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

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Running Head: ITC tolerance in *Ralstonia solanacearum*

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Abstract

Crop losses to plant pathogens are a growing threat to global food security, and hence, 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 synthetic 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. Mechanistically, tolerance was associated with parallel mutations in a gene linked to glucose/sorbonose dehydrogenase, resulting in cross-tolerance to ampicillin beta-lactam antibiotic. Interestingly, pathogen adaptation to the growth media also indirectly selected for increased ITC tolerance through potential metabolic adaptations linked with cell wall structure (serine/threonine kinase) and DNA replication, recombination and repair (deoxyribonucleases). 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.
Importance

Several alternatives to agrochemicals have been proposed to reduce crop losses to plant pathogens. One such strategy is called biofumigation, which is a biocontrol technique based on natural plant allelochemicals, such as isothiocyanates (ITCs), that have antimicrobial activity against plant pathogens. Here we show that while one such compound, allyl-ITC, is effective at suppressing the growth of notorious *Ralstonia solanacearum* plant pathogenic bacterium, increased tolerance evolves rapidly, leading to cross-tolerance to the beta-lactam antibiotic, ampicillin. Mechanistically, ITC tolerance was linked to mutations in a putative glucose/sorbonose dehydrogenase gene, which has previously been linked to resistance to clinical antibiotics in other pathogens. Together, our results suggest that plant pathogenic bacteria can rapidly evolve tolerance to antimicrobial compounds produced by plants via similar mechanisms by which they evolve resistance to clinical antibiotics. Potential pathogen tolerance evolution should thus be considered when developing new biocontrol methods based on pathogen growth inhibition.
Introduction

Plant pathogens are a growing threat to global food security, accounting for up to 40% of crop losses annually (1). The phasing out of environmentally toxic chemical fumigants, such as methyl bromide, has directed attention towards alternative biocontrol strategies (2). Plant-derived antimicrobial allelochemicals, such as phenolic acids, terpenes and volatile isothiocyanates (ITCs), are naturally exuded by the roots of legumes (3, 4), cereals (5, 6) and other crops such as Brassica (7, 8). These compounds could potentially be used to control pathogens by biofumigation, which involves mulching of plant tissues into soils to release biocidal allelochemicals. While biofumigation has previously been shown to suppress the growth of soil-borne fungal (8–10), nematode (11, 12) and bacterial pathogens (13, 14), outcomes are still varied, ranging from clear pathogen suppression (15, 16) to having no effect (17–19). 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 (16). The biocidal effects of Brassica-based biofumigation are believed to result primarily from the release of toxic ITCs from their glucosinolate (GSL) pre-cursors (11, 16, 20). Moreover, other allelochemicals such as dimethyl sulfide and methyl iodide might contribute to the biocidal activity of biofumigant plants (21, 22). Even though ITC-liberating GSL levels can potentially reach as high as 45.3 mM/m² following initial mulching and incorporation of plant material into the soil (23), their concentrations often decline rapidly due to high volatility, sorption to organic matter, leaching from the soil and microbial degradation (16, 24–27). As ITCs often have short half-lives of up to sixty hours (28, 29), 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 (30) and leakage of cell metabolites (31). 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 (33). It is also possible
that some ITCs, such as allyl-ITC, could have multiple targets, making them relatively more
toxic to pathogenic bacteria (34). 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 (35). 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 (36). Such tolerance has recently been shown to evolve
de novo in Pseudomonas protegens CHA0 bacterium against scopoletin antimicrobial
secreted by Arabidopsis thaliana (37). 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 competitive 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 (8), no direct experimental evidence of ITC tolerance evolution by 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. Ralstonia solanacearum was chosen as a model species because it is a globally important pathogen, affecting over 200 different plant species including various important crops (38, 39). Furthermore, 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 (40–42). Indian mustard (Brassica juncea) was chosen as a model biofumigant plant due to its well-established allelochemical properties (8, 43–45), which are predominantly caused by the release of allyl, sec-butyl and 2-phenylethyl ITCs (46–48). 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 (16, 23, 27, 49, 50).

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. No clear competitive costs were observed. 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.

**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. 1B). However, this effect was mainly driven by allyl-ITC, which significantly reduced bacterial densities compared to the non-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. 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. 1B).

However, a significant interaction between ITC type and ITC concentration in both single and combination treatments was found (ITC concentration x 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. 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. 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. 1B), and the presence of allyl-ITC had a clearer effect at the high ITC concentration (Allyl-ITC presence x ITC concentration: \(F_{1, 57} = 7.51, p < 0.01; \) Fig. 1B). Together these results suggest that allyl-ITC was the most inhibitory ITC and its antimicrobial activity was not enhanced by the presence of other ITCs.

(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 \(\mu\)M) 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). 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), bacterial growth was least affected in the low transfer frequency treatment (ITC presence x Transfer frequency: \(F_{2, 42} = 4.36, p < 0.05; \) \(p < 0.001\) for all pairwise comparisons; Fig. 2). We also observed that the inhibitory activity of allyl-ITC varied over time: while 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 x Transfer frequency x ITC...
presence: $F_{2, 673} = 7.33, p < 0.001$; Fig. 2). Together these results suggest that the long-term
ITC activity varied temporally and depended on the ITC exposure and serial transfer
frequency.

(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. 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. 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. 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. 3B). As expected, 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: $F_{2,40} = 2.66, p =$
In all pairwise comparisons, Tukey: p< 0.05; Fig. 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. 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. 3C), and this effect was mainly driven by adaptation in the low transfer frequency ITC exposure treatments (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. 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. 3D), which was clearest in the low transfer frequency exposure treatment (Evolutionary history x Transfer frequency: $F_{2, 49} = 23.17$, p< 0.001; Fig. 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. 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 x Transfer frequency: $F_{2, 49} = 3.04$, p< 0.05; Fig. 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.

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

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 (51). 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 relatively more inhibitory, Fig. S3). However, the evolved clones originating 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: $p < 0.05$; Fig. S3), while evolved clones derived from low and intermediate transfer frequency control treatment (no prior ITC exposure) were inhibited to the same extent (Tukey: $p > 0.05$; Fig. S3). In more detail, ampicillin tolerance in the low transfer frequency clones was only observed under the high ampicillin concentration (Evolutionary history in high ampicillin concentration: $F_{3, 28} = 8.22, p < 0.001$; Fig. S3C), and no improved tolerance was observed under low ampicillin concentration (Evolutionary history in low ampicillin concentration: $F_{3, 28} = 1.551, p = 0.223$; Fig. S3B). 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.
ITC tolerance is linked to parallel mutations in a gene encoding glucose/sorbonose dehydrogenase.

A subset of clones which were phenotyped regarding ITC and ampicillin tolerance were selected for genome sequencing (N= 25). Specifically, we focused on comparing parallel mutations between populations that had evolved in the low transfer frequency treatments (clones evolved in the absence and presence of ITC, showing some evidence of tolerance evolution) with ancestral and control populations from the intermediate transfer frequency treatment (no evidence of tolerance evolution). 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, 9 were non-synonymous and 4 synonymous (Table S2). We mainly focused on comparing differences in parallel non-synonymous mutations and indels which likely have the greatest effect on bacterial phenotypes. Some of these mutations were observed across all treatments, indicative of adaptation to the 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 (Fig 5; Table S2). 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 (between 1/8 to 5/8 replicate clones) were found across all treatments (Fig. 5; Table S2). 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 one other clone originating from this treatment had a mutation in the putative TIS1421-transposase orfA protein-encoding gene (Fig. 5; Table S2).

Interestingly, one non-synonymous parallel mutation in putative glucose/sorbonose...
The glucose/sorbonose dehydrogenase gene was exclusively identified in all the low transfer frequency ITC exposure clones (present in 8/8 clones; Fig. 5; Table S2) and this gene was also mutated in 4/8 replicate clones in the control (no ITC exposure) low transfer frequency treatment (present in 4/8 clones; Fig. 5; Table S2). However, those low transfer frequency control clones possessing the glucose/sorbonose dehydrogenase mutation did not show significantly higher ITC tolerance compared to non-mutated clones when grown in LB supplemented with ITC (the effect of glucose/sorbonose dehydrogenase mutation presence in low transfer frequency control clones: F₁, 6 = 3.05, p= 0.13). This suggests that a combination of mutations may be required for ITC tolerance. Together, these results suggest that bacteria potentially adapted to experimental conditions across all treatments, while ITC tolerance was associated with parallel mutations in the putative glucose/sorbonose dehydrogenase encoding gene.

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 show that long-term allyl-ITC exposure selected for ITC tolerance in the low transfer frequency ITC exposure treatment, which was associated with cross-tolerance to ampicillin. At the genetic level, ITC tolerance was linked with parallel mutations in the putative glucose/sorbonose dehydrogenase gene. 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 (52). 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 (8). Moreover, it is possible that different *R. solanacearum* strains respond differently to ITCs, 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 (53) and fungi (54), 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 (8). Previous studies have shown greater pathogen suppression by ITCs with aliphatic compared to aromatic sidechains in fungal pathogens (8, 55), insect pests (56), and weeds (57). With bacteria, the toxicity of allyl-ITC could be attributed to its high volatility, very short R-side chains and high reactivity (23, 58, 59). These properties could enable rapid diffusion through the liquid media before ITC is lost in the gaseous phase (21). This is supported by a study by Sarwar *et al.* (8), 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. 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 (60, 61), 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 (Fig. S1). Alternatively, stationary phase growth conditions could have triggered expression of stress tolerance genes, enabling selection for mutants with relatively higher ITC tolerance (61). For example, expression of $\text{RpoS}$ sigma factor in $P. \text{aeruginosa}$ has previously been linked to elevated antibiotic resistance and biofilm formation at stationary phase (62, 63). 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 (64) and
presence of clinical antibiotics (65). 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 molecular level, ITC tolerance was associated with parallel mutations in a gene
predicted to govern glucose/sorbonose dehydrogenase activity. Crucially, all ITC-exposed
(8/8) and half of the control clones (4/8) from the low transfer frequency treatment
exclusively harboured this mutation. Dehydrogenase enzymes have previously been
associated with both metabolism and antibiotic resistance (66, 67). For example, a gain of
function mutation in a gene encoding glutamate dehydrogenase has been linked with beta-
lactam resistance in *Bacillus subtilis* through altered expression of an extra-cytoplasmic
sigma factor (67). Furthermore, in *E. 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 (68, 69). These
genes have also been linked with beta-lactam resistance in human opportunistic
*Staphylococcus aureus* pathogen through increased cell wall thickness and reduced cell wall
antibiotic permeability (70). In line with this, we found that ITC tolerance conferred cross-
tolerance to ampicillin beta-lactam antibiotic in clones originating from the low transfer
frequency ITC exposure treatment. However, the glucose/sorbonose dehydrogenase did not
confer increased tolerance for clones that had evolved in low transfer frequency control
treatment in the absence of allyl-ITC. This suggests that other mutations might have also
been important for ITC tolerance via potential epistatic interactions.

Three clones from the intermediate transfer frequency transfer 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 (71). In addition, we observed mutations in genes encoding putative
serine/threonine protein kinases, amino acid biosynthesis ($hisH1$ gene) and DNA replication,
recombination and repair (putative $RhsC$ gene) in clones from all treatments. While more
work is required to directly test the role of these mutations, they were likely associated with
bacterial growth and metabolism, which could have resulted from adaptation to the growth
media during the selection experiment. Accumulating evidence suggests that antimicrobial
resistance can be indirectly driven by metabolic adaptations (72) and through stepwise
accumulation of multiple mutations that confer high-level antibiotic resistance via epistasis
(73, 74). While little is known about ITC tolerance mechanism in bacteria, it has previously
been shown that upregulation of three glutathione S-transferase encoding genes (linked to
protecting cells from oxidative damage) (75) were required for the induction of ITC
tolerance in the stem rot fungal plant pathogen $Sclerotinia sclerotiorum$ (76). Concurrently,
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 $Alternaria brassicicola$ (77), and bacterial pathogen $Escherichia coli$ (34). It
would also be interesting to study potential trade-offs between tolerance and other
pathogen life-history traits in the future. While we did not observe clear growth costs
associated with ITC tolerance, mutations in dehydrogenase genes have previously been
associated with loss of virulence in *R. solanacearum* (78). Such trade-offs could potentially counteract the drawbacks of tolerance evolution by constraining disease outbreaks through reduced virulence.

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. Furthermore, it is not clear if the ITC concentrations used in this experiment are achievable through biofumigation and whether they would have negative effects also 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 the highest using finely chopped plant material, which maximises cell disruption and ITC release to the soil (79). 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.

**Materials and Methods**

(a) **Pathogen strain and culture media**
We used a *Ralstonia solanacearum* strain which was originally isolated from the river Loddon (phylotype II sequevar 1) in the UK as our 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$_2$O) 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$_2$O) was also used as a ‘naïve’ growth media to control the effects of *R. solanacearum* adaptation to CPG media during the selection experiment.

(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 non-ITC exposed 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 (Fig. S2). 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 148 hours and bacterial densities were measured every 24 hours as optical density (OD) at 600 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 (Fig. S2). 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 (Table S1).

Furthermore, these concentrations are known to be achievable at least transiently during biofumigation in the field (16, 23, 27, 50, 80). 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) and the experiment was run for three days (72 hours), with population density measurements recorded every 24 hours as optical density at 600 nm.

(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; Fig. S2). 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 subcultured to fresh CPG media in the absence (control) and presence of allyl-ITC. ITC treatments thus manipulated both resource renewal and exposure to fresh ITCs. 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 the potential costs of ITC tolerance and adaptation to the growth media (CPG) by growing the strains in the absence of ITCs. 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. We also measured if ITC exposure led to cross-tolerance to the beta-lactam antibiotic ampicillin, 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). 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 was whole genome sequenced (Illumina 30x coverage) to identify potential single nucleotide polymorphisms (SNPs) and genomic rearrangements (small
insertions and deletions) linked with \textit{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), the ancestral strain (1 clone), and as a control, 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). 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 (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 \textit{et al.}, 2014).

Assembly was performed on samples using SPAdes version 3.7 (Bankevich \textit{et al.}, 2012) and contigs were annotated using Prokka 1.11 (81). Genomes were analysed using a standard analysis pipeline, where reads were first mapped to a high quality and well annotated UY031 reference genome (82) which showed 99.95% similarity with our \textit{R. solanacearum} strain at the chromosome level and 97.87% similarity at the mega-plasmid level. Variant calling was performed using Snippy, a rapid haploid variant calling pipeline (Snippy v3.2).
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. Additionally, we used phage identification software Phaster (83), and mobile genetic element integration identification software MGEfinder (84) to identify potential phenotypic changes via mobile genetic elements but did not observe any clear differences between ITC-exposure treatments. 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*, Fig. S1) 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 Version (3. 5. 1), Packages: ggplot, tidyverse, ggpubr, lme4, rcompanion).
Acknowledgements

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References


Figures and figure legends

A

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B

Low ITC Concentration  
High ITC Concentration

**Figure 1. The antimicrobial activity of different ITCs against *Ralstonia solanacearum***

Figure 1. The antimicrobial activity of different ITCs against *Ralstonia solanacearum* pathogen when applied alone and in combination. The chemical properties of the three different ITCs predominantly released from Indian mustard biofumigant plant (*Brassica juncea*) (A), and their effects on *R. solanacearum* growth after 48h exposure when applied alone and in combination in liquid microcosms at low (500 \(\mu\)M) and high (1000 \(\mu\)M) concentrations (B). In (B) boxplot colours represent different ITC treatments that are labelled on X-axes as follows: (0): non-ITC exposed (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).
Figure 2. *Ralstonia 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.
Figure 3. Comparison of *Ralstonia 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 48 hours of 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 4.** Comparison of *Ralstonia 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 (OD$_{600nm}$) after 48 hours of 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).
Figure 5. Mutations associated with evolved *Ralstonia solanacearum* clones. In each panel, each ring represents a replicate *R. solanacearum* clone in different colours (N= 8, see key). Panels show replicate clones isolated from low transfer frequency ITC-exposure treatment (A), low transfer frequency control treatment (B) and intermediate transfer frequency...
control treatment (C). Circles on the left and right show mutations identified in chromosome and megaplasmid, respectively (see key), and black dots on the rings represent mutations at different loci. Labels indicate the gene product and the locus tag or gene name. Within each ring is a distance marker in Mb.
Supplementary Table 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 Supplementary Fig. 2.

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Supplementary Table 2. Mutated *Ralstonia 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.

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Int No ITC 5
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Low ITC 1
Low ITC 2
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Low ITC 5
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Supplementary Figure. 1. 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).
Supplementary Figure 2. 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 (OD$_{600\text{nm}}$), 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).
Supplementary Figure 3. *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.