Dickeya solani D s0432-1 produces an arsenal of secondary metabolites with anti-prokaryotic and anti-eukaryotic activities against bacteria, yeasts, fungi, and aphids.

Running title: *Dickeya solani* secondary metabolites

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SUMMARY

The necrotrophic plant pathogenic bacterium *Dickeya solani* is a new invader of potato agrosystem in Europe. All isolated strains of *D. solani* contain several large polyketide/fatty acid/non-ribosomal peptide synthetase clusters. Analogy with genes described in other bacteria, suggests that two clusters are involved in the production of secondary metabolites of the oocydin and zeamine family. In this study, we constructed by an approach of reverse genetics mutants affected in the three secondary metabolite clusters *ssm*, *ooc* and *zms* in order to compare the phenotype of the *D. solani* strain D s0432-1 with its derived mutants. We demonstrated that the zeamine cluster *zms* inhibits growth of gram-positive and gram-negative bacteria. It is also implicated in a toxicity against aphids. The oocydin cluster *ooc* inhibits growth of fungi of the phylum *Ascomycota*. Finally, we unveiled the function of a new secondary metabolite cluster *ssm* (for *solani* secondary metabolite), only conserved in some *Dickeya* species. This cluster produces a secondary metabolite inhibiting yeasts. *D. solani* therefore produces several molecules that are toxic to a wide range of living and potentially interacting organisms, from bacteria to insects. The expression of these secondary metabolite pathways could contribute to the rapid spread of *D. solani* in Europe.

INTRODUCTION

Bacterial phytopathogens of the genus *Dickeya* and *Pectobacterium* are pectinolytic necrotrophic bacteria with a broad host plant spectrum (Charkowski *et al.*, 2012; Hugouvieux-Cotte-Pattat *et al.*, 2020). These members of the family *Pectobacteriaceae* (Van Gijsegem, Toth, *et al.*, 2021) cause substantial agricultural losses worldwide by affecting many vegetables, ornamentals and crops, of which the potato is the most important economically. These bacteria are able to invade and degrade the plant tissues through the coordinated expression of genes encoding virulence factors, with a major role of pectate lyases that dissociate the plant cell wall constituents (Hugouvieux-Cotte-Pattat *et al.*, 2014).

The *Dickeya* genus was established in 2005 (Samson *et al.*, 2005), resulting from the reclassification of *Pectobacterium chrysanthemi* (formerly *Erwinia chrysanthemi*). To date, twelve species of *Dickeya* have been described, *D. aquatica*, *D. chrysanthemi*, *D. dadantii D. dianthicola* , *D. fangzhongdai*, *D. lacustris*, *D. oryzae*, *D. paradisiaca*, *D. poaceiphila*, *D. solani*, *D. undicola*, and *D. zeae* (Samson *et al.*, 2005; Brady *et al.*, 2012; Parkinson *et al.*, 2014; van der Wolf *et al.*, 2014; Tian *et al.*, 2016; Hugouvieux-Cotte-Pattat *et al.*, 2019, 2020; Oulghazi *et al.*, 2019; Wang *et al.*, 2020).

The species *D. solani* was officially established in 2014 (van der Wolf *et al.*, 2014) but *D. solani* isolates have attracted attention since its emergence on the potato agrosystem in Europe in

the early 2000s. It appeared to be highly aggressive in both subtropical and temperate climates. Recently, seed companies have adopted a zero-tolerance policy to *D. solani* due to its invasive and aggressive nature (Van Gijsegem, van der Wolf, *et al.*, 2021). Many scientific efforts have been made to provide information on this phytopathogen, resulting in 76 *D. solani* genomes available in May 2021 (Blin *et al.*, 2021). Comparative genomics was performed to identify the genetic basis for the high virulence level of *D. solani* (Garlant *et al.*, 2013; Pédron *et al.*, 2014; Khayi *et al.*, 2015; Golanowska *et al.*, 2018; Motyka-Pomagruk *et al.*, 2020; Blin *et al.*, 2021). Most *D. solani* strains Isolated from different regions show a low level of genetic variation, suggesting a clonal origin (Khayi *et al.*, 2015). The genomes of *D. solani* share a high similarity and synteny with those of the model strain *D. dadantii* 3937, prompting comparison between the two species. Only a few hundred genes were specific to each species, including a few dozen distinctive genomic regions (Garlant *et al.*, 2013; Pédron *et al.*, 2014). Three of these regions have been shown to encode polyketide synthases (PKS), non-ribosomal peptide synthetases (NRPS) and amino acid adenylation domain proteins, suggesting that they encode proteins involved in the production of secondary metabolites (Garlant *et al.*, 2013).

PKSs and NRPSs are able to synthesize molecules by sequential condensation of carboxylic acids and amino acids, respectively. PKS and NRPS modules can combine together to form hybrid PKS/NRPS systems capable of producing compounds of great structural diversity (Cane and Walsh, 1999). The molecules synthesized may have siderophore, antibiotic or phytotoxic properties that promote the virulence of a plant pathogen. Three PKS/NRPS clusters are present in all sequenced *D. solani* strains and found in a few other *Dickeya* species (Duprey *et al.*, 2019). In *Serratia plymuthica,* the cluster *ooc* is involved in the synthesis of oocydin A, a halogenated macrolide with antifungal and anti-oomycete activity (Matilla *et al.*, 2012). The cluster *zms*, previously found in the genomes of *S. plymuthica* and *Dickeya oryzae,* leads to the biosynthesis of a polyamino-amide antibiotic, zeamine (Zhou *et al.*, 2011; Masschelein *et al.*, 2013). A third cluster is found in a few *Dickeya* species but the nature and function of the molecule synthesized are unknown.

Mutagenesis is one of the most powerful genetic tools for analyzing the function of a gene and its involvement in bacterial virulence. A random mutagenesis approach was used in the *D. solani* type strain IPO2222 with a transposon Tn5 harboring a promoterless *gusA* reporter gene to obtain mutants affected under given conditions (Fikowicz-Krosko and Czajkowski, 2017; Lisicka *et al.*, 2018; Czajkowski *et al.*, 2020). Some mutants affected for a selected gene have also been constructed in various strains of *D. solani* using a generalized transduction method with phage, to transfer a mutation previously constructed in *D. dadantii* 3937 (Potrykus *et al.*, 2014, 2018). However, this method is strictly limited to genes common to *D. dadantii*

101 and *D. solani*. Given the need for in-depth studies on this economically important species, we
102 have tested a general method to precisely inactivate a selected gene using the SacB-based
103 reverse genetics method. We used this method to construct mutants affected for the three loci
104 involved in the biosynthesis of secondary metabolites in the highly virulent *D. solani* strain
105 Ds0432-1. This method is potentially applicable to all genes and in all strains of *D. solani*. We
106 showed that *D. solani* Ds0432-1 clusters *zms* and *ooc,* encoding zeamine and oocydin
107 biosynthesis, are involved in growth inhibition of bacteria and fungi, respectively. We also
108 showed evidence that the *D. solani* Ds0432-1 cluster *ssm* (for *solani* secondary metabolite)
109 produces a third secondary metabolite that prevents yeast growth.

110

111 **RESULTS**

112

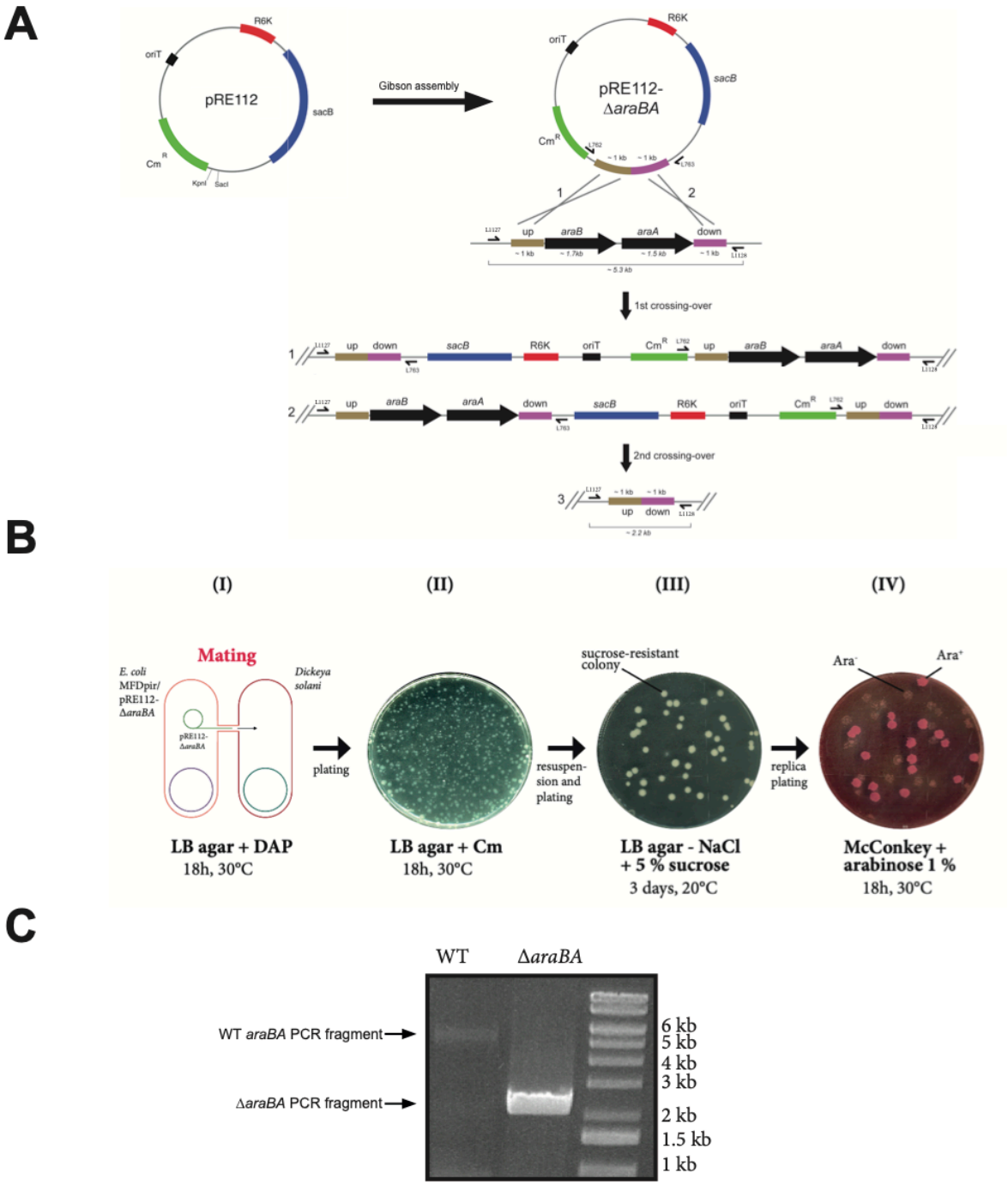
113 **Use of the SacB-based reverse genetics method to delete genes of *D. solani***

114

115 A technique commonly used in bacterial genetics to delete a gene from a Gram-negative
116 bacterium requires a suicide plasmid with the R6K origin of replication and the counter-
117 selection gene *sacB1*, which confers sucrose sensitivity (Edwards *et al.*, 1998). The vector
118 pRE112 had previously been successfully used for allelic exchange in *D. dadantii* 3937
119 (Koskiniemi *et al.*, 2013; Royet *et al.*, 2019). We tried to use it with *D. solani*.
120 To evaluate the effectiveness of the reverse genetic approach with *D. solani*, we first tried to
121 delete the *araBA* operon of the *D. solani* strain Ds0432-1. *araA* encodes the L-arabinose
122 isomerase and *araB* a ribulokinase. A Δ*araBA* mutant, deleted for this operon, is unable to
123 catabolize arabinose and it forms white colonies on MacConkey-arabinose agar plates. This
124 mutant is thus easily distinguishable from the wild-type Ara^+ strain forming red colonies. Two
125 1-kb fragments upstream and downstream *araBA* were assembled and cloned directly into
126 linearized pRE112 vector (Fig. 1A). The plasmid pRE112-Δ*araBA* was transferred from *E. coli*
127 MFDpir to *D. solani* Ds0432-1 by conjugation (Fig. 1B). Plasmid integrants were selected by
128 their chloramphenicol resistance (Cm^R) on LB agar plates supplemented with
129 chloramphenicol. On this selective medium, the *E. coli* donor strain cannot survive because
130 LB agar does not contain diaminopimelic acid (DAP). The plasmid is integrated either
131 upstream or downstream of the *araBA* locus (Fig. 1A). The integrated plasmid can be lost by
132 homologous recombination between the tandemly duplicated *ara* sequences (Fig. 1A).
133 Plasmid loss is stimulated in the presence of sucrose, *i.e.* by spreading the integrants onto LB
134 agar without NaCl containing 5% sucrose. The sucrose-resistant colonies were then replica-
135 plated onto MacConkey-arabinose agar, allowing the identification of Ara^- segregants, and on
136 LB agar-Cm, to confirm the plasmid loss by their Cm^S phenotype (Fig. 1B). Finally, the correct

137 structure of the mutants was confirmed by colony PCR analysis (Fig. 1C). By this first
138 experiment, we were able to verify that this method works perfectly and is easy to implement
139 in *D. solani*. In addition, this method was shown to work by cloning only 500 bp DNA fragments
140 upstream and downstream of *araBA*, instead of 1000 bp. We also used this protocol to
141 construct a Δ*araBA* mutant of the *D. solani* type strain IPO2222 with comparable efficiency
142 (data not shown).

Figure 1

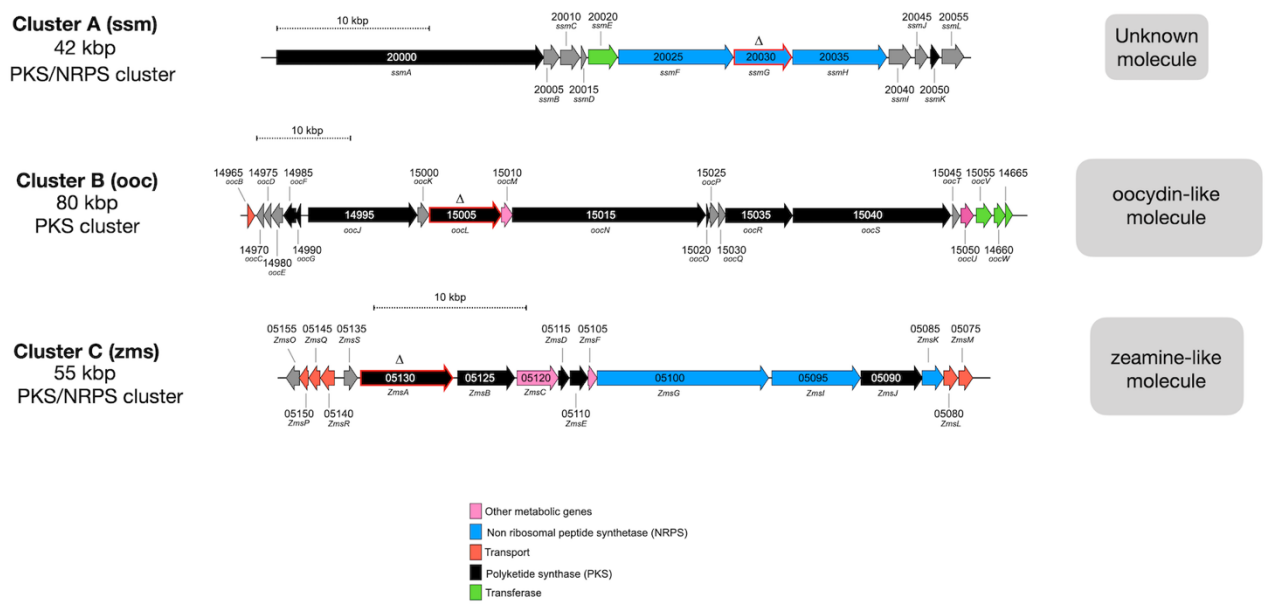


143

144 **Figure 1. Summary of the method used to obtain in-frame deletion mutants.** (A) Genetic
145 steps to obtain a Δ*araAB* deletion mutant of *Dickeya solani*. The 1 kb upstream and
146 downstream DNA regions of the *araBA* genes are cloned between the KpnI and SacI
147 restriction sites of the suicide plasmid pRE112 by Gibson assembly. The resulting plasmid
148 pRE112-Δ*araBA* is then transferred into *D. solani* by conjugation. The plasmid is integrated
149 into the chromosome by homologous recombination either upstream or downstream the *araBA*
150 locus. The structures of both possible integrants are shown as structures 1 and 2. Next,
151 sucrose-resistant segregants lose the integrated plasmid pRE112-ΔaraBA by homologous
152 recombination between the tandemly duplicated upstream or downstream *ara* sequences.
153 This either regenerates a WT *araBA* locus or an *araBA* deletion on the chromosome, shown
154 as structure 3.
155 (B) Microbiological steps to obtain a Δ*araBA D. solani* mutant. The *E. coli* donor strain
156 MFDpir(pRE112-Δ*araBA*) was mated with *D. solani* (step I). *D. solani* recombinants were
157 selected onto LB agar medium supplemented with chloramphenicol (step II). *D. solani*
158 recombinant were then spread onto LB agar without NaCl and supplemented with 5% sucrose.
159 Sucrose resistant colonies (step III) were transferred onto MacConkey agar plate with 1 %
160 arabinose (step IV). White colonies are formed by bacteria unable to catabolize arabinose
161 because they acquired the Δ*araBA* mutation. (C) Their genotype was confirmed by PCR on
162 colonies using oligo pairs L1127/L1128. The desired mutants give a PCR product of 2 kb,
163 whereas PCR done on WT *D. solani* gives a PCR product > 5 kb. Detail procedure is indicated
164 in the material and methods section.
165
166
167 Using this reverse genetic approach, we decided to study the three secondary metabolite
168 clusters of *D. solani* which are absent in *D. dadantii*. We focused our work on *D. solani* D
169 s0432-1 since it is one of the most aggressive *D. solani* strain (Khayi *et al.*, 2015; Golanowska
170 *et al.*, 2018). This strategy should allow us to assess the contribution of these three clusters
171 on the competitiveness of *D. solani* against eukaryotic and prokaryotic organisms.
172
173 **Description of the three selected PKS/NRPS secondary metabolite clusters of *D. solani*.**
174
175 The clusters A, B and C encoding complex NRPS and PKS involved in the production of
176 secondary metabolites were named *ssm*, *ooc* and *zms,* respectively (Fig.2).
177 The ~42-kbp cluster A contains the 12 genes *ssmABCDEFGHIJKL* (Fig.2). It is widely
178 conserved in the genus *Dickeya*, *i.e.*, in all sequenced *D. solani, D. aquatica, D. fangzhondai,*
179 *D. poaceiphila* and *D. zeae* genomes, in some *D. dadantii* strains (NCPPB 898, NCPPB 3537,
180 but not the model strain 3937), in some *D. undicola* strains (FVG1, FVG10), and in some *D.*
181 *oryzae* strains (EC2, NCPPB 3531 and CSLRW192). The role and the structure of the
182 metabolite produced from this cluster have not been elucidated yet.
183 The ~80-kbp cluster B is highly similar to the *oocBCDEFGJKLMNOPQRSTUVW* cluster of *S.*
184 *plymuthica* A153. It is present in all the sequenced genomes of *D. solani* and *D. dianthicola*,
185 in four *D. oryzae* strains (ZYY5, EC1, DZ2Q and ZJU1202), and in two *D. paradisiaca* strains,
186 Ech703 and NCPPB2511. In *S. plymuthica* A153, disruption of this gene cluster abolished
187 bioactivity against the fungi *Verticillium dahliae* and the oomycetes *Pythium ultimum*. This

188 cluster produces oocydin A (Matilla *et al.*, 2012), a chlorinated macrolide, powerfully active
189 against plant pathogenic oomycetes (Strobel *et al.*, 1999). Since various *D. solani* strains
190 inhibit *V. dahliae* and *P. ultimum* growth (Matilla *et al.*, 2015), it was suggested, on the basis
191 of gene sequence homologies and similar cluster organization, that *D. solani* also produces
192 oocydin A.
193 The ~55-kbp cluster C encodes mixed fatty acid synthase (FAS)/PKS and hybrid NRPS/PKS
194 enzymes. Its genomic organization is identical to the *D. oryzae* EC1 zeamine cluster and
195 related to the *S. plymuthica* AS12 zeamine cluster (Zhou *et al.*, 2011, 2015). The *zms* cluster
196 is conserved in all the sequenced *D. solani* and *D. fangzhongdai* genomes. After
197 reclassification of several *D.zeae* strains in the novel species *D. oryzae* (Wang *et al.*, 2020),
198 this cluster appeared to be absent in the genomes of true *D. zeae* strains. It is present in some,
199 but not all, *D. oryzae* rice strains (ZYY5, EC1, DZ2Q and ZJU1202). The zeamine biosynthetic
200 clusters from *D. oryzae* EC1 and *D. solani* Ds0432-1 share from 59 to 94% identity at individual
201 protein level (Zhou *et al.*, 2015).
202

Figure 2



203

204 **Figure 2. Organization of the secondary metabolite clusters of *D. solani* D s0432-1.**
205 Genes are indicated using the NCBI nomenclature of the NCBI reference genome sequence
206 NZ_CP017453.1 of *D. solani* D s0432-1. XXXXX are the digital number in BJD21_RSXXXXX,
207 which corresponds to the locus tag. Arrowheads show gene orientations. Color code indicates
208 gene function. The red-framed arrows indicate genes targeted for in-frame deletion performed
209 in this study. Cluster function is noted on the right.
210
211

212 While the *zmsABCDEFGIJKLMNPQRS* cluster directing zeamine biosynthesis is present only
213 in some *D. oryzae* strains isolated from rice, it is conserved in all *D. fangzhongdai* and *D.*
214 *solani* strains, which suggest secondary acquisition by horizontal gene transfer in *D. oryzae*
215 (Zhou *et al.*, 2015; Duprey *et al.*, 2019). Zeamine-related antibiotics are polyamino-amide
216 molecules toxic to a wide range of pro- and eukaryotic organisms such as bacteria, fungi,
217 oomycetes, plants, and nematodes (Masschelein *et al.*, 2017). Mutation of the zeamine
218 synthase gene *zmsA* in *D. oryzae* EC1 attenuates the inhibition of rice seed germination (Zhou
219 *et al.*, 2011) and suppresses antibacterial activity against *E. coli* (Zhou *et al.*, 2011). Zeamine
220 produced by *S. plymuthica* kills nematodes and yeast (Hellberg *et al.*, 2015). *D. solani*
221 IPO2222 can also kill *C. elegans* but not as quickly as *S. plymuthica* (Hellberg *et al.*, 2015).
222 To interrupt the synthesis of the secondary molecules produced by these three clusters, we
223 constructed in-frame deletion mutants inactivating a key gene of each cluster. The mutants
224 Δ*ssmG* (cluster A), Δ*oocL* (oocydin cluster B) and Δ*zmnA* (zeamine cluster C) were
225 constructed using the described reverse genetic approach with the pRE112 suicide plasmid.
226 Inhibitory effects of these mutants against fungi, bacteria, yeasts, aphids and plants were
227 compared with that of the wild-type strain.
228
229 **The *D. solani* oocydin cluster inhibits *Ascomycota* growth**
230
231 We compared the ability of the *D. solani* Ds0432-1 DS58 strain (a Nal^R Gm^R derivative of the
232 WT strain) and its derived mutants to inhibit the growth of *Botrytis cinerea*, *Magnaporthe*
233 *oryzae* and *Sclerotinia sclerotiorum*, three fungi-like eukaryotes of the phylum *Ascomycota*. A
234 potato dextrose agar (PDA) plate was inoculated at the periphery of the Petri dish with fungal
235 mycelium and 10 µl of overnight bacterial culture of each *D. solani* strain was deposited at the
236 center of the plate (Fig. 3). After incubation at 25°C for several days, we observed a growth
237 inhibition of the three fungi by the *D. solani* WT and the Δ*ssmG* and Δ*zmsA* mutants. In
238 contrast, the Δ*oocL* mutant did not inhibit mycelium growth (Fig. 3). Thus, we conclude that
239 the gene *oocL* is involved in the production of an oocydin-like secondary metabolite that has
240 an anti-fungal activity.

Figure 3

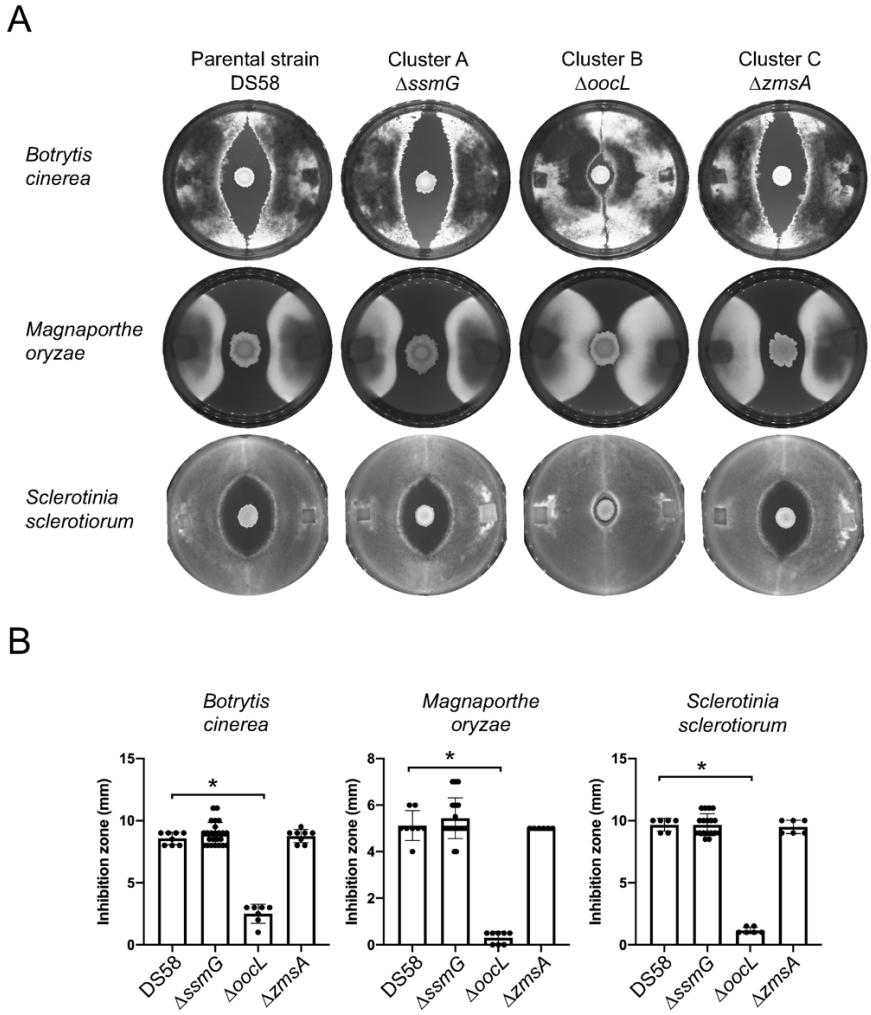


Figure 3. Radial diffusion assays to assess fungal growth inhibition on PDA plates with *D. solani* D s0432-1 or derived mutants. 5 µl of *D. solani* D s0432-1 at OD_{600nm} of 0.1 was deposited in the center of a 90 mm Petri dish inoculated with plugs of *Sclerotinia sclerotiorum*, *Botrytis cinerea* or *Magnaporthe oryzae* mycelium. Plates were incubated at 25 °C until the mycelium covers the plate. Lengths of fungi inhibition zone were measured in 3 independent experiments. A statistical difference was significant only between the WT and Δ*oocL* mutant (Mann-Whitney test; * P value < 0.05).

The *D. solani* zeamine cluster inhibits bacterial growth.

The zeamine cluster of *D. oryzae* EC1 is responsible of the bactericidal activity against *E. coli* DH5α (Zhou *et al.*, 2011). Because *D. oryzae* EC1 and *D. solani* zeamine biosynthetic genes share a high degree of similarity (Zhou *et al.*, 2015), we evaluated the capacity of *D. solani* WT DS58 and mutants to inhibit the growth of gram-positive and gram-negative bacteria. It was previously shown that D. *oryzae* EC1 produced 128 µg.mL^{-1} of zeamine in the minimum medium LS4+yeast extract, while zeamine production was not detected in LB (Liao *et al.*, 2014). Based on this work, we first used LS4+yeast extract medium for production of zeamine

259 by *D. solani*. The *D. solani* strains were grown in LS4+yeast extract minimal medium before
260 addition of 5 µl of the overnight culture onto a LB agar plate pre-inoculated with *B. subtilis*.
261 Only a very slight zone of *B. subtilis* inhibition was observed around the drop of *D. solani* in
262 our bioassay (Fig. S1). We then tested *D. solani* growth in minimal medium M63 supplemented
263 with sucrose and yeast extract. A large diameter of inhibition was observed, allowing
264 differentiation between strains capable or not of inhibiting *B. subtilis* growth (Fig. S1). Thus,
265 we used this medium for overnight growth of *D. solani* in all the following experiments. The *D.*
266 *solani* WT strain DS58 and the three mutants were tested (Fig. 4A). Only the Δ*zmnA* mutant
267 was unable to inhibit *B. subtilis* growth, indicating that *D. solani* produces an active zeamine
268 antibiotic (Fig 4A). We also tested the ability of *D. solani* to inhibit growth of other bacteria (Fig
269 4A) such as *E. coli* DH5α, *Streptomyces scabiei* CFBP 4517, *D. dadantii* 3937 and *P.*
270 *atrosepticum* SCRI 1043. The *D. solani* zeamine cluster was also the sole responsible for the
271 growth inhibition of *S. scabiei*, a plant pathogen causing the potato disease common scab
272 (Loria *et al.*, 2006) (Fig 4A). The *zms* cluster also inhibits the growth of *E. coli*, but with less
273 efficiency than that observed for the two Gram$^+$ bacteria tested. No inhibition of the two other
274 pectinolytic bacteria *D. dadantii* and *P. atrosepticum* growth was observed. In conclusion, out
275 of the three clusters analyzed, only the zeamine-like molecule has an anti-bacterial activity.

276 **The *D. solani* cluster *sms* but not the zeamine cluster inhibits yeast growth.**

277 Since zeamine produced by *S. plymuthica* A153 has previously been shown to be toxic to the
278 ascomycete yeast *Saccharomyces cerevisiae* (Hellberg *et al.*, 2015), we tested the capacity
279 of *D. solani* D s0432-1 and its mutants to inhibit the growth of the yeasts *S. cerevisiae* and
280 *Kluyveromyces lactis*. *K. lactis* has been isolated from milk and constitutes the predominant
281 eukaryote during cheese productions (Rodicio and Heinisch, 2013). *S. cerevisiae* and *K. lactis*
282 were grown in the rich medium YPD. We observed the inhibition of *S. cerevisiae* and *K. lactis*
283 growths by the *D. solani* WT strain DS58 (Fig. 4B), grown either in M63+sucrose+yeast extract
284 or in YPD medium (Fig S1). The anti-eukaryotic activity against these yeasts was caused by
285 the cluster *sms* since only the Δ*smsG* mutant did not inhibit yeast growth (Fig 4B). Therefore,
286 it appears that zeamine is not the primary factor responsible for yeast inhibition in *D. solani* D
287 s0432-1. Conversely, we identified a novel secondary metabolite cluster that has never been
288 studied previously and produces a molecule with anti-eukaryotic activity.

Figure 4

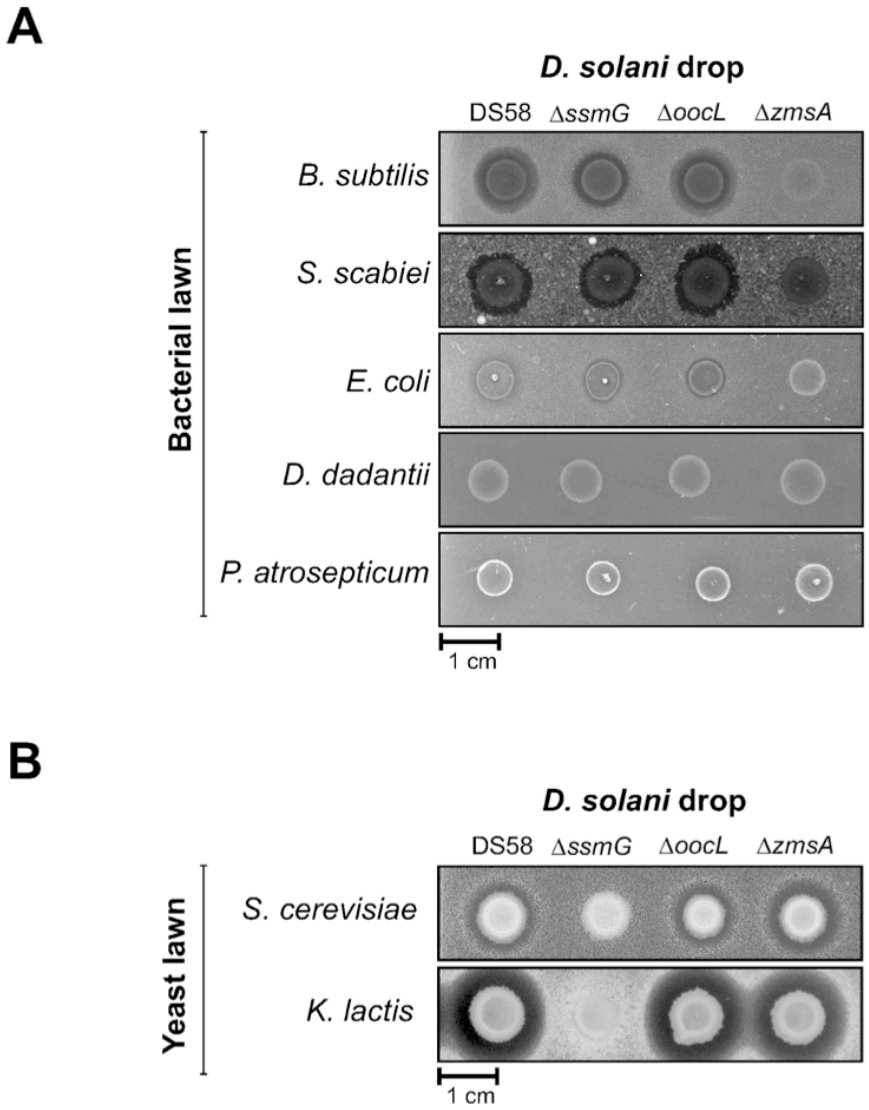
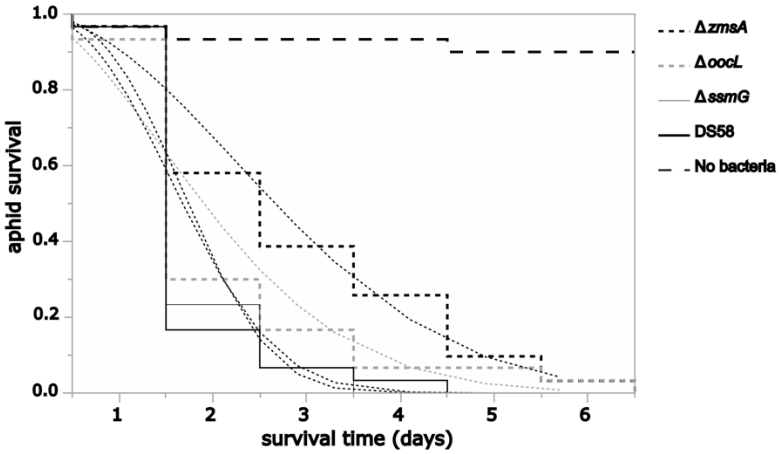


Figure 4. Plate-based bioassays for metabolite production by *D. solani* and mutant derivatives. Bioassay plates were prepared by mixing different bacteria culture (*B. subtilis, S. scabiei, E. coli, D. dadantii* or *P. atrosepticum*) with warmed LB agar. (A) 5 µl of overnight bacteria culture from *D. solani* D s0432-1 or derivative mutants were spotted onto the bioassay plate. (B) Bioassay with the yeasts *Saccharomyces cerevisiae* and *Kluyveromyces lactis* in YPD agar plates. The experiment was repeated at least three times. The photograph was taken 24 h after incubation at 30°C.

303 **The *D. solani* zeamine cluster contributes to the killing of aphid.**
304
305 Since *D. dadantii* 3937 was previously shown to be able to kill aphids (Grenier *et al.*, 2006),
306 we tested the effect of *D. solani* D s0432-1 on these insects. Aphid survival was heavily
307 affected by infection with *D.solani*, similarly to what was observed with *D.dadantii*. Among the
308 three mutants analyzed, the mutant Δ*zmsA* was clearly altered in its virulence towards the pea
309 aphid, with a significant survival outcome over the WT strain (p=0.0008, Log-rank test) (Fig.
310 5). Mean lethal time was increased from 1.68 days ± [1.41-1.99] (mean ± *conf. interv.*) to 2.66
311 days ± [2.16-3.28]. The mutant Δ*oocL* seemed slightly affected for aphid survival, (Fig. 5), but
312 the results were not significant with the non-parametric tests used throughout (p=0.23), only
313 showing significance for a global Weibull model (p=0.027), with a mean survival time increased
314 to 1.90 days ± [1.50-2.41]. The mutant Δ*ssmG* was not affected in its capacity to kill aphids.
315 Globally however, and as this occurred for the *cyt* proteins toxin cluster in *D. dadantii* 3937
316 (Costechareyre *et al.*, 2010), the capacity of both mutants Δ*zmsA* and Δ*oocL* to kill aphids
317 remained high as all aphids were dead at the end of the 7 days period. Thus, the analyzed
318 clusters only supported partial causality for the aphid-killing phenotype harbored by *D. solani*
319 D s0432-1. In conclusion, these results showed that the zeamine cluster is partially implicated
320 in aphid killing by *D. solani* D s0432-1.
321

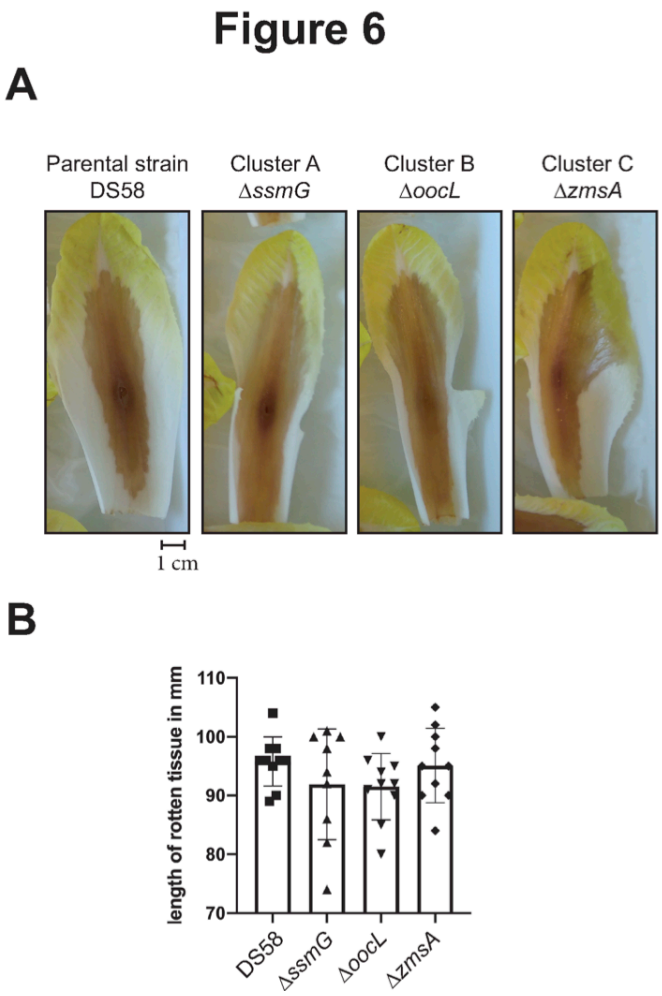
Figure 5



322

323 **Figure 5. Bioactivity assay on pea aphids.** Survival curves of pea aphid individuals (n=30)
324 after 24h exposure to *D. solani* WT and mutants. Control survival with no infection, in green
325 (top). Curves fitted with Weibull distributions (dashed lines), showing Δ*zmsA* mutant with
326 significantly altered infection capacity (see text for statistical data).

327 **Plant cell wall maceration capacity of the *D. solani* mutants affected for secondary**
328 **metabolite production .**
329
330 In *D. oryzae* EC1, the mutation of *zmsA* attenuates the inhibitory activity observed on rice
331 seed germination (Zhou *et al.*, 2011). Thus, zeamine was suggested to be a phytotoxin that
332 could affect virulence against plants.
333 We examined the role of clusters *ssm*, *ooc*, and *zms* on the ability of *D. solani* to infect chicory
334 leaves. The *D. solani* WT strain and its derived mutants were inoculated on a small wound
335 made on a chicory leaf. After overnight incubation, no difference in maceration was observed
336 (Fig. 6), suggesting that the wild-type strain of *D. solani* does not require any of the three
337 PKS/NRPS secondary metabolites to efficiently infect plants.
338



339

340 **Figure 6. Maceration of chicory leaves by *D. solani* and mutant derivatives. (A)** The
341 photograph was taken 24 h after incubation at 30°C. (B) The length of rotten tissue was
342 measured from 9 infected leaves. No statistical difference was measured between the different
343 strains (Mann–Whitney U-test).

344

345
346
347 **DISCUSSION**
348
349 Secondary metabolite pathways are a great source of molecules with anti-eukaryotic or anti-
350 prokaryotic activity giving the bacteria that synthesize them a competitive advantage over
351 other organisms. The development of targeted mutagenesis of the *D. solani* chromosome has
352 allowed us to specifically study the involvement of three secondary metabolite synthesis
353 clusters encoded by all the *D. solani* strains for which genome sequence is available. We
354 focused our work on D s0432-1, one of the most virulent *D. solani* strains (Golanowska *et al.*,
355 2018). We have demonstrated that this *D. solani* strain is able to inhibit the growth of a variety
356 of living organisms. We tested bio-activities against Gram-negative bacteria (*E. coli*, *P.*
357 *atrosepticum*, *D. dadantii*), Gram-positive bacteria (*B. subtilis*, *S. scabiei*), yeasts (*S.*
358 *cerevisiae*, *K. lactis*), fungi (*B. cinerea*, *M. oryzae* and *S. sclerotiorum*), and pea aphids (*A.*
359 *pisum*). The targeted mutagenesis approach revealed that each of the three secondary
360 metabolite synthesis pathways studied has the capacity of inhibiting a particular type of
361 organism. Cluster B , or *ooc*, encodes an enzymatic pathway that produces an oocydin-like
362 molecule, a chlorinated macrocyclic lactone molecule having antifungal, anti-oomycete and
363 antitumor activities in *S. plymuthica* (Matilla *et al.*, 2012, 2015). In contact with fungi *B. cinerea*,
364 *M. oryzae* and *S. sclerotiorum*, *D. solani* D s0432-1 prevents their growth. It was previously
365 shown that the *D. solani* strains MK10, MK16, IPO 2222, 3337, D-s0432-1 and GBBC 2040,
366 all encoding the oocydin cluster, inhibit the growth of the fungus *V. dahliae* and the oomycete
367 *P. ultimum* (Matilla *et al.*, 2015). Our study showed that inactivation of the *oocL* gene of this
368 cluster in *D. solani* D-s0432-1 suppressed the inhibition of the three fungi but it had no
369 apparent effect on the other organisms tested.
370 Our data also demonstrated that *D. solani* D s0432-1 has an anti-bacterial activity linked to
371 cluster C, or *zsm*, which encodes a zeamine biosynthetic pathway. Zeamine produced by *S.*
372 *plymuthica* kills *B. subtilis,* the yeasts *S. cerevisiae* and *Schizosaccharomyces pombe,* and
373 the nematode *C. elegans* (Hellberg *et al.*, 2015). In our study, we showed that *D. solani* D
374 s0432-1, but not the *zmsA* mutant, can clearly inhibit the growth of two Gram-positive bacteria,
375 *B. subtilis* and *S. scabies*. We tested *S. scabiei* because it is the principal causal agent of the
376 common scab disease of potato (Loria *et al.*, 2006). Since *D. solani*, is also a potato pathogen,
377 *D. solani* and *S. scabiei* might be in competition for the same ecological niche. It is thus
378 interesting to note that *D. solani* can inhibit the growth of another potato pathogen. We also
379 observed a slight inhibition exerted by *D. solani* D s0432-1 against *E. coli*, but no inhibition
380 towards *D. dadantii* and *P. atrosepticum*. Zeamine resistance of *D. dadantii* could be explained
381 by the presence in its genome of the genes *desAB* encoding the RDN pump DesAB involved

382 in zeamine efflux (Liang *et al.*, 2019). In-frame deletion of *desA* or *desB* in *D. oryzae* EC1
383 leads to a zeamine sensitive phenotype (Liang *et al.*, 2019). In *in planta* Tn-seq experiments
384 with *D. dadantii* 3937 (Royet *et al.*, 2019), mutants of the genes *desA* and *desB*
385 (Dda3937_00787 and Dda3937_00786 respectively) did not display a significant negative or
386 positive variation (Log2 fold-changes -0.09 and +0.44, respectively), suggesting that this RND
387 efflux pump does not play a significant role during *D. dadantii* plant infection, contrary to the
388 *D. dadanti* RND efflux pump AcrAB that appeared to be essential for virulence (Royet *et al.*,
389 2019). *P. atrosepticum* does not have the genes *desAB* but it is not inhibited by *D. solani* D
390 s0432-1. Another RND efflux pump could allow zeamine efflux, or zeamine resistance could
391 be provided by a different mechanism.
392 We also showed that zeamine is involved in insect killing. We tested pea aphids because they
393 are susceptible to infection by *D. dadantii* (Grenier *et al.*, 2006). The *D. solani* strain D s0432-
394 1 is also able to kill pea aphids and the zeamine synthesis pathway (absent in *D. dadantii*) is
395 partially implicated in pea aphid killing. We did not test inhibition of other multicellular
396 organisms. Hellberg *et al.* tested nematodes with *S. plymuthica* A153 and a few *D. solani*
397 strains (Hellberg *et al.*, 2015), they showed that *S. plymuthica* A153 but not its zeamine-
398 deficient mutants can kill *C. elegans* in a few hours. In contrast, *D. solani* strains MK10, MK16,
399 and IPO2222 that also carry the zeamine cluster behaves like the zeamine-deficient mutants
400 of *S. plymuthica*. The authors suggested the *D. solani* zeamine cluster might be cryptic under
401 their conditions or that it produces a zeamine molecule with somewhat different biological
402 properties (Hellberg *et al.*, 2015).
403 Zeamine produced by *S. plymuthica* A153 is bioactive against *S. cerevisiae* and *S. pombe*
404 (Hellberg *et al.*, 2015). We observed that *D. solani* D s0432-1 is bioactive against *S. cerevisiae*
405 and *K. lactis*. However, the zeamine-deficient mutant of *D. solani* behaves exactly like the WT
406 strain, ruling out the involvement of zeamine in yeast inhibition. By testing other mutants of
407 the secondary metabolites clusters, we observed that the mutant s*smG* was totally unable to
408 prevent the growth of *S. cerevisiae* and *K. lactis*. Therefore, the *ssm* cluster appears to be
409 responsible for yeast inhibition. This cluster was not required for bioactivities against bacteria,
410 aphids, and fungi. This result opens the way to the discovery of a new molecule with very
411 specific activity against yeasts. The *ssm* cluster is only conserved in a few *Dickeya* genomes
412 but not in other bacterial genera. The structure of the molecule and its target remains to be
413 elucidated.
414 Finally, we assessed the virulence of the *D. solani* mutants Δ*ssmG*, Δ*oocL* and Δ*zsmA* by
415 testing the maceration of chicory leaves. No difference was observed between the WT strain
416 D s0432-1 DS58 and the mutants affected for the production of a secondary metabolite,
417 indicating that the three secondary metabolites tested play no significant role in the
418 degradation of the plant cell wall. Zeamine was described as a major virulence determinant of

419 *D. oryzae* since it is involved in inhibition of rice seed germination (Zhou *et al.*, 2011). Zeamine
420 could have a phytotoxic activity mostly efficient at the level of seed germination.
421 In conclusion, *D. solani* D s0432-1 produces an arsenal of bioactive secondary metabolites
422 against a variety of living organisms. The cluster *ssm* produces an unkown molecule active
423 against yeasts; the cluster *ooc* produces an oocidin-like molecule active against fungi and the
424 cluster *zms* produces a zeamine-like molecule active against bacteria and insects. The
425 complementary activities of this set of molecules may have favored the rapid spread of *D.*
426 *solani* in Europe by giving the capacity to compete with other microorganisms.
427
428 **EXPERIMENTAL PROCEDURES**
429
430 **Bacterial and fungal strains, plasmids and growth conditions.**
431 The *E. coli* and *Dickeya* bacterial strains, plasmids and oligonucleotides used in this study are
432 described in Table S1 and S2. The genome accession number of *D. solani* D S0432-1 is
433 NZ_CP017453. The following strains have been also used in the study: *Sclerotinia*
434 *sclerotiorum* S5, *Botitris cinerea* B05.10, *Magnaporthe oryzae* strain Guy11, *Saccharomyces*
435 *cerevisiae* BY4743 (*MATa/α his3Δ1/his3Δ1 leu2Δ0/leu2Δ0 LYS2/lys2Δ0 met15Δ0/MET15*
436 *ura3Δ0/ura3Δ0)*, *Kluyveromyces lactis* MWL9S1 (Wésolowski-Louvel, 2011), *Dickeya dadantii*
437 3937, *Pectobacterium atrosepticum* SCRI1043*, Streptomyces scabiei* CFBP4517, *Bacillus*
438 *subtilis* PY79. *E. coli* was grown routinely at 37°C in LB. Fungus strains were grown at 25°C
439 onto Potato Dextrose Agar (PDA). *S. scabiei* was cultivated in tryptic soy broth (TSB) medium
440 at 28°C. *B. subtilis*, *P. atrosepticum* and the *Dickeya* strains were cultivated in LB unless
441 specified. Yeast cells were grown at 30°C in rich medium consisting of complete yeast extract-
442 peptone (YP) medium containing 1% Bacto yeast extract, 1% Bacto peptone (Difco)
443 supplemented with 2% glucose (yeast extract-peptone-dextrose [YPD] medium). For the
444 bacteria and yeast inhibition assay, M63 medium supplemented with sucrose and yeast
445 extract (2 g $(NH_4)_2SO_4$, 13.6 g KH_2PO_4, 2.5 mg $FeSO_47H_2O$, 0.2 g $MgSO_47H_2O$, 10 g sucrose,
446 0.1 g yeast extract, per liter) or LS4 medium supplemented with yeast extract (9.25 g K_2HPO_4,
447 3.3 g KH_2PO_4, 1.4 g NH_4NO_3, 12.7 g sucrose, 1 g KCl, 0.1 g yeast extract and 0.25 g $MgSO_4$,
448 pH 7.0, per liter) (Liao *et al.*, 2014) were employed to grow the *D. solani* strains.
449 When required, antibiotics were added at the following concentrations: ampicillin (Amp), 100
450 µg/L; kanamycin (Kan), 50 µg/mL; nalidixic acid (Nal), 10 µg/mL; chloramphenicol (Cm), 20
451 µg/mL or 4 µg/mL for *E. coli* or *D. solani,* respectively. Diaminopimelic acid (DAP) (57 µg/mL)
452 was added for the growth of the *E. coli* MFDpir strain. Media were solidified with 12 g/L agar.
453
454 **PCR conditions**

455 To amplify DNA for cloning, a bacterial suspension of the *D. solani* was prepared by boiling
456 an isolated colony resuspended into 40 µl of sterilized water for 10 minutes at 95°C. The PCR
457 was carried out in a 50 µl reaction mix containing 1 µl of bacterial suspension, 1.5 µl of 10 µM
458 of each primer (Eurofins), 25 µL of primestar master mix 2x (Takara) and demineralized water.
459 Thermocycling for amplification of a 1 kb upstream and downstream fragment of operon *araBA*
460 consisted of 34 cycles of 98°C for 15 seconds, 55°C for 15 seconds and 72°C for 15 seconds.
461 To check the correct deletion of a gene of interest in *D. solani*, a bacterial suspension was
462 made by boiling an isolated colony resuspended into 40 µl of sterilized water for 10 minutes
463 at 95°C. The suspension was centifuged 1min at 12000 g to remove cellular debris. The
464 supernatant was used as a template for PCR. To amplify up to 3-kb DNA fragments, colony
465 PCR was performed with DreamTaq Green PCR Master mix (2X) (Thermofisher, Waltham,
466 MA, USA). However, the amplification from colonies with the dreamtaq kit did not work when
467 the amplified fragments was larger than 3-kb. In this case the colony PCR was carried out with
468 the Q5 High-Fidelity DNA Polymerase (Biolabs) by following manufacturer recommendations.
469
470 **Construction of the Δ*araBA D. solani* mutants.**
471
472 To construct the in-frame deletion Δ*araBA* of *D. solani*, the *sacB* counter-selection method
473 was used (Edwards *et al.*, 1998). pRE112 is an R6K-based suicide plasmid carrying the *sacB*
474 gene and the cat gene (Cm^R). The plasmid pRE112-Δ*araBA* was constructed by cloning
475 simultaneously two PCR fragments corresponding to the upstream and downstream 1-kbp
476 DNA of the *araBA* genes into SacI/KpnI digested pRE112 using the Gibson's assembly
477 method. Chemical ultracompetent DH5α λpir cells were prepared with the Mix & Go! *E.coli*
478 Transformation Kit using standard procedures (Zymo Research). They were transformed with
479 5 µl of the Gibson reaction. Transformants were selected onto LB plate supplemented with
480 chloramphenicol. Transformants with the correct plasmid were found by colony PCR with oligo
481 pairs L762/L763 and Dreamtaq DNA polymerase. Plasmids were extracted with the
482 NucleoSpin Plasmid kit (Macherey-Nagel) and checked by restriction digestion (NEB) and
483 sequencing (Eurofins).
484 Then, plasmids were transferred into competent *E. coli* strain MFDpir (Ferrières *et al.*, 2010)
485 prepared with the TSS method (Chung *et al.*, 1989). *E. coli MFDpir* produces the RP4
486 conjugation machinery, which allows the transfer of the suicide plasmid into *D. solani* by
487 conjugation. To do that, a few colonies of *D. solani* and MFDpir were mixed in the same
488 proportion in 500 µl LB and centrifuged for 2 min at 8000 rpm. The pellet was resuspended in
489 90 µl LB with 5 µl DAP at 57 mg/mL, and deposited onto a LB agar plate incubated at 30°C.
490 After 18h, the bacteria were resuspended in 1 ml LB, diluted in 10-fold series from 10^{-1} to 10^{-}
491 ^{7} and spread onto LB agar supplemented with chloramphenicol at 4 µg/l to select the first event

492 of recombination. Transconjugants re-isolated on this medium were then spread onto LB agar
493 without NaCl supplemented with 5% sucrose and incubated at 19°C for 2-3 days to allow the
494 second event of recombination. Sucrose-resistant colonies were then replicated onto Mac
495 Conkey medium supplemented with 1% arabinose incubated for 18h at 30°C to check the
496 arabinose catabolism, and LB-Cm plate to check plasmid loss.
497
498 **Construction of the secondary metabolite in-frame deletion mutants of *D. solani*.**
499
500 A spontaneous mutant of *D. solani* D s0432-1 resistant to nalidixic acid (Nal^R) was obtained
501 by growing the WT strain at 30°C in LB medium supplemented with Nal at 5 µg/mL for 18 h
502 before spreading the liquid culture onto LB agar plates with Nal at 10 µg/mL. The Nal^R strain
503 was named DS50 (Table S1). Then to discriminate in-frame deletion mutants from the wild-
504 type DS50 strain, a Gm^R derivative of strain DS50 was constructed by insertion of a mini-Tn7-
505 Gm cassette into the attTn7 site of *D. solani,* as previously performed with *D. dadantii* 3937
506 (Royet *et al.*, 2019) by using the plasmids pTn7-M (Zobel *et al.*, 2015) and pTnS3 (Choi *et al.*,
507 2008). The correct integration of the Gm^R cassette was then checked by colony PCR using
508 oligo pairs L365/L848 (amplification of 350 bp for the correct integration) (Table S2). This gave
509 the Nal^R Gm^R strain DS58. Subsequently, the in-frame deletion mutations Δ*ssmG* (NCBI
510 Reference Sequence: WP_022634121.1; cluster A), Δ*oocL* (NCBI Reference Sequence:
511 WP_023638021.1; cluster B) and Δ*zsmA* (NCBI Reference Sequence: WP_022632849.1;
512 cluster C) were constructed in strain DS58. Derivatives of the pRE112 suicide plasmids
513 containing upstream and downstream 500-bp DNA of the gene to delete were also constructed
514 (Table S2). In-frame deletions were checked by PCR as previously described (Table S2).
515
516 **Growth inhibition assay of bacteria and yeast.**
517 First, *B. subtilis*, *E. coli*, *D. dadantii* and *P. atrosepticum* were grown overnight in LB at 30°C
518 with shaking. LB agar was cooled until the temperature reached about 50°C (just before the
519 agar re-solidified). The OD_{600} of the overnight culture of *B. subtilis*, *E. coli*, *D. dadantii* or *P.*
520 *atrosepticum* were adjusted to 1 with fresh LB. 100 mL of the LB agar in surfusion were mixed
521 with 100 µl of the OD_{600} 1 culture of *B. subtilis*, *E. coli*, *D. dadantii* or *P. atrosepticum* . 25 mL
522 of inoculated LB agar was poured in 10- by 10-cm square plates. The plates were dried for a
523 few hours. *D. solani* strains were grown for 24h at 30°C with shaking in M63 medium
524 supplemented with sucrose and yeast extract or in LS4 medium supplemented with yeast
525 extract. The OD_{600} of the *D. solani* cultures were adjusted to 1 and 5 µl were spotted onto the
526 inoculated square plates which were incubated at 30°C for 24-48 h before visualization of the
527 inhibition zone.

528 The same experiment was conducted with *Streptomyces scabiei*, except that it was grown for
529 3 days in TSB at 28°C and TSB agar was poured in the square plates. With the yeasts *S.*
530 *cerevisiae* and *K. lactis*, YPD medium was used.
531
532 **Growth inhibition of fungal strains.**
533 *S. sclerotiorum*, *B. cinerea* and *M. oryzae* were grown onto PDA plate at 25°C for 5, 7 and 10
534 days respectively. At the start of the experiments, Colonies of *D. solani* D s0432-1 strains were
535 resuspended in M63 medium and OD_{600} was adjusted to 0.1. Then, 10 µl of the bacterial
536 suspensions were deposited at the center of a 90-mm Petri dish with two 5 mm agar plugs of
537 fungus at 4.5-cm around. The radius of fungus inhibition zone was measured.
538
539 **Pea aphid survival assay**
540 Aphid survival assays were performed at 20-25°C as previously described (Grenier *et al.*,
541 2006). Pea aphid (*Acyrthosiphon pisum*, LL01 clone, alfalfa strain reared on faba bean)
542 individuals aged 0-24 h were transferred on Ap3 diet (Febvay *et al.*, 1988) loaded with
543 10^7/mL cells of *D. solani* D S0432-1 or its corresponding mutants. After a full day on diet,
544 aphids were transferred on single *V. faba* plantlets and scored daily for survival up to the 7th
545 day. Survival data were analysed with JMP software (SAS Inc) and its survival platform, and
546 data adjusted through Kaplan-Meier graphing and analysis, followed by a Weibull fit (best of
547 tested log-normal or exponential fits). Data are fully compatible with previously published
548 work on *D. dadantii* (Costechareyre *et al.*, 2010), except that temperature was less
549 stringently controlled.
550
551 **Chicory inoculation experiments**
552 The *D. solani* WT strain and the different mutants were grown overnight in LB medium.
553 Bacteria were washed in M63 medium and OD_{600} was adjusted to 1. One microliter of the
554 bacterial suspension was inoculated into a small hole made into the leaves by a yellow pipette
555 tip. The wound was covered with mineral oil and the leaves were incubated at 30°C at high
556 humidity for 24 h. The length of rotten tissue was measured.
557
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AUTHOR CONTRIBUTION

E.G., B.T, R.Y and G.E carried out the experiments. G.E and H-C-P.N wrote the manuscript with support from E.G, B.T and R.Y. H-C-P.N and G.E conceived the original idea. G.E supervised the project. All authors provided critical feedback and helped shape the research, analysis and manuscript.

CONFLICT OF INTEREST

The authors state that the research was conducted in the absence of any commercial or financial relationship that could be interpreted as a potential conflict of interest.

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739 **SUPPLEMENTAL DATA**
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743 **Figure S1. Plate-based bioassays against *B. subtilis* and *K. lactis* for metabolite**
744 **production by *Dickeya solani* WT cultivated in various medium.**
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746 **Table S1. Strains and plasmids used in the study.**
747 **Table S2. oligonucleotides used in the study.**
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