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2-Tridecanone impacts surface-associated bacterial behaviours and hinders plant-bacteria interactions

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Running title: 2-tridecanone: a new chemical cue for bacteria

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Originality-Significance Statement: Our work assigns new roles to the volatile methylketone 2-tridecanone (2-TDC), which was known previously as a natural insecticide. We demonstrate that 2-TDC is an airborne cue that affects surface motility and biofilm formation in phylogenetically distant bacteria, including plant and animal pathogens, without any effect on growth. Moreover, we found that 2-TDC protects plants from bacterial infections. These observations open new perspectives not only for fundamental research, but also for the development of biotechnological solutions to prevent/control bacterial infections, minimizing the risk of generating antimicrobial resistance.

Summary

Surface motility and biofilm formation are behaviours which enable bacteria to infect their hosts and are controlled by different chemical signals. In the plant symbiotic alpha-proteobacterium *Sinorhizobium meliloti*, the lack of long-chain fatty acyl-coenzyme A synthetase activity (FadD) leads to increased surface motility, defects in biofilm development, and impaired root colonization. In this study, analyses of lipid extracts and volatiles revealed that a *fadD* mutant accumulates 2-tridecanone (2-TDC), a methylketone known as a natural insecticide. Application of pure 2-TDC to the wild-type strain phenocopies the free-living and symbiotic behaviours of the *fadD* mutant. Structural features of the methylketone determine its ability to promote *S. meliloti* surface translocation, which is mainly mediated by a flagella-independent motility. Transcriptomic analyses showed that 2-TDC induces differential expression of iron uptake, redox, and stress-related genes. Interestingly, this methylketone also influences surface motility and impairs biofilm formation in plant and animal pathogenic bacteria. Moreover, 2-TDC not only hampers alfalfa nodulation but also the development of

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tomato bacterial speck disease. This work assigns a new role to 2-TDC as an infochemical that affects important bacterial traits and hampers plant-bacteria interactions by interfering with microbial colonization of plant tissues.

Introduction

The behaviour of bacteria on surfaces is crucial for host-microbe interactions. For successful host colonization, the ability to spread over and colonize preferred niches as well as to establish a long-lasting community, are important attributes. Different mechanisms are used by bacteria to translocate over surfaces (Harshey, 2003; Jarrell and McBride, 2008). Swarming, twitching and gliding are active bacterial motilities mediated by flagella, type IV pili and focal adhesion complexes, respectively. Bacteria can also spread on surfaces using sliding, a passive translocation mechanism in which surfactants and other compounds that diminish friction between cells and surfaces play a crucial role (Hölscher and Kovács, 2017). Amongst the different types of surface translocation, swarming motility has been the most extensively studied because it is a trait closely connected with virulence in pathogenic bacteria (Verstraeten *et al.*, 2008). Swarming requires flagellar action as does the well-known swimming motility. The latter is the movement of individual bacteria in liquid environments. Unlike swimming, swarming is a coordinated multicellular migration of bacteria on top of solid surfaces (Kearns, 2010). Several studies have reported an intimate link between swarming and the formation of surface-attached communities of bacteria known as biofilms (O'Toole *et al.*, 2000). Both processes are multicellular surface-associated behaviours which confer resistance to different stressors. In addition, they are often controlled by the same regulatory pathways (Verstraeten *et al.*, 2008; Prüβ, 2017), and influenced by the same chemical cues, which include quorum sensing signals and volatile compounds.

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Quorum sensing (QS) is a cell-to-cell communication system that allows bacteria to collectively modify behaviours by regulating gene expression in response to the population density (Papenfort and Bassler, 2016; Whiteley *et al.*, 2017). It depends on the synthesis and detection of different extracellular chemical signals. *N*-acylhomoserine lactones (AHL), *cis*-2-unsaturated fatty acids of the Diffusible Signal Factor (DSF) family or α-hydroxyketones are some of the QS signals recognized to control motility and biofilm formation (Papenfort and Bassler, 2016). More recently, several volatile compounds emitted by bacteria have also been found to impact motility and biofilm formation with different effects depending on the bacterial species or the volatile concentration (Audrain *et al.*, 2015).

Swarming and biofilm formation occurs also in beneficial microorganisms such as rhizobia. These soil bacteria can induce the formation of nitrogen-fixing nodules in legume plant roots following a complex molecular interchange (Gibson *et al.*, 2008; Oldroyd and Downie, 2008). Data about genetic determinants and environmental cues that control surface motility and biofilm formation in rhizobia, as well as the role that these two traits play in the plant-microbe interaction, are scarce. Swarming motility has been reported for the alfalfa symbiont *Sinorhizobium meliloti* strains GR4 and Rm1021, although the latter can also translocate over surfaces using a type of flagellum-independent motility, which requires the amphiphilic siderophore rhizobactin 1021 (Nogales *et al.*, 2010; Nogales *et al.*, 2012; Bernabéu-Roda *et al.*, 2015). Recent studies performed with these rhizobial strains revealed the existence of mechanisms which regulate swarming and biofilm formation inversely to promote efficient plant root colonization (Amaya-Gómez *et al.*, 2015). One of these mechanisms involves the fatty acid catabolism-related enzyme FadD. In *S. meliloti*, *fadD* inactivation promotes surface motility, obstructs normal biofilm development, and impairs colonization and

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nodulation efficiency on alfalfa roots (Soto *et al.*, 2002; Nogales *et al.*, 2010; Amaya-Gómez *et al.*, 2015; Bernabéu-Roda *et al.*, 2015). FadD is an acyl-coenzyme A (CoA) synthetase which converts long-chain fatty acids into acyl-CoAs which can enter β-oxidation (Rock, 2008). Recently, FadD has been associated with the turnover of QS signals of the DSF family (Zhou *et al.*, 2015; Zhou *et al.*, 2017).

The phenotypes exhibited by *S. meliloti fadD* mutants led us to hypothesize that lipidic compounds differentially produced in the wild-type and mutant strains, may act as cues that control bacterial behaviours on surfaces and influence the interaction with the plant host. To identify such a compound, in a previous work, the lipidic composition of *S. meliloti* wild-type and *fadD* mutant strains was investigated. It was determined that the *fadD* mutant accumulated significant amounts of free fatty acids in the stationary phase of growth (Pech-Canul *et al.*, 2011). However, none of the fatty acids analysed was able to induce surface motility of the wild-type strain. In the present study, we found that the *fadD* mutant accumulates 2-tridecanone (2-TDC), and that overproduction of this compound might be responsible for the phenotypes exhibited by the mutant strain. 2-TDC is a volatile methylketone (MK) known as a natural insecticide that is produced in prodigious amounts by specialized trichomes of wild tomato plants (*Solanum habrochaites* ssp *glabratum*), which makes these plants naturally resistant to a wide spectrum of arthropod pests (Williams *et al.*, 1980; Fridman *et al.*, 2005). Production of 2-TDC has also been reported for several bacterial species (Forney and Markovetz, 1971; Elgaali *et al.*, 2002; Blom *et al.*, 2011; Lemfack *et al.*, 2014; Raza *et al.*, 2016), but not for rhizobia. More importantly, the impact of this methylketone on bacterial behaviour and on the establishment of plant-bacteria interactions has not been hitherto described. Here, we provide evidence for a new role of 2-TDC that affects bacterial lifestyles.

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Results

A *Sinorhizobium meliloti fadD mutant accumulates 2-tridecanone*

To identify the putative FadD-related compound responsible for the pleiotropic phenotype of a *S. meliloti fadD* mutant, lipidic extracts obtained from cells and spent culture media of *S. meliloti* wild-type GR4 and the *fadD* mutant QS77 were analysed by Gas Chromatography/Mass Spectrometry (GC/MS). Besides the already reported accumulation of free fatty acids (Pech-Canul *et al.*, 2011), the methylketone (MK) 2-tridecanone (2-TDC) and the aldehyde dodecanal, were detected in the spent media from the *fadD* mutant (Supporting Information Table S1 and Fig. S1 and S2). Peaks corresponding to dodecanal or 2-TDC were not detected in the spent media obtained from the wild-type strain or from the *fadD* mutant QS77 complemented with the *fadD*-harbouring plasmid pBBRD4. Dodecanal and 2-TDC were present in similar amounts and each represented about 3% of the total lipidic compounds detected in the spent media from the *fadD* mutant (Supporting Information Table S1).

Since dodecanal and 2-TDC are known as volatile compounds, we decided to also analyse by Solid Phase MicroExtraction (SPME)-GC/MS the compounds emitted into the headspace by the wild-type and *fadD* mutant strains grown on MM (1% agar). Whereas dodecanal was absent in these analyses, peaks corresponding to three methylketones (2-TDC, 2-pentadecanone, and 2-heptadecanone) were identified in both strains, albeit at very low levels and close to the detection limit in the case of the wild-type. We specifically focused on 2-TDC since this was also detected in the analyses of lipidic extracts from liquid cultures. As shown in Fig. 1, the peak area corresponding to 2-TDC in the *fadD* mutant was 7.6-fold larger than that of the wild-type strain (49021±7532 *vs* 6411±1793 counts). These results indicate that *S. meliloti* produces

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148 volatile 2-TDC upon growth on semisolid surfaces and that the absence of FadD activity
149 increases production of the MK, both in liquid and surface-grown cultures.

150

151 *2-TDC promotes surface migration and reduces biofilm formation in S. meliloti*

152 In order to investigate whether accumulation of 2-TDC in the *fadD* mutant could be
153 responsible for the phenotypes exhibited by this strain, the behaviour of *S. meliloti* wild-
154 type strains in response to the application of 2-TDC was analysed. Firstly, possible
155 effects of the MK on bacterial growth were tested. It was found that *S. meliloti* cannot
156 use 2-TDC as the sole carbon source (Supporting Information Fig. S3). Moreover,
157 bacterial growth rates in liquid MM remained unaffected in the presence of different
158 concentrations of 2-TDC up to 500 µM (Supporting Information Fig. S4A). In contrast
159 to the lack of swarming-inducing activity observed for several fatty acids (Pech-Canul
160 *et al.*, 2011), addition of 2-TDC into semisolid MM at 5 µg/ml (25 µM) or 10 µg/ml
161 (50 µM), which correspond to total amounts of 0.5 or 1 µmol in the medium,
162 respectively, induced surface translocation in the wild-type strain GR4. Interestingly,
163 the same effect was observed in response to airborne 2-TDC generated from solutions
164 applied onto the lid of the Petri dish. The stimulatory effect was more pronounced using
165 less permissive conditions for surface migration MM (1% agar) (Bernabéu-Roda *et al.*,
166 2015). Airborne 2-TDC generated from solutions containing from 0.2 to 10 µmol of the
167 MK was able to stimulate surface motility with similar dose-dependent effects on GR4
168 and Rm1021 (Fig. 2A), two *S. meliloti* strains which exhibit different modes of surface
169 translocation (Bernabéu-Roda *et al.*, 2015). In contrast, swimming motility remained
170 unaffected in the presence of 2-TDC (Supporting Information Fig. S4B), indicating that
171 2-TDC acts by specifically activating surface translocation in *S. meliloti*.

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Next, we investigated whether 50 μM 2-TDC could also impact the ability of *S. meliloti* to form surface-attached communities. The defect shown by *S. meliloti fadD* mutants on biofilm development on glass surfaces has been shown previously (Amaya-Gómez *et al.*, 2015) and was exhibited after long periods of incubation. Due to the volatile nature of 2-TDC, its effect was analysed at earlier stages of biofilm formation (3 days). Under control conditions and as previously reported (Amaya-Gómez *et al.*, 2015), GR4 and Rm1021 developed flat, unstructured biofilms that covered most of the glass surface. In the presence of 2-TDC, biofilm formation in both *S. meliloti* strains was significantly impaired and significant reductions in surface area colonization and biofilm mass were observed (Figure 2B and Supporting Information Fig. S5A). These data show that 2-TDC affects surface-associated behaviours in *S. meliloti*, without affecting bacterial growth.

2-TDC impairs nodulation of alfalfa plants

The effects caused by exogenous application of 2-TDC on surface translocation and biofilm formation in *S. meliloti* wild-type strains suggested the possibility that the phenotypes exhibited by the *fadD* mutant were triggered by 2-TDC accumulation. To investigate whether 2-TDC also accounts for the symbiotic deficiency demonstrated by this mutant, the nodulation kinetics of alfalfa plants inoculated with the *S. meliloti* wild-type strain GR4 were determined in the absence or presence of 2-TDC added to the plant mineral solution at the time of inoculation. Compared with control conditions, the application of 2-TDC led to an important delay (2-3 days) in the appearance of the first nodules and to a significant reduction in the number of nodules formed per plant, an effect that was dose-dependent (Fig. 3A).

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Impaired nodulation of alfalfa roots was also detected when plants were treated with 2-TDC days before inoculation but also when the MK was applied 2 days after rhizobial inoculation (Fig. 3B and Supporting Information Fig. S6A-C). However, no significant effect was observed when 2-TDC was applied 4 days after rhizobial inoculation (Fig. 3B and Supporting Information Fig. S6D) indicating that 2-TDC adversely interferes in the early stages of the *Rhizobium*-legume interaction. Nodulation inhibition caused by 2-TDC cannot be explained by either a direct bacteriostatic or bactericidal effect (Supporting Information Fig. S4A) or impaired nodulation (*nod*) gene expression (Supporting Information Table S2). Nevertheless, 2-TDC was found to negatively interfere with the bacterial ability to efficiently colonize plant roots (Fig. 3C). The difference in colonization ability was especially remarkable 24 h postinoculation when the presence of 2-TDC reduced by 100-fold the number of bacteria that were associated to roots compared to control plants. This effect, which is in line with the inhibition of biofilm formation caused by 2-TDC, might account for the interference exerted by the MK on the *Rhizobium*-legume symbiosis.

Structural features of 2-TDC are important to promote surface motility in S. meliloti

To evaluate whether the effect caused by 2-TDC on *S. meliloti* surface translocation is a general property of aliphatic ketones, nine different compounds, which varied from 2-TDC either in the acyl chain length or in the position of the carbonyl group, were applied at three different amounts and tested in surface motility assays with strain GR4 (Fig. 4A). In contrast to 2-TDC, treatments with 3-tridecanone (3-TDC), 4-tridecanone (4-TDC), or 7-tridecanone (7-TDC) did not promote surface translocation, indicating that the position of the carbonyl group is crucial for biological activity. Our data indicate that the acyl chain length is another structural attribute that influences

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biological activity of MKs. With the exception of 2-heptanone (2-Hp), which did not affect surface motility at any of the amounts tested, the different MKs used in our assay exhibited certain motility-promoting activity. In general, biological activity decreased with MKs of shorter acyl chain length. For a given MK, the activity increased when raising the concentration at which it was applied. A remarkable exception to this rule was 2-pentadecanone (2-PDC), whose activity at the lowest amount tested (0.2 μmol) was even higher than that obtained with 1 μmol 2-TDC (Fig. 4A). Significant promotion of GR4 surface motility (24.9 ± 3 and 8.8 ± 0.6 mm) was still observed in response to 40 nmol and 20 nmol 2-PDC, respectively, amounts at which 2-TDC did not show any effect.

Interestingly, a competitive interaction was observed between 2-TDC and 7-TDC. GR4 surface translocation promoted by 2-TDC decreased proportionally as the amount of 7-TDC applied to the assay increased, being completely abolished by the highest amount of 7-TDC used (Fig. 4B). Altogether, these data suggest that 2-TDC triggers surface motility in *S. meliloti* upon specific recognition.

Exogenous application of 2-TDC promotes surface migration in S. meliloti by stimulating flagella-dependent and independent mechanisms

We sought to investigate the mechanism by which 2-TDC specifically promotes surface migration in *S. meliloti*. It is known that inactivation of the *fadD* gene promotes surface motility in *S. meliloti* GR4 and Rm1021 by stimulating a flagella-independent mechanism exhibited by the surface spreading displayed by the flagella-less GR4fadDflaAB and 1021fadDflaAB strains (Bernabéu-Roda *et al.*, 2015; Fig. 5). We found that application of volatile 2-TDC promoted surface motility not only in GR4 and Rm1021 but also in the flagella-less derivative strains GR4flaAB and 1021flaAB (Fig.

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246 5A and 5B, respectively). Therefore, like FadD loss-of-function, application of volatile
247 2-TDC triggers a flagella-independent surface translocation in *S. meliloti*. Addition of 2-
248 TDC had a greater influence on promoting surface motility in GR4 and Rm1021 than
249 inactivation of the *fadD* gene in the corresponding genetic backgrounds, suggesting a
250 concentration-dependent effect of the MK.

251 To the best of our knowledge, the only flagellum-independent surface migration
252 described in *S. meliloti* requires production of rhizobactin 1021 (Rhb1021), a lipid-
253 containing siderophore with surfactant properties (Nogales *et al.*, 2010, 2012;
254 Bernabéu-Roda *et al.*, 2015). Rhb1021 is produced by *S. meliloti* strain Rm1021 in
255 response to low iron concentrations but is not produced by strain GR4 (Nogales *et al.*,
256 2010), which explains the different surface motility behaviour of the two strains on
257 semisolid MM. The defect in surface translocation exhibited by a siderophore-defective
258 mutant (1021rhbD) is restored when the *fadD* gene is inactivated and also with
259 application of volatile 2-TDC (Fig. 5B). These results demonstrate that the surface
260 motility triggered by 2-TDC is not mediated by Rhb1021 siderophore.

261 Data shown in Fig. 5 indicate that application of 2-TDC induces mainly a flagella-
262 independent surface translocation in *S. meliloti*. Nevertheless, the increased surface
263 migration exhibited by wild-type strains compared to their corresponding non-
264 flagellated strains in response to 2-TDC suggests that flagella might contribute to the
265 enhanced motility triggered by 2-TDC. In a previous study, we found that expression of
266 the flagellin *flaA* gene was higher in the *fadD* mutant than in the wild-type strain GR4
267 under swarming-inducing conditions (Soto *et al.*, 2002). Here, we investigated whether
268 2-TDC could have a similar effect on *flaA* gene expression by analysing the β-
269 galactosidase activity of GR4 cells harbouring a *flaA*::*lacZ* transcriptional fusion and
270 grown in MM (1% agar) in the absence or presence of volatile 2-TDC. In parallel,

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271 expression of the kanamycin promoter was also tested in cells carrying a *km::lacZ*
272 transcriptional fusion, in order to detect unspecific changes in gene expression caused
273 by 2-TDC treatment. No differences in the expression of the *lacZ* gene driven by the
274 kanamycin promoter were detected between cells grown in the presence (559 ± 60
275 Miller Units) and in the absence (432 ± 75 Miller Units) of 2-TDC. In contrast,
276 expression of the *flaA*::*lacZ* fusion was 2.6-fold higher in cells exposed to volatile 2-
277 TDC than that exhibited by cells grown under control conditions (29085 ± 3296 *versus*
278 11169 ± 2176 Miller Units; ANOVA test P≤0.05). These results indicate that, like FadD
279 loss-of-function, 2-TDC promotes surface translocation in *S. meliloti* by stimulating
280 both flagella-dependent and independent mechanisms.

281

282 *Genome wide analysis of 2-TDC-regulated genes in S. meliloti*

283 To gain insights into the molecular mechanisms responsible for the effects caused by 2-
284 TDC in *S. meliloti*, the transcriptomes of Rm1021 cells grown in broth and semisolid
285 MM in the absence or presence of 2-TDC were compared. While no differential gene
286 expression was detected in response to 2-TDC treatment in *S. meliloti* cells grown in
287 liquid media, 88 genes altered their transcription levels when cells were grown on the
288 surface of semisolid media exposed to volatile 2-TDC (49 genes up-regulated, 39 genes
289 down-regulated; Supporting Information Table S3). Previously, we verified that volatile
290 2-TDC treatment did not cause any effect on growth.

291 The up-regulation of up to 9 genes coding for proteins with putative functions in
292 redox reactions was remarkable. Indeed, the two most up-regulated genes in response to
293 2-TDC code for oxidoreductases: *smb20343* coding for a putative isoquinoline 1-
294 oxidoreductase, and *azoR* (*smc01329*) which codes for a predicted FMN-dependent

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NADH-azoreductase, an enzyme involved in resistance to thiol-specific stress in *Escherichia coli* (Liu *et al.*, 2009). In addition to oxidoreduction-related genes, the presence of 2-TDC increased the expression of 6 genes involved in the synthesis of the amino acids glutamate, methionine and cysteine, and 5 genes coding for transcriptional regulators that might control the expression of drug resistance genes. Interestingly, 56% of the genes down-regulated in response to 2-TDC treatment (22 genes) were related to iron uptake and metabolism, including siderophore Rhb1021 biosynthesis genes. These microarray data, which were validated by RT-qPCR (Supporting Information Table S4), suggest that volatile 2-TDC reduces iron uptake while inducing stress-related responses.

Surface-associated behaviours in plant and animal pathogenic bacteria are also influenced by 2-TDC

Different airborne chemical signals have been implicated in interspecific bacterial signalling, regulating important microbial behaviours such as motility, biofilm formation, drug resistance or virulence (Kim *et al.*, 2013; Audrain *et al.*, 2015; Schmidt *et al.*, 2015). To test if 2-TDC could also influence the behaviour of bacteria other than *S. meliloti*, the surface motility phenotypes of several plant and animal pathogenic bacteria were assessed in the presence of the MK. As shown in Fig. 6A, volatile 2-TDC increased surface motility in the plant pathogens *Pseudomonas syringae* pathovars *syringae* (Pss) and *tomato* (Pst). In contrast, 2-TDC inhibited and reduced swarming motility in *Salmonella enterica* serovar Typhimurium and *Pseudomonas aeruginosa*, respectively (Fig. 6B). The effect of swarming reduction in *P. aeruginosa* was not caused by alterations in rhamnolipid production (Supporting Information Fig. S7). Furthermore, twitching motility was also significantly inhibited by increasing

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319 concentrations of 2-TDC in *P. aeruginosa* (Fig. 6C), suggesting that type IV pili may be
320 affected by this molecule. However, no changes in *pilA* gene expression were observed
321 in response to 2-TDC (Supporting Information Fig. S8). As in *S. meliloti*, no effect of 2-
322 TDC on bacterial growth rate or swimming motility was detected for any of the
323 pathogenic bacteria used in this study.

324 We also examined the influence of 2-TDC on biofilm formation in *P. syringae* and
325 *P. aeruginosa*. The presence of 2-TDC in the culture medium significantly decreased
326 the ability of the bean and soybean pathogen Pss BT111 to develop biofilm firmly
327 attached to the glass surface (Fig. 7A). Likewise, static biofilms developed on glass
328 surfaces by *P. aeruginosa* were significantly inhibited by 2-TDC (Fig. 7B and
329 Supporting Information Fig. S5B). Interestingly, 2-TDC did not have an apparent
330 impact on cyclic dimeric guanosine monophosphate (c-di-GMP) or exopolysaccharide
331 levels when *P. aeruginosa* was grown on LB or NB agar plates using a transcriptional
332 reporter fusion to *cdrA* (regulated by c-di-GMP), or translational fusions to *pelA* and
333 *pslA* (exopolysaccharide biosynthetic genes) (Supporting Information Fig. S9). This
334 suggests that the negative effect of 2-TDC on *P. aeruginosa* biofilms is not mediated
335 via these determinants of biofilm formation. In contrast, a significant decrease in
336 expression of the quorum sensing *rhlI* and *pqsA* genes was detected in response to 2-
337 TDC (Fig. 7C), which could explain the negative effect of the MK on *P. aeruginosa*
338 biofilms since it has been observed that these two QS systems stimulate biofilm
339 formation (Duan and Surette, 2007; Guo *et al.*, 2014) and are required for biofilm
340 maturation (Rampioni *et al.*, 2010).

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342 *Application of 2-TDC to tomato plants prevents infection by the phytopathogen P.*
343 *syringae pv. tomato*

344 Considering the above mentioned results, the hypothesis that 2-TDC could interfere not
345 only with the *Rhizobium*-legume symbiosis but also with the establishment of
346 pathogenic interactions was worth testing. With this aim, we analysed the effect of 2-
347 TDC treatment on the pathogenicity of *P. syringae* pv. *tomato* DC3000 (Pst DC3000)
348 on tomato plants. Pst DC3000 is the causative agent for bacterial speck on tomato and it
349 is used as a relevant model in plant pathology. Interestingly, we found that the
350 development of disease symptoms (brown necrotic spots in leaves) (Fig. 8A) as well as
351 growth of Pst DC3000 populations within leaf tissues (Fig. 8B) were significantly
352 reduced in tomato plants which were sprayed with 2-TDC at the time of bacterial
353 inoculation. The protective effect was more notable in plants which were sprayed with
354 the highest concentration of 2-TDC. Therefore, the presence of 2-TDC during early
355 stages of plant-bacteria interactions protects plants from bacterial infections.

356

357 **Discussion**

358 The aim of this study was to identify putative lipidic compounds responsible for the
359 pleiotropic phenotype exhibited by a *fadD* mutant of *S. meliloti*. The results show that
360 the mutant overproduces 2-tridecanone (2-TDC), and that the accumulation of this
361 methylketone (MK) might account for the increased surface motility, decreased biofilm
362 formation and impaired alfalfa root colonization shown by the *fadD* strain. This is the
363 first time that 2-TDC has been shown to be produced by rhizobia. Likewise, to the best
364 of our knowledge, this is the first report that relates FadD inactivation with levels of 2-
365 TDC production in bacteria. More importantly, we demonstrate for the first time that 2-
366 TDC acts as a chemical cue that affects bacterial behaviours and the establishment of

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plant-microbe interactions. Therefore, our study assigns new biological roles to 2-TDC, which previously was known only as a strong pesticide responsible for the natural resistance against insect herbivory exhibited by 2-TDC-hyperproducing wild tomato plants (Williams *et al.,* 1980).

Production of 2-TDC was initially detected exclusively in the spent media from the *S. meliloti fadD* mutant cultures. Later, we found that the wild-type strain also produces volatile 2-TDC during growth on the surface of MM (1% agar), although the amount emitted was only 13% of that of the *fadD* mutant. These results demonstrate that *S. meliloti* can produce 2-TDC and indicate that growth conditions and especially FadD activity somehow modulate 2-TDC synthesis in this bacterium. Why the *fadD* mutant accumulates 2-TDC remains unclear because the biochemical pathways implicated in the synthesis of MKs are largely unknown. In tomato plants, 2-TDC is derived from 3-ketomyristoyl-ACP, an intermediate formed during fatty acid biosynthesis (Yu *et al.*, 2010), but information on how bacteria produce MKs is scarce (Forney and Markovetz, 1971; Patel *et al.*, 1980). We hypothesize that, in a *fadD* mutant, 2-TDC may derive from substrates and enzymatic reactions similar to those described for tomato (Supporting Information Fig. S10). We are currently investigating the pathway for 2-TDC formation in *S. meliloti* and how a deletion in *fadD* leads to a significant increase in 2-TDC production.

The concentrations of 2-TDC used in our assays are higher than those detected by SPME-GC/MS in the *fadD* mutant. However, it is very likely that only a fraction of the total amount of 2-TDC synthesized by this strain was detected due to limited volatility of the MK and partition into the cell membranes. For the same reasons, not all of exogenously applied 2-TDC might reach its target in the cell to trigger biological effects. Nevertheless, our study shows that the application of volatile 2-TDC promotes

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surface translocation in several strains of *S. meliloti*, phenocopying the effect caused by a *fadD* mutation (Fig. 5). Likewise, without affecting growth, the presence of 2-TDC impairs biofilm formation and alfalfa root colonization by *S. meliloti* wild-type strains, resembling the effect of *fadD* inactivation. These results suggest that the accumulation of 2-TDC is responsible for the altered behaviour in the *fadD* mutant. Notably, the effects caused by 2-TDC are not exclusive to *S. meliloti* as application of the MK impacts surface motility and biofilm formation in phylogenetically distant bacteria, including plant and animal pathogens. Whereas the application of 2-TDC stimulates bacterial surface motility in plant-interacting bacteria (rhizobia and *P. syringae* pathovars), the presence of the volatile MK hampers surface translocation in the two animal pathogenic bacteria tested (*P. aeruginosa* and *S. enterica*). Importantly, the application of 2-TDC impaired biofilm formation in all the different bacteria tested, without affecting bacterial growth. These properties could be harnessed as a potential synergistic treatment against biofilm-forming pathogenic bacteria.

The mechanisms by which bacteria perceive and respond to volatiles are mostly unknown (Kim *et al.*, 2013; Audrain *et al.*, 2015; Schmidt *et al.*, 2015). In this study, different approaches have been used to unveil how 2-TDC impacts bacterial surface behaviours. We found that promotion of surface translocation is not a general property of aliphatic ketones since structural features of the molecule determine the compound's bioactivity, which suggests specific recognition. The bacterial response to 2-TDC cannot be explained by an effect on key motility mechanisms, since swimming motility remains unaffected. This result contrasts with the effect observed for two volatiles produced by *Bacillus subtilis,* glyoxylic acid and 2,3-butanedione*,* which were found to reduce both swimming and swarming motilities in different bacteria (Kim *et al.*, 2013). In *S. meliloti*, the increased surface motility triggered by 2-TDC is mainly the result of

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promoting a flagellum-independent migration. The nature of this motility, which cannot be explained by 2-TDC acting as a surfactant (Kotowska and Isidorov, 2011), and that is not mediated by the surfactant activity of the Rh1021 siderophore, warrants further investigation.

The phenotypes altered by 2-TDC are often under quorum sensing (QS) regulation (Verstraeten *et al.*, 2008). In *S. meliloti*, we could not detect changes in the expression level of the QS genes *sinI* and *wgeA* (Charoenpanich *et al.*, 2013) in response to the MK (Supporting Information Fig. S11). However, in *P. aeruginosa*, swarming and biofilm inhibition caused by 2-TDC could be explained by the decreased transcription of the QS genes *rhlI* and *pqsA*, as these two QS systems stimulate biofilm formation and are required for biofilm maturation (Diggle *et al.*, 2003; Duan and Surette, 2007; Guo *et al.*, 2014; Rampioni *et al.*, 2010). The negative impact of 2-TDC on twitching may also explain the reduction on biofilm formation since this type of motility has been associated with the early stages of biofilm formation (Oliveira *et al.*, 2016). Under the conditions tested, no changes in transcription of the type IV major pilin gene *pilA* could be detected. Nevertheless, decreased *pqsA* expression could influence transcriptional activity of the cognate regulator PqsR, which is required for twitching motility (Guo *et al.,* 2014). Considering the results obtained in *S. meliloti* and *P. aeruginosa*, and that surface translocation and biofilm formation are controlled by different mechanisms depending on the bacterial species, studies performed in different model bacteria are required in order to identify common and/or specific targets of 2-TDC.

The transcriptomic analyses performed in *S. meliloti* revealed that 2-TDC alters gene expression in cells grown in semisolid but not in liquid media. This could indicate that cell responses to 2-TDC might require the physiological conditions conferred upon growth on surfaces (Nogales *et al.*, 2010). Differential expression of redox and iron

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uptake-related genes were detected in response to volatile 2-TDC. Curiously, in the cotton bollworm *Helicoverpa armigera*, 2-TDC increases the expression of a transferrin gene coding for a putative iron-binding protein and of several genes coding for heme-containing P450 enzymes which catalyse different oxidative reactions. Whereas increased expression of P450 enzymes was related to the retardant effect on insect development, transferrin was associated with moth tolerance to 2-TDC by controlling iron and H_2O_2 levels (Zhang *et al.,* 2015; Zhang *et al.,* 2016). Future research will be focused on investigating whether in bacteria a link also exists between iron homeostasis, oxidative stress and 2-TDC. The control of iron levels is not a novel mechanism underlying the effect of bacterial volatiles. The inorganic volatile H_2S renders bacteria highly resistant to oxidative stress through a mechanism involving H_2S-mediated sequestration of free iron, thus avoiding damaging Fenton reactions (Shatalin *et al.*, 2011; Mironov *et al.*, 2017).

The present study not only describes for the first time the impact of 2-TDC on bacterial behaviours that are relevant for niche colonization and persistence, but also its effect on the establishment of plant-microbe interactions. The presence of 2-TDC hampers the bacterial ability to efficiently colonize plant tissues. This effect, which correlates with the reduced biofilm formation on abiotic surfaces observed with 2-TDC, might account for the negative effect that the MK exerts on the development of plant-bacteria interactions. However, whether 2-TDC exerts an effect directly on the plant cannot be ruled out since it is known that some bacterial volatiles can elicit plant responses, including induced systemic resistance (Ryu *et al.*, 2004; Audrain *et al.*, 2015; Chung *et al.*, 2016). In any case, we demonstrate that in addition to the protective role against insect pests, 2-TDC can also protect plants from detrimental bacteria. As a natural, non-growth limiting chemical that targets bacterial traits which influence the

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virulence of pathogenic bacteria, 2-TDC opens perspectives for the development of biotechnological solutions to control bacterial infections, minimizing the risk of generating antimicrobial resistance. The identification of genes involved in 2-TDC metabolic pathways, as well as deciphering specific mechanisms through which 2-TDC impacts bacterial behaviours and plant-bacteria interactions will undoubtedly contribute to the development of new approaches for environmental-friendly agricultural practice.

Experimental procedures

Bacterial strains, plasmids, and growth conditions

Bacterial strains and plasmids used in this work are listed in Supporting Information Table S5. *S. meliloti* strains were grown in either complex tryptone yeast (TY) medium, in Bromfield medium (BM), or in Robertsen minimal medium (MM) as described previously (Nogales *et al.*, 2012). *E. coli*, *S. enterica* and *Pseudomonas* spp. strains were routinely grown in Lysogeny Broth (LB) medium. Nutrient Broth (NB) medium (Rashid and Kornberg, 2000) containing 0.8% Nutrient broth No.2 (Oxoid) and 0.5% D glucose was also used for *P. aeruginosa*. When required, antibiotics were added to final concentrations (µg/ml) of: 200 ampicillin, 50 kanamycin, 10 tetracycline for *E. coli*; 200 kanamycin, 10 tetracycline and 75 hygromycin for *S. meliloti*; 10 rifampicin for *P. syringae* pv. *tomato* DC3000; 10 gentamicin for *P. aeruginosa*. *S. meliloti* and *P. syringae* were routinely grown at 30°C. *E. coli*, *S. enterica* and *P. aeruginosa* strains were grown at 37ºC.

Analyses of lipid extracts by Gas Chromatography/Mass Spectrometry (GC/MS)

One litre-cultures of *S. meliloti* strains were grown for 26 h (OD_{620}= 1.2) at 30°C in Sherwood minimal medium as described previously (Pech-Canul *et al.*, 2011). Lipids

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492 from cell pellets were extracted following the method of Bligh and Dyer (Bligh and
493 Dyer, 1959), and spent media were extracted twice with 400 ml of acidified ethyl
494 acetate (0.01 % acetic acid). Each of the extracts was dried by rotary evaporation at
495 45°C and dissolved in 1 ml of 1:1 methanol:chloroform. Each extract (1 ml) was
496 fractionated on a 5 g silica gel (Merck) column (1.5 cm diameter) by eluting with ethyl
497 acetate:hexanes:acetic acid (60:40:5). Twenty fractions of 2 ml were collected and
498 aliquots of each fraction were analysed by thin layer chromatography (TLC) using as
499 mobile phase ethyl acetate:hexanes:acetic acid (60:40:5). TLC plates were revealed with
500 iodine vapours and fractions showing a similar pattern were pooled, dried with a stream
501 of nitrogen, and dissolved in 600 µl of methanol:chloroform (1:1). Extractions were
502 carried out from two biological repetitions. One µl of the extract was used for analysis
503 by GC (GC 6890 Agilent Technologies, Santa Clara, CA, U.S.A.) coupled to MS
504 detection (Quadrupole MSD HP 5973, Agilent Technologies, Santa Clara, CA).
505 Samples were analysed on a 5% phenyl, 95% methylpolysiloxane capillary column
506 (HP-5MS, Agilent Technologies, Santa Clara, CA, 25 m x 0.20 mm x 0.33 µm film
507 thickness). The oven temperature was set to 40°C for 2 min followed by a temperature
508 gradient of 40ºC to 269ºC at 10ºC min^{-1} and held at 269ºC for 20 min. Helium was used
509 as the carrier gas, with a flow rate of 40 cm s^{-1} at 100ºC. The molecules were ionized at
510 70 eV by electron impact and the NIST database version 1.7a used for identification.
511
512 *Analyses of volatiles by Solid Phase MicroExtraction (SPME)-GC/MS*
513 Ten µl of washed, 10-fold-concentrated of *S. meliloti* cultures grown in TY broth to the
514 late exponential phase were inoculated onto the surface of 3 ml of MM (1% agar) in 10
515 ml glass vials equipped with silicone septa and incubated at 30°C for two days. For
516 control samples 10 µl of liquid MM were applied onto the medium. Volatiles were

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517 collected from the headspace of the vials by SPME (Zhang and Pawliszyn, 1993). For
518 that, a Divinylbenzene/Carboxen/Polydimethylsiloxane (DVB/CAR/PDMS) coated
519 StableFlex SPME fiber of 50/30µm (Supelco 57298-U), was conditioned for 1 h at
520 250ºC in a stream of helium and then introduced into the headspace of the vial and
521 incubated at 30°C for 60 min. Then the fiber was inserted in the injector of GC/MS
522 (Varian 450GC 240MS, Ion Trap) and desorbed at 280°C (6 min). Samples were
523 separated in a DB5MS-UI column (30 m, 0.25-mm inside diameter, 0.25 µm). The
524 program used for oven temperature was 40°C (3 min), 140°C (15°C min^{-1}) and 300°C
525 (15°C min^{-1}), and held for a further 5 min. Helium was used as the carrier gas, with a
526 flow rate of 1.2 ml min^{-1}. The mass spectrometer was run at 220ºC in the positive
527 electron ionization mode at 70 eV and 220°C with scans performed from 40 to 600 *m/z*,
528 which allow detection of characteristic ions of medium and long-chain aldehydes and
529 MKs. The mass spectra of detected peaks were compared with the NIST database. The
530 production of 2-TDC was confirmed by comparison with a pure 2-TDC standard
531 prepared and analysed using SPME fibers in the same manner as the biological samples.
532
533 *Methylketone (MK) treatments*

534 MK solutions were prepared in ethanol. When the treatment was applied in the culture
535 medium, a 1000-fold concentrated solution was diluted accordingly. Control treatment
536 contained 0.1% ethanol. For treatments as a volatile, 20 µl of either solutions containing
537 the desired amount of the MK (e.g. 20 µl of 50 mM 2-TDC contain 1 µmol of the MK)
538 or ethanol (control) were applied onto the lid of the plate just before incubation. Plates
539 were sealed with parafilm and incubated face-down. Furthermore, treatments with
540 different MKs were kept in independent compartments to avoid possible interferences
541 between them.

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543 *Motility assays*

544 Swimming motility was assayed on BM plates with 0.3% Bacto agar as reported
545 previously (Nogales *et al.*, 2012). Surface motility assays for *S. meliloti* and *P. syringae*
546 were carried out according to Soto *et al*. (2002) in MM containing 1% Noble Agar
547 (Difco, BD). Swarming assays for *S. enterica* were carried out according to Harshey and
548 Matsuyama (1994) on LB medium containing 0.4% glucose and 0.6% Bacto Agar
549 (Difco). Swarming assays for *P. aeruginosa* were performed by inoculating 3 µl of an
550 overnight culture on the surface of NB medium containing 0.5 % Bacto Agar (Difco).
551 Twitching motility in *P. aeruginosa* was assessed by stab inoculation of overnight
552 cultures right to the bottom of plates containing 10 ml of LB (1% agar). After 48 h of
553 incubation at 37ºC, the agar was eliminated and plates were stained with 1% crystal
554 violet.

555

556 *Biofilm formation assays*

557 Biofilms of *S. meliloti* and *P. aeruginosa* strains expressing the gene coding for the
558 green fluorescence protein (GFP) were established under static growth conditions on
559 chambered cover glass slides (Lab-Tek® II Chamber Slide™ no. 70379-82, Electron
560 Microscopy Sciences, Hatfield, PA, USA). Late exponential phase cultures in MM
561 broth of *S. meliloti* strains harbouring the *gfp*-expressing vector pHC60 were washed
562 and diluted to an OD_{600nm} of 0.2 in the same culture medium containing either 50 µM 2-
563 TDC or 0.1% ethanol (control). In the case of *P. aeruginosa*, an overnight culture of the
564 GFP producing strain PADP44 was diluted to an OD_{600nm} of 0.05 in NB broth
565 containing 100 µM 2-TDC or 0.1% ethanol (control). Five hundred microliters of the
566 corresponding bacterial suspensions were inoculated into each chamber and incubated at

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30ºC for three days (*S. meliloti*) or at 37ºC overnight (*P. aeruginosa*). Prior to visualization, supernatants were carefully removed by pipetting to avoid biofilm detachment. Biofilms were observed using a confocal laser inverted microscope Zeiss LSM 700 (Carl Zeiss, Oberkochen, Germany). To quantify the mass of biofilms established on glass surfaces, total fluorescence emitted by each biofilm was determined using ImageJ (Abràmoff *et al.*, 2004). At least three replicates were analysed to obtain a mean value. To assess biofilm formation of *P. syringae* pv *syringae,* strain BT111 was grown in LB broth with 50 µM 2-TDC or 0.1% ethanol (control) for 24 h at 30ºC. After the incubation period, liquid from the tubes was removed and bacterial biofilm stained with 0.1% crystal violet during 20 minutes.

Transcriptomics

Total RNA was isolated from cells grown either in MM broth or on the surface of semisolid MM in the presence or absence of 2-TDC. 2-TDC (50 µM) or ethanol (0.1%) were added to broth cultures at mid-exponential phase (OD_{600nm} = 0.5), and cells were collected after 1 and 4 h of treatment. RNA was also extracted from cells grown for 7 h on the surface of semisolid MM as described previously (Nogales *et al.*, 2010) in the presence of volatile 2-TDC (1 µmol) or ethanol (control). RNA extraction, cDNA synthesis from 10 µg of total RNA, Cy3- and Cy5-labelling, hybridization to Sm14kOLI microarrays, image acquisition and data analysis were performed as previously reported (Calatrava-Morales *et al.*, 2017). Normalization and t-statistics were carried out using the EMMA 2.8.2 microarray data analysis software (Dondrup *et al.*, 2009). Data were collected and analysed from three independent biological replicates. Genes were regarded as differentially expressed if they showed a *P* value ≤ 0.05, average signal to noise value ≥ 7 and $\log_2$ experiment/control ratio value ≥ 1 or ≤ -1. Raw data resulting

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592 from the microarray experiments and detailed protocols have been deposited in the
593 ArrayExpress database with accession number E-MTAB-3893.
594

595 *Reverse transcription quantitative real-time PCR (RT-qPCR)*
596 One µg of RNA was reversely transcribed using Superscript II reverse transcriptase
597 (Invitrogen) and random hexamers (Roche) as primers. qPCR was performed on an
598 iCycler iQ5 (Bio-Rad, Hercules, CA, USA) according to Calatrava-Morales *et al.*
599 (2017). The primer sequences for qPCR are listed in Supporting Information Table S6.
600 Data normalization with 16S rRNA was performed with the critical threshold (ΔΔCT)
601 method (Pfaffl, 2001).
602

603 *Measurement of β-galactosidase activity*
604 β-galactosidase activity of *S. meliloti* cells containing *lacZ* fusions and grown in either
605 liquid or semisolid MM, was assayed as described previously (Soto *et al.*, 2002).
606

607 *Construction of a pilA-lux transcriptional fusion*
608 To construct a *pilA-lux* transcriptional fusion, a ~0.3 kb fragment generated from
609 PAO1-L chromosomal DNA by PCR with primers PPpilAFw (5´-
610 TATAAGCTTCTTTTCGTCGAGTAGATTGG-3´) and PPpilARv (5´-
611 ATAGAATTCGTTGATTATGTATAGGCCTA-3´), was cloned into a *Hind*III and
612 *Eco*RI-cut miniCTX-*lux*(Gm^R) plasmid. The plasmid obtained was mobilized from *E.*
613 *coli* S17-1 λpir and mini-CTX elements inserted in the chromosome of PAO1-L wild-
614 type by mating.
615

616 *Analysis of bioluminescence activity*

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To study the effect of 2-TDC on the expression of QS regulators and type IV pili, *P. aeruginosa* PAO1 containing the corresponding transcriptional reporter fusion was grown overnight, and cultures were spotted onto NB-agar plates in the presence and absence of 2 µmol of 2-TDC inoculated on the plate lid (20 µL). After an incubation time of 8 h or 16 h at 37 °C, cells were harvested from the plate, dissolved in phosphate buffer saline (PBS), and analysed for bioluminescence output activity over growth using a TECAN Genios Pro spectrophotometer.

Rhamnolipid determination by TLC

Three µl from overnight *P. aeruginosa* cultures grown in LB were diluted in water and then spread on swarming plates with the help of glass beads. The next day, the whole agar content was recovered and mixed with 20 ml of 1 % $KHCO_3$, pH=9, and the mixture was incubated 1 day at 4ºC. The agar was then removed by centrifugation and filtration with 0.2 µm pore filters (Sartorius), and the liquid phase was extracted 3 times with equal volumes of ethyl acetate.

Normal phase TLC was used to study rhamnolipid production, based on a previously published method (Wittgens *et al.*, 2011). The mobile phase consisted of chloroform:methanol:glacial acetic acid in a 65:15:2 ratio. Once the samples were run, mono- and di-rhamnolipids were put in contact with a detection agent comprised of 0.15 g orcinol monohydrate, 8.2 ml 60 % H_2SO_4 and 42 ml water, and subsequently incubated at 110 ºC for 10 min. The areas corresponding to rhamnolipids on the TLC plates were measured using GNU Image Manipulation Program (GIMP) version 2.8.10.

Nodulation and colonization assays

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Alfalfa (*Medicago sativa* L. cv. Aragón) seeds were surface-sterilized, germinated and the seedlings were grown in hydroponic cultures under axenic conditions in glass tubes as described previously (Olivares *et al.*, 1980). For nodulation assays, ten day-old plants (1 plant/tube) were inoculated with 1 ml of a rhizobial suspension containing 5 x 10^6 cells (Bernabéu-Roda *et al.*, 2015). After inoculation, the number of nodules per plant was recorded daily. For colonization assays, one-week-old alfalfa plants (5 plants in each tube) were inoculated with 1 ml of a rhizobial suspension as described above. At defined times, 15 roots from each treatment were collected and processed to determine colony-forming units (CFU) as described previously (Amaya-Gómez *et al.*, 2015).

Tomato infection assays

Solanum lycopersicum "Moneymaker" seedlings were grown in a growth chamber with 70% relative humidity, 16/8 h light/dark photoperiod and 24/20ºC day/night temperature. *P. syringae* pv. *tomato* DC3000, grown on LB plates containing rifampicin for 48 h at 28ºC, was resuspended in 10 mM $MgCl_2$ and the OD_{600} was adjusted to 0.3-0.4 to obtain a bacterial suspension of 10^8 CFU/ml. Fifty µM 2-TDC, 500 µM 2-TDC, or 0.1% ethanol as control, were added to the bacterial suspension. Four- to five-week-old plants were inoculated with each of the three different bacterial suspensions (4 plants/treatment) using an airbrush. The evolution of symptoms and sampling were performed 3 h after inoculation (time 0) and several days (3, 6 and 10) after inoculation. To determine the number of bacteria in infected leaves, five 10 mm diameter-disks (3.9 cm^2) were obtained per leaf with a cork borer, homogenized by mechanical disruption into 1 ml of 10 mM $MgCl_2$ and the CFU determined by plating serial dilutions. At least 4 different homogenized samples obtained from 2 leaves from 2 plants were analysed per treatment.

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841 **Figure legends**

842 **Fig. 1.** Identification of volatile 2-TDC in *S. meliloti* by SPME-GC/MS.

843 A. Representative GC/MS chromatograms in the selected ion monitoring (SIM) mode
844 for ion 58 of SPME trapped volatiles emitted into the headspace by *S. meliloti fadD* and
845 wild-type strains grown on MM (1% agar), or a pure 2-TDC standard applied onto the
846 same medium after 48 h of incubation at 30ºC. The scale at the Y axis is in arbitrary
847 units. The mean peak area ± standard error (given in counts) corresponding to 2-TDC
848 produced by the two *S. meliloti* strains and calculated from at least four biological
849 measurements, is indicated with A.

850 B. Mass spectrum of the compound identified in *S. meliloti fadD* or wild-type strains
851 and the 2-TDC standard. The arrow points to the molecular ion (*m/z* 198).

852

853 **Fig. 2.** 2-Tridecanone promotes surface motility and impairs biofilm formation in *S.*
854 *meliloti*.

855 A. Surface motility assays on MM (1% agar) with *S. meliloti* wild-type GR4 and
856 Rm1021 strains in the presence of volatile 2-TDC. Twenty µl of either ethanol (control)
857 or solutions containing the desired amount (µmol) of 2-TDC were applied to the lid of
858 the plates just before incubation. Representative pictures of the motilities exhibited after
859 48 h of incubation are shown. Values below each picture represent average surface
860 migration (given in millimetres). Means and standard errors were obtained from at least
861 3 replicates in three independent experiments (n=9). Different letters indicate significant
862 differences according to an analysis-of-variance (ANOVA) test ($P \leq 0.05$). * Indicates
863 that the treatment was applied as a volatile.

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B. Three-dimensional reconstructions of overlapped confocal image stacks obtained from 3-day old biofilms developed by GFP-marked GR4 and Rm1021 after growth in MM in the presence of 0.1% ethanol (control) or 50 µM 2-TDC under static conditions.

Fig. 3. 2-TDC impairs nodulation of alfalfa plants.

A. Nodulation kinetics of alfalfa plants inoculated with *S. meliloti* GR4 in the absence or presence of 2-TDC. 2-TDC was added to final concentrations of 5 µM or 25 µM to the mineral solution at the moment of rhizobial inoculation. Data represent the average number of nodules per plant. The bars represent standard errors from twenty independent replicates. Asterisks indicate significant differences compared to the control according to an ANOVA test ($P \leq 0.05$). One representative experiment out of at least three repetitions is shown.

B. Percentage of nodules formed by alfalfa plants 10 days after inoculation with *S. meliloti* GR4 and treated with 25 µM 2-TDC at 4 days before inoculation (4dbi), 2 days before inoculation (2dbi), at the same time as inoculation (0), 2 days postinoculation (2dpi), and 4 days postinoculation (4dpi). Data represent the average percentage calculated relative to the corresponding mean of the control treatment at the same moment as 2-TDC application. Error bars indicate the standard error. Values followed by the same letter do not differ significantly according to an ANOVA test ($P \leq 0.05$). Additional data can be found in Fig. S6.

C. Efficient alfalfa root colonization by *S. meliloti* is hindered by the presence of 2-TDC. Colony forming units (CFU) recovered from alfalfa roots were determined 2, 24, 48 and 72 h postinoculation with *S. meliloti* GR4. Data are expressed per gram of root fresh weight (RFW). Error bars indicate the standard error from the mean. According to ANOVA test ($P \leq 0.05$), values followed by the same letter do not differ significantly.

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889

890 **Fig. 4.** Structural features of aliphatic ketones determine their biological activity.

891 A. Surface motility of *S. meliloti* GR4 48 h after application of different ketones in
892 volatile form. Twenty µl of either ethanol (control) or concentrated solutions containing
893 the desired amount of each ketone were applied to the lid of the plates just before
894 incubation. Data represent the means and standard errors from at least nine
895 measurements obtained in three independent experiments. Different letters indicate
896 significant differences compared with control and 2-TDC treatments according to an
897 ANOVA test ($P \leq 0.05$). 2-TDC, 2-tridecanone; 3-TDC, 3-tridecanone; 4-TDC, 4-
898 tridecanone; 7-TDC, 7-tridecanone; 2-Hp, 2-heptanone; 2-DC, 2-decanone; 2-UDC, 2-
899 undecanone; 2-DDC, 2-dodecanone; 2-PDC, 2-pentadecanone; 2-HxDC, 2-
900 hexadecanone.

901 B. Suppression of 2-TDC-induced surface motility by different amounts of 7-TDC. Data
902 are shown as the proportion of the surface motility exhibited by GR4 when only 2-TDC
903 (1 µmol) was applied.

904

905 **Fig. 5.** Effects on surface translocation caused by the *fadD* mutation or volatile 2-TDC
906 in flagella-less and Rhb1021-defective *S. meliloti* strains.

907 Surface motility assays on MM (1% agar) with GR4 derivative strains (A) and Rm1021
908 derivative strains (B). The genotype of each strain is indicated. Twenty µl of either
909 ethanol (control) or a solution containing 1 µmol 2-TDC were applied to the lid of the
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911 strain are shown. Values represent average surface migration (given in millimetres)
912 obtained after 48 h of incubation at 30°C. Means and standard errors were obtained

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913 from at least 3 replicates in three independent experiments (n=9). * Indicates that the
914 treatment was applied as a volatile.
915
916 **Fig. 6.** 2-TDC impacts surface motility of plant and animal pathogenic bacteria.
917 Swarming of (A) the plant pathogens *Pseudomonas syringae* pv. *syringae* (Pss) BT111
918 and *P. syringae* pv. *tomato* (Pst) DC3000 and (B) the animal pathogens *Salmonella*
919 *enterica serovar* Typhimurium (St) LT2 and *P. aeruginosa* PAO1-L in response to
920 volatile 2-TDC. Twenty µl of ethanol (control) or a solution containing 1 µmol 2-TDC
921 were applied to the lid of the plates just before incubation. * Indicates that the treatment
922 was applied as a volatile.
923 C. Twitching motility of *P. aeruginosa* PAO1-L assayed in the presence of volatile 2-
924 TDC generated from solutions containing different amounts of the MK and applied to
925 the lid of the plates. The zone of twitching is indicated with arrows. Error bars represent
926 one standard deviation from three independent replicates. A one-way ANOVA was used
927 to assess statistical significance; ****, p value<0.0001.
928
929 **Fig. 7.** Biofilm formation in plant and animal pathogenic bacteria is hampered by 2-
930 TDC.
931 A. Effect of 2-TDC (50 µM) on the crystal violet-stained biofilm formed by Pss BT111
932 on glass tubes after 24 h of growth in LB broth.
933 B. Three-dimensional reconstructions of overlapped confocal image stacks obtained
934 from biofilms developed by *gfp*-expressing *P. aeruginosa* PAO1-L after growth in NB
935 in the presence of ethanol (control) or 2-TDC (100 µM) under static conditions.
936 Representative images of the biofilms developed after incubation overnight at 37ºC are
937 shown.

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C. Impact of 2-TDC on the expression of *P. aeruginosa* quorum sensing regulatory genes. Strains harbouring transcriptional fusions were spotted onto NB plates alongside the corresponding promoterless construct, in the absence or presence of volatile 2-TDC (2 µmol applied onto the plate lid). Plates were incubated for 8 h or 16 h at 37ºC. * Indicates that the treatment was applied as a volatile. Relative luminescent units (RLU) were divided by the OD_{600}. Results are presented standardised by the RLU/OD and by the background RLU/OD of the promoterless pminiCTX-lux PADP228. Error bars represent one standard deviation of the means. Multiple t-tests, with the Holm-Sidak correction (cut off value=0.05) were performed; a, p-value<0.0001.

Fig. 8. Tomato plants treated with 2-TDC are more resistant to the development of bacterial speck disease.

A. Bacterial speck symptoms developed on tomato leaves 10 days after spray-inoculation with a suspension of *P. syringae* pv. tomato DC3000 (10^8 CFU/ml) in the absence or presence of 2-TDC (50 or 500 µM). The different treatments were applied at the time of inoculation with the pathogen. Control plants were treated with an equivalent amount of ethanol (0.1%).

B. Time course of *P. syringae* pv. tomato DC3000 growth in leaves of tomato plants. CFUs were determined 3 h (day 0) and 3, 6, and 10 days postinoculation. Data represent the average and standard errors obtained from at least three different measurements. Asterisk indicates significant differences compared with control treatment according to an ANOVA test (P≤0.05).

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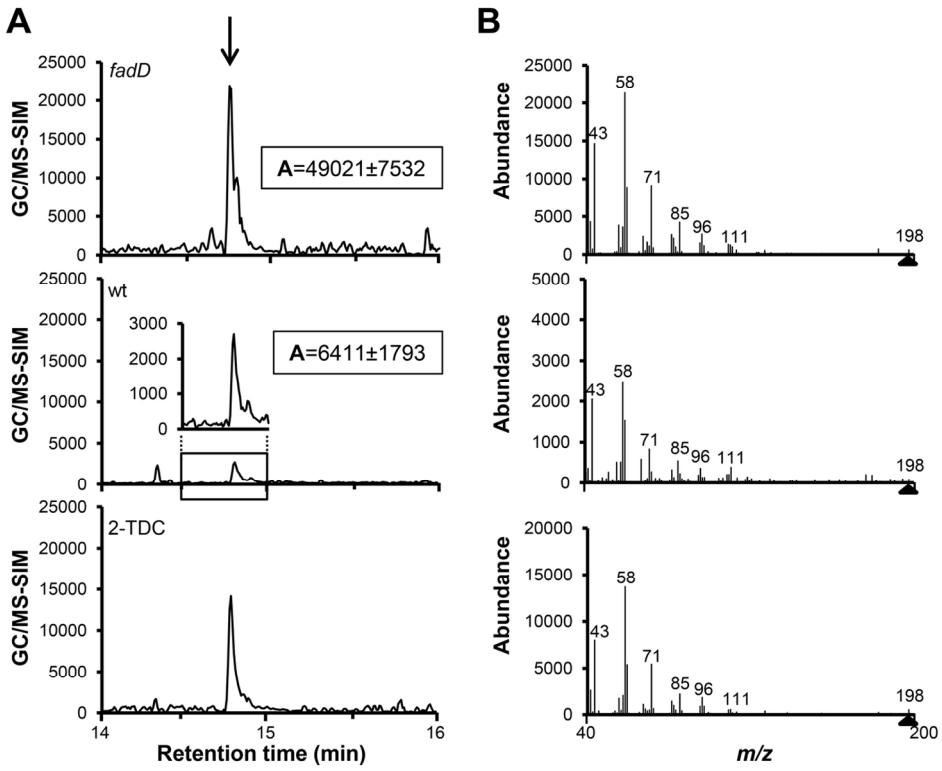

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B. Mass spectrum of the compound identified in S. meliloti fadD or wild-type strains and the 2-TDC standard. The arrow points to the molecular ion (m/z 198).

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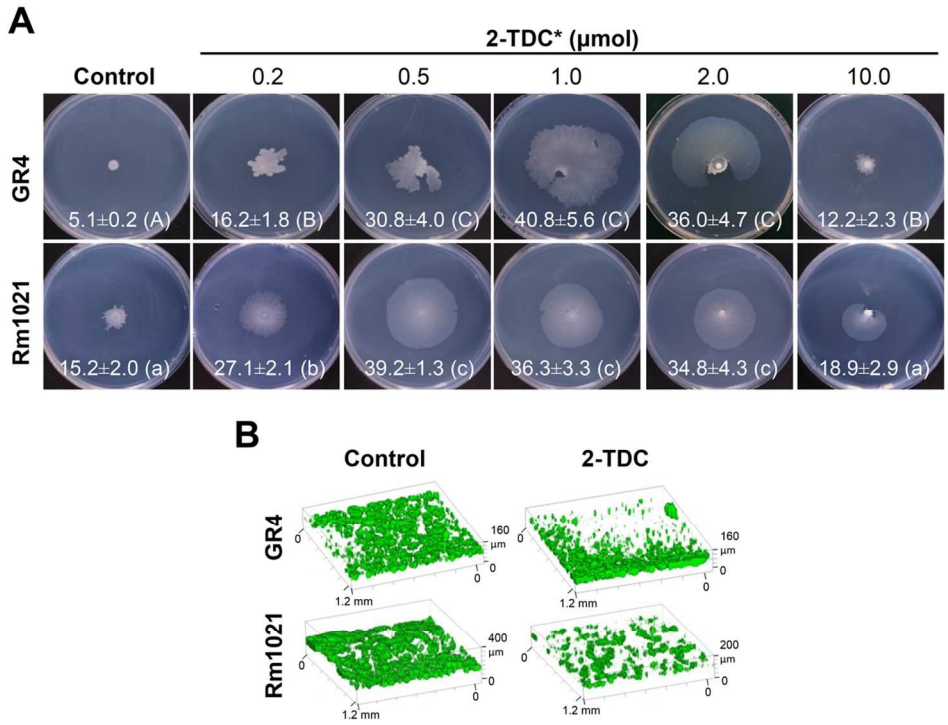

Fig. 2. 2-Tridecanone promotes surface motility and impairs biofilm formation in S. meliloti.
A. Surface motility assays on MM (1% agar) with S. meliloti wild-type GR4 and Rm1021 strains in the presence of volatile 2-TDC. Twenty µl of either ethanol (control) or solutions containing the desired amount (µmol) of 2-TDC were applied to the lid of the plates just before incubation. Representative pictures of the motilities exhibited after 48 h of incubation are shown. Values below each picture represent average surface migration (given in millimetres). Means and standard errors were obtained from at least 3 replicates in three independent experiments (n=9). Different letters indicate significant differences according to an analysis-of-variance (ANOVA) test (P≤0.05). * Indicates that the treatment was applied as a volatile.
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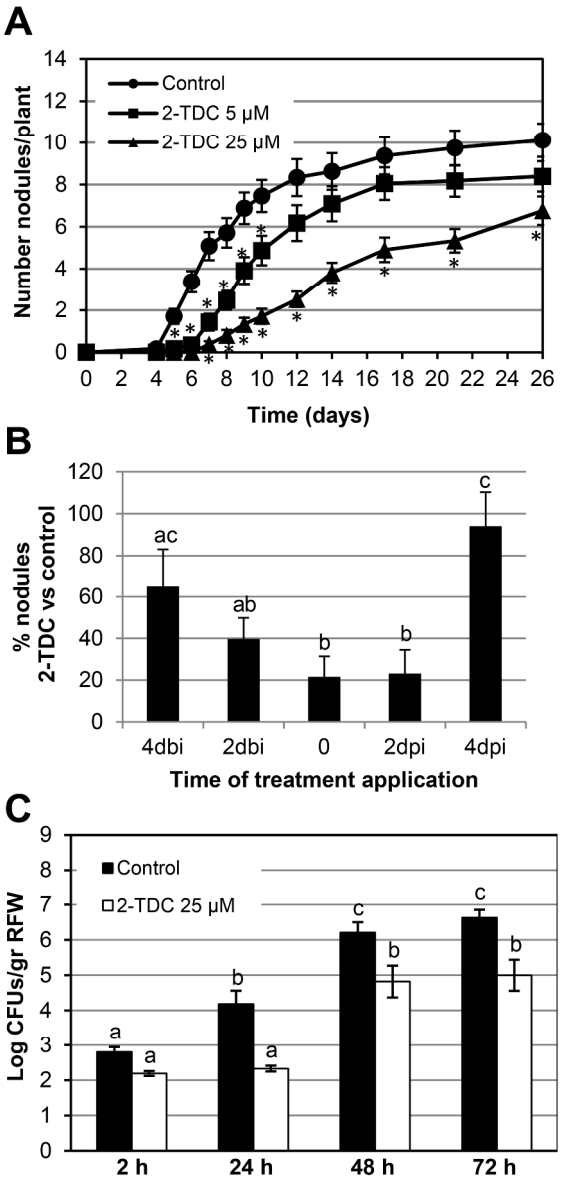

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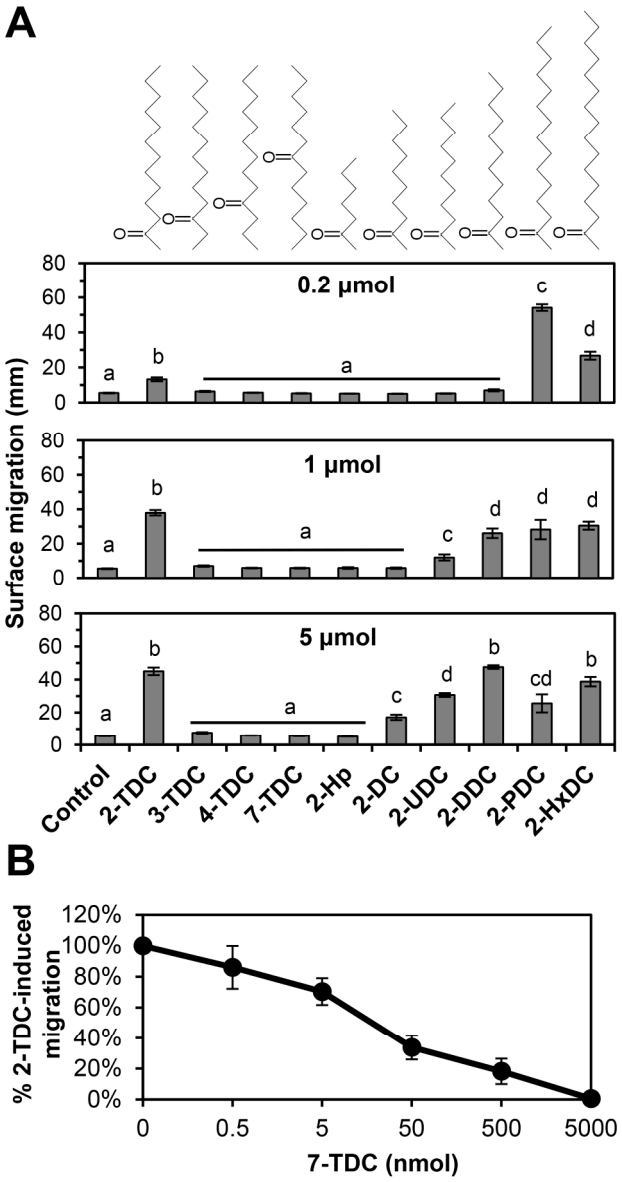

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A. Surface motility of S. meliloti GR4 48 h after application of different ketones in volatile form. Twenty µl of either ethanol (control) or concentrated solutions containing the desired amount of each ketone were applied to the lid of the plates just before incubation. Data represent the means and standard errors from at least nine measurements obtained in three independent experiments. Different letters indicate significant differences compared with control and 2-TDC treatments according to an ANOVA test ($P \leq 0.05$). 2-TDC, 2-tridecanone; 3-TDC, 3-tridecanone; 4-TDC, 4-tridecanone; 7-TDC, 7-tridecanone; 2-Hp, 2-heptanone; 2-DC, 2-decanone; 2-UDC, 2-undecanone; 2-DDC, 2-dodecanone; 2-PDC, 2-pentadecanone; 2-HxDC, 2-hexadecanone.
B. Suppression of 2-TDC-induced surface motility by different amounts of 7-TDC. Data are shown as the proportion of the surface motility exhibited by GR4 when only 2-TDC (1 µmol) was applied.

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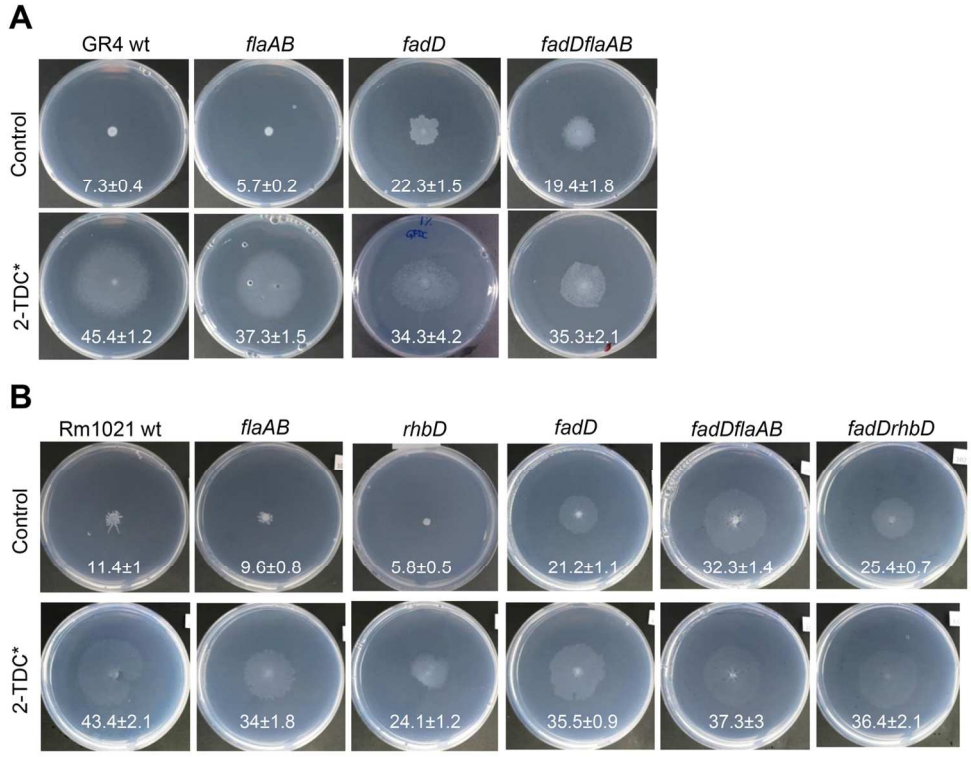

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Surface motility assays on MM (1% agar) with GR4 derivative strains (A) and Rm1021 derivative strains (B). The genotype of each strain is indicated. Twenty µl of either ethanol (control) or a solution containing 1 µmol 2-TDC were applied to the lid of the plates just before incubation. Representative pictures of the motilities exhibited by each strain are shown. Values represent average surface migration (given in millimetres) obtained after 48 h of incubation at 30°C. Means and standard errors were obtained from at least 3 replicates in three independent experiments (n=9). * Indicates that the treatment was applied as a volatile.

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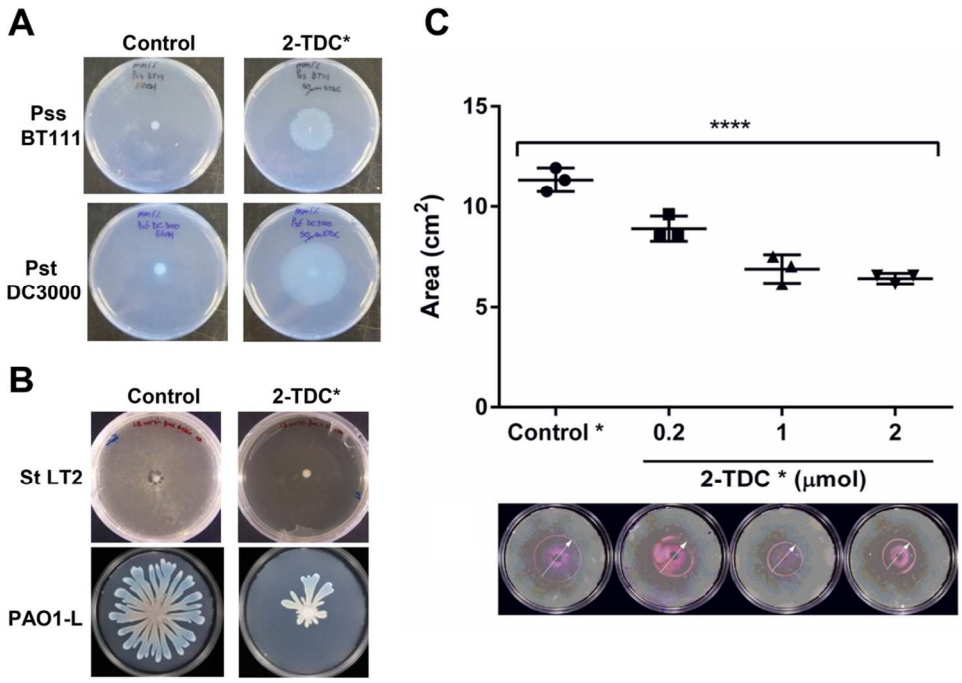

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Swarming of (A) the plant pathogens Pseudomonas syringae pv. syringae (Pss) BT111 and P. syringae pv. tomato (Pst) DC3000 and (B) the animal pathogens Salmonella enterica serovar Typhimurium (St) LT2 and P. aeruginosa PAO1-L in response to volatile 2-TDC. Twenty µl of ethanol (control) or a solution containing 1 µmol 2-TDC were applied to the lid of the plates just before incubation. * Indicates that the treatment was applied as a volatile.
C. Twitching motility of P. aeruginosa PAO1-L assayed in the presence of volatile 2-TDC generated from solutions containing different amounts of the MK and applied to the lid of the plates. The zone of twitching is indicated with arrows. Error bars represent one standard deviation from three independent replicates. A one-way ANOVA was used to assess statistical significance; ****, p value<0.0001.

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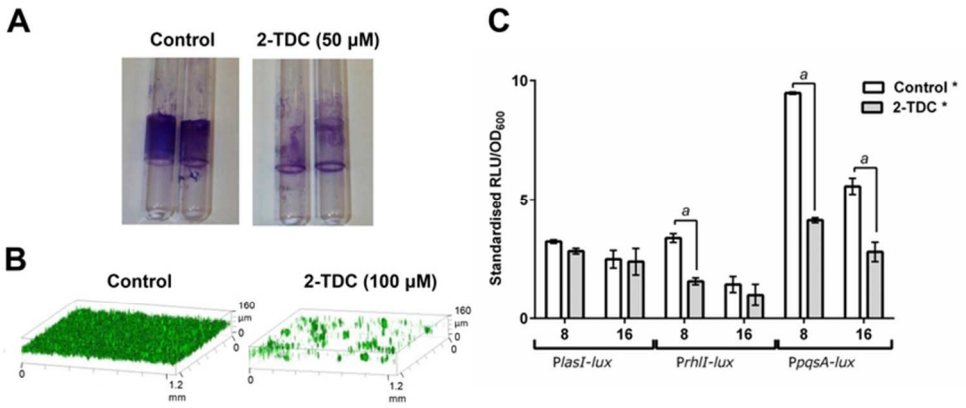

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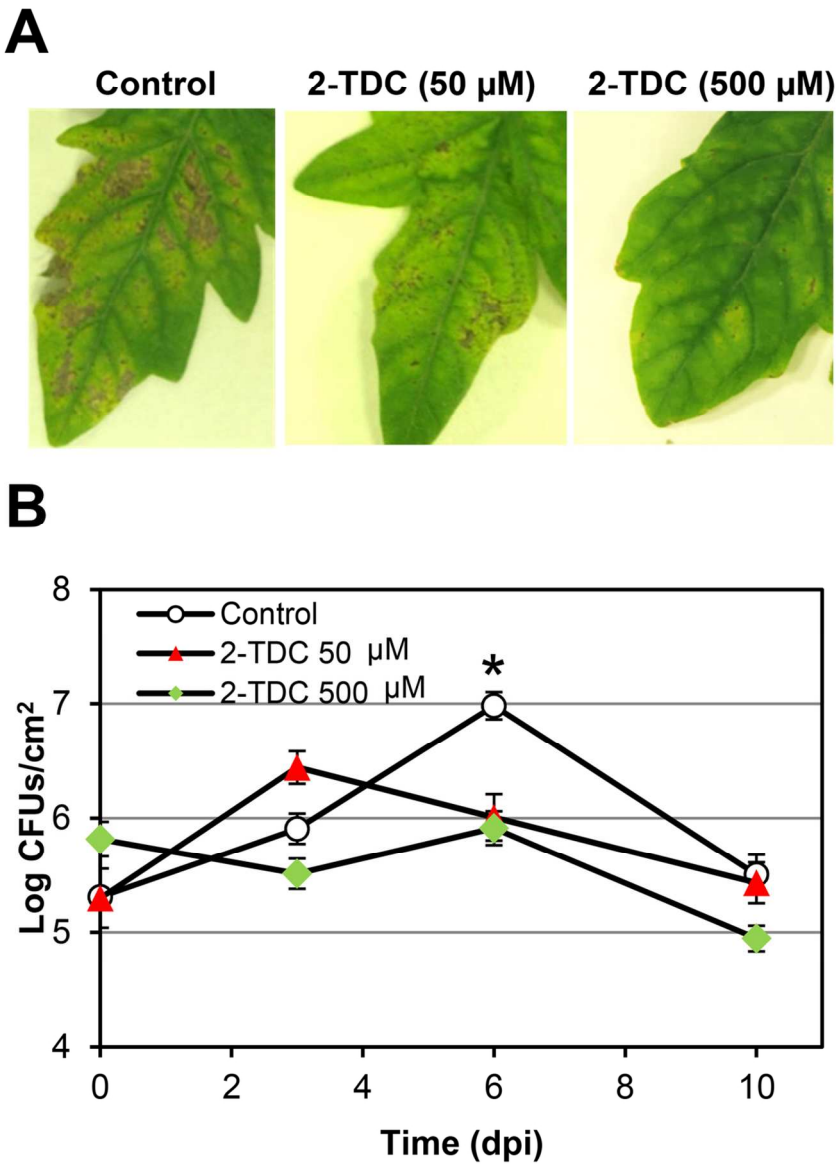

Fig. 8. Tomato plants treated with 2-TDC are more resistant to the development of bacterial speck disease. A. Bacterial speck symptoms developed on tomato leaves 10 days after spray-inoculation with a suspension of P. syringae pv. tomato DC3000 (108 CFU/ml) in the absence or presence of 2-TDC (50 or 500 µM). The different treatments were applied at the time of inoculation with the pathogen. Control plants were treated with an equivalent amount of ethanol (0.1%).
B. Time course of P. syringae pv. tomato DC3000 growth in leaves of tomato plants. CFUs were determined 3 h (day 0) and 3, 6, and 10 days postinoculation. Data represent the average and standard errors obtained from at least three different measurements. Asterisk indicates significant differences compared with control treatment according to an ANOVA test ($P \leq 0.05$).

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