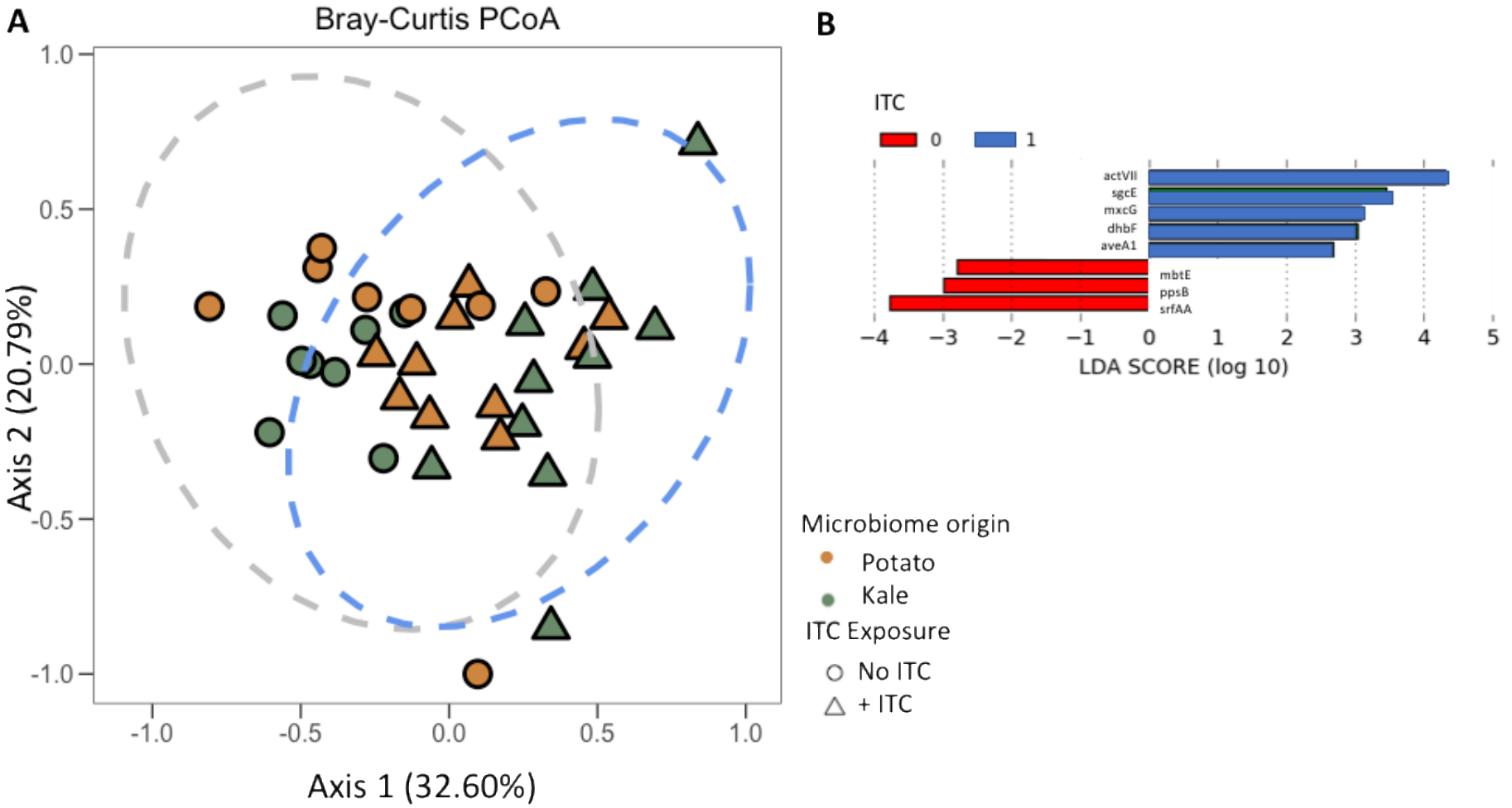
E

Unique to Potato	Unique to Potato+Rsc	Unique to Potato+ITC	Unique to Potato+Rsc+ITC
Qipengyuania	*Dyella*	*Agromyces*	*Alicyclobacillus*
Defluviicoccus	*Thermus*	*Blastocatellaceae_1*	*Sphingopyxis*
Nitrolancea	*Acidibacter*	*Neochlamydia*	*Pedosphaera*
Streptococcus	*Micropepsaceae_1*	*Candidatus Omnitrophus*	*Dyadobacter*
Glycomyces	*Aquisphaera*		*Bacteriovorax*
Lacunisphaera	*Caulobacter*		*Fluviicola*
Lactococcus	*Pantoea*		*Lentimicrobiaceae_1*
	Labrys		
	Sporolactobacillus		
	Acidicaldus		
	Leptographium lundbergii		
	Nostocaceae_1		
	Chryseobacterium		

Appendix Figure B2. Effects of ITC, microbiome origin and *R. solanacearum* presence on the shared microbiome. Panels show the number of genera unique to each treatment and the number shared between different treatments, including those occurring in the presence and absence of ITC (A), in the kale or potato rhizosphere microbiome (B) and in the presence and absence of *R. solanacearum* (C) (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC). We also show genera unique to each treatment in kale (D) and in potato (E). The genera names in the centre are the microbial associations common to all treatments, listed in the tables below. Where the taxonomic resolution did not include genera level, the family name is given with '_1'.

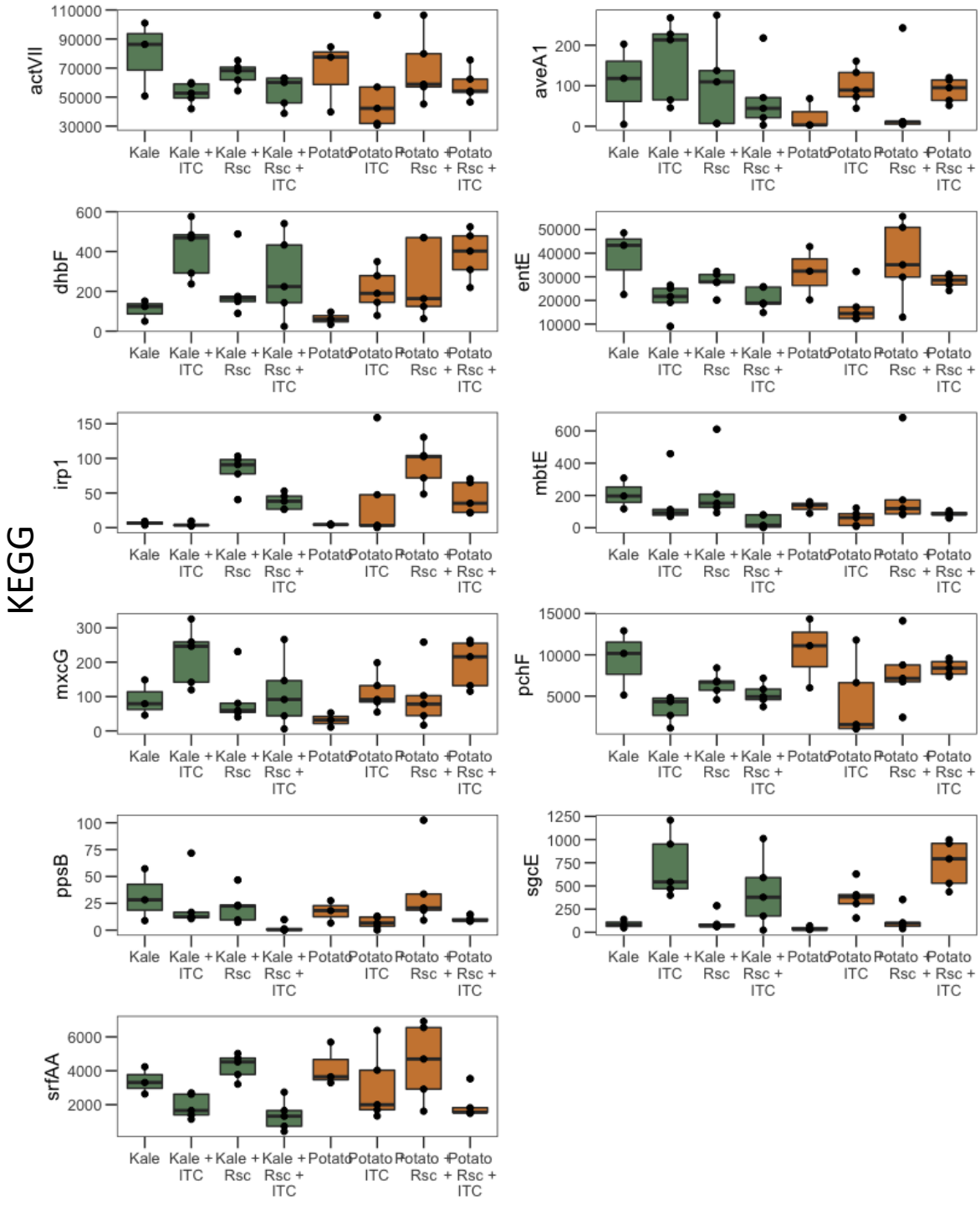


Appendix Figure B3. Effect of microbiome origin and *R. solanacearum* presence on genera abundances. Panel (A) shows genera who were enriched in the kale (red bars) or potato (blue bars) microbiota, while panel (B) shows genera who were enriched the presence or absence of *R. solanacearum* (blue and red bars, respectively) based on LEfSe output (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC). The length of the bar represents a log10 transformed LDA score. Data show post-treatment samples only.



Appendix Figure B4. Effects of microbiome origin and ITC on community metabolic function based on PICRUSt analyses. Panel (A) shows Bray-Curtis PCoA based on community diversity of pathway abundance and panel (B) shows differential abundance of KEGGs linked to

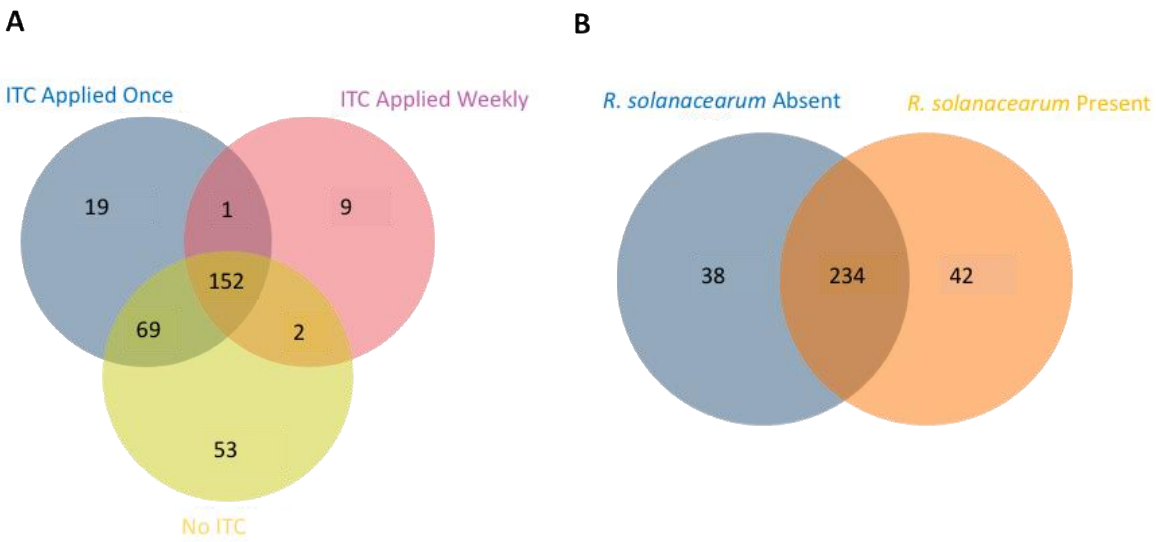
antibiotic resistance (NRPs and polyketides) identified by LEfSe in the presence and absence of ITC. Confidence ellipsoids are clustered based on ITC exposure (grey=no ITC, blue=ITC-exposed samples). Bars indicate effect size (LDA) for a particular genus in the presence (green bars) and absence (blue bars) of ITC. The length of the bar represents a log10 transformed LDA score. The colours represent which group that genus was found to be more abundant in compared to the other group (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).



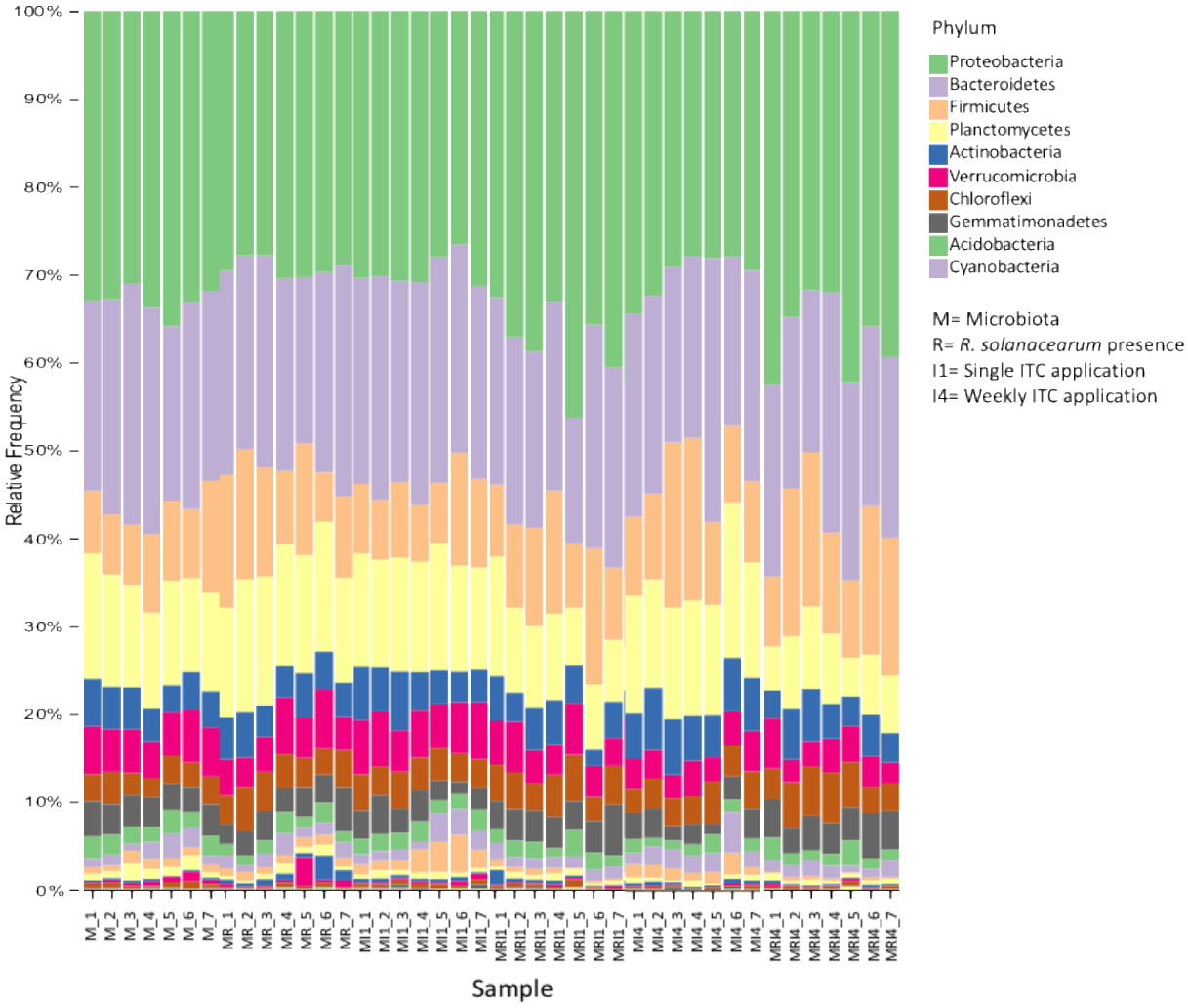
Appendix Figure B5. Effects of ITC, microbiome origin and *R. solanacearum* presence on the abundance of KEGGs linked with antibiotic synthesis genes (NRPs and polyketides). Panels show KEGGs identified based on PICRUSt. Green boxplots denote for kale rhizosphere microbiota and orange for potato rhizosphere microbiota. Boxplots show the

minimum, maximum, interquartile range and the median (black line), while points indicate individual replicates (N=3 for no-ITC and no *R. solanacearum* treatments, while N=5 for treatments with *R. solanacearum* and ITC).

Appendix C: Chapter 5



Appendix Figure C1. Effects of ITC and *R. solanacearum* presence on the shared microbiota. Panels show the number of genera unique to each treatment and the number shared between different treatments, including those occurring in the presence and absence of ITC (A) and in the presence and absence of *R. solanacearum* pathogen (B).



Appendix Figure C2. Effects of ITC exposure on microbiota phyla abundances (ten most abundant phyla) in the absence and presence of *R. solanacearum*. Figure shows relative frequency (%) of phyla abundances. Each bar indicates a replicate sample. X-axis labels indicate the sample number and treatment. Bars are colour coded by the phylum (see key; N=7 for all other treatments).

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