Salmonella exploits the reactive oxygen species generated by the plant immune system to enhance colonization

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Abstract

Understanding bacterial interaction with the plant immune system is essential for building a sustainable food system. Discouraging pathogens enhancing beneficial bacteria and preventing food spoilage and contamination by human enteric pathogens. Immune system activation triggers a burst of toxic reactive oxygen species (ROS). We discovered that the food-poisoning bacterium *Salmonella* exploits the plant ROS to enhance biofilm formation and colonization. Scavenging the ROS with antioxidants or inhibiting their production reduces colonization. Further, combining the antioxidant vitamin C and immune system activation with salicylic acid synergistically inhibited *Salmonella* colonization of the plant model Arabidopsis and edible plants. This safe-to-use combination is an eco-friendly method to prevent plant contamination by *Salmonella*, *Listeria* and enteropathogenic *E. coli.* Finally, we have found that exploiting the plant immunity for enhancing colonization is not exclusive to *Salmonella* and diverse beneficial *pseudomonas* species isolated from plants can also exploit the plant immune system, suggesting that it is a general plant colonization strategy.

Introduction

The increase in the world population, global warming and the reduction in the efficiency of chemical fertilizers and pesticides necessitate the building of sustainable food systems[1]. Understanding plant microbe interaction at the molecular level is an avenue to reach this goal. Better understanding will enable us to discourage pathogens, enhance beneficial bacteria and prevent food spoilage and contamination by human enteric pathogens[2].

Food poisoning by enteric pathogens is a leading cause of morbidity and mortality worldwide[3]. The increased consumption of fresh fruits and vegetables (fresh produce), due to awareness of their importance to a healthy lifestyle and the extension of the supply chains, made it a significant source of food-borne illnesses. The Gram-negative bacteria *Salmonella* and *E. coli* and the Gram-positive bacterium *Listeria monocytogenes* are the three main

33 bacteria contaminating fresh produce[3-5]. Chemical disinfection of fruits and vegetables
34 adversely affects workers' and consumers' health. Thus, eco-friendly methods to ensure food
35 safety are highly needed[6].
36 Research from recent years revealed that human pathogens can colonize plants and may even
37 utilize plants as a secondary host[5,7]. They exhibit a complex interaction with plants and
38 activate plant immune responses. Thus, understanding their plant colonization process could
39 inform rational approaches to fight food contamination. In this regard, utilizing plant
40 immunity to fight salmonella contamination is extensively explored[6,8]. We have analyzed the
41 molecular interaction between the enteropathogenic bacteria *Salmonella enterica* and the
42 plant model Arabidopsis thaliana. As previously shown, the *Salmonella* activates the plant
43 immune system[9-11], despite exhibition of beneficial lifestyle on Arabidopsis with no obvious
44 pathogenic symptoms. The first level of plant immunity comprises receptors that recognize
45 conserved microbial molecules, e.g., flagella, elongation factor Tu, prevalent in both plant
46 pathogens and non-pathogenic bacteria. Receptor activation leads to a burst of reactive
47 oxygen species (ROS) and a signal transduction cascade[12]. Specialized plant pathogens employ
48 a plethora of effectors that target plant immunity, mainly delivered through the type III
49 secretion system. Thus, they can evade the first protection line and grow to a high number[13].
50 However, if and how 'non-specialized' plant pathogens including commensal and beneficial
51 bacteria evade immune system toxicity is much less understood[14].
52 Bacteria synthesize extracellular matrix to facilitate surface adhesion[15]. The matrix comprises
53 sugar polymers, protein fibres, extracellular DNA and cell debris. Bacteria encased in a matrix
54 are known as biofilm[16]. The matrix protects the bacteria from chemical assaults and, as such,
55 poses a serious challenge for antibiotic treatment[17]. Biofilm formation is essential for root
56 colonization[15,18]. However, the effect of the biofilm matrix on bacteria dealing with the plant
57 immune system is not explored in detail. Our research revealed that biofilm formation
58 protects *Salmonella* from the reactive oxygen species (ROS) produced upon plant immune
59 system activation. Further, the bacteria exploited the ROS to enhance biofilm formation and
60 colonization. Thus, immune system recognition paradoxically enhances root colonization.
61 Revealing the importance of ROS for facilitating biofilm formation and colonization enabled
62 us to outsmart the bacteria. Scavenging the ROS with antioxidants reduced bacterial
63 colonization and sensitized the bacteria to the plant immune system activity. The combination
64 of the antioxidant ascorbic acid (vitamin C) and excessive immune system activation by the

65 plant hormone salicylic acid (SA) exhibited synergistic inhibition of bacterial contamination.
66 Further, the combination of vitamin C and SA inhibits plant colonization of various *salmonella*
67 serovars, *listeria monocytogenes* and enteropathogenic *E. coli*, both in Arabidopsis and in
68 edible plants, making it a promising method to ensure food safety. Finally, we bring initial
69 evidence that exploitation of the plant immunity for enhanced colonization is utilized by other
70 bacteria from the pseudomonas genus.

71 **Results**

72 ***Salmonella* exhibited reduced colonization of immune mutant plants.**

73 We inoculated seedlings with 10^6 CFU/ml of *Salmonella* (the concentration of inoculated
74 bacteria from here on unless otherwise stated), we used *Salmonella enterica Serovar*
75 *Typhimurium 14028s* throughout the paper except for specific experiments. Notably, at this
76 number the bacteria exhibited beneficial effect and significantly enhanced primary root
77 length, while 10^8 CFU/ml inhibited root length (Figure S1A), consistent with previous results
78 showing plant pathogenic effect of *Salmonella* at high concentration [e.g.[11]]. Despite the
79 beneficial effect *Salmonella* stimulated immune system activation in Arabidopsis (Figure 1A
80 and Figure S1B), as judged by FrK1 promoter expression, a downstream target of the FLS2
81 receptor[19] (Figure 1A) and CYP71A12 promoter expression, a gene involve in biosynthesis of
82 the secondary metabolite camalexin[20] (Figure S1B). To understand how the plant immune
83 system modulates bacterial colonization, we inoculated bacteria into bbc (*bak1,bkk1,cerk1*)
84 triple mutant plants, lacking all three co-receptors of the pattern-triggered immunity[21]. We
85 anticipated overgrowth of *Salmonella* on plants perturbed in immune recognition. However,
86 when inoculated with 10^6 CFU/ml bacteria exhibited no significant reduction [12% reduction
87 on bbc compared to Col0 but P = 0.07) (Figure 1B)]. Immune system activation triggers a burst
88 of ROS production to kill the invading pathogen[22,23]. However, inoculating *Salmonella* into
89 *rbohd rbohf* plants perturbed in ROS production in response to immune system activation[24]
90 revealed a significant reduction rather than an increase in root colonization (Figure 1B),
91 [compare for example to[23] where Bacilli overgrown on *rbohd rbohf* plants]. Of note, when
92 seedlings inoculated with 10^8 CFU bacteria exhibited significant overgrowth on bbc mutant
93 plants (Figure S1C). It has been previously shown that *Salmonella* colonizes the endophyte of
94 Arabidopsis[11,25]. However, we failed to detect any CFU from crashed roots after surface-
95 sterilization (we performed 2 experiments with 4 roots in each with zero bacteria detected in
96 all the roots).

97 To understand why bacteria failed to properly colonize *rbohd rbohf* plants, we monitored root
98 colonization by GFP-expressing bacteria. On WT roots, bacteria appeared in long strips (Figure
99 1C). However, on *rbohd rbohf* plants, bacteria failed to form strips (Figure 1C). Thus, our
100 results suggest that at low number of bacteria *Salmonella* can resist the activity of the plant
101 immune system and that ROS production by the plant can even enhance the bacterial
102 colonization (we discuss the differences between low and high number in the Discussion
103 section).

104 **Biofilm formation is stimulated by the plant immune system derived ROS and protects the**
105 **bacteria from the ROS toxicity.**

106 Biofilm formation is crucial for bacteria to adhere to plants[18]. We examined the colonization
107 of three biofilm mutant strains: 1. *ΔcsgD*, a master regulator of curli polymers[26]2. *ΔbcsA,*
108 encoding a cellulose synthase and 3. *ΔcsgB* a minor curli subunit plus *ΔbcsA* double mutant
109 cells[26-28]. We found that mutant cells exhibited a significant reduction in root colonization
110 (Figure 2A and Figure S2A-S2B), while PcsgD cells constitutively expressing CsgD[26] exhibited a
111 significant increase (Figure 2A), confirming the importance of biofilm formation for root
112 colonization. Notably, the inability of *Salmonella* to form large stripes and patches on *rbohd*
113 *rbohf* plants was reminiscent of bacteria perturbed in biofilm formation (Figure S2B). We
114 hypothesized that plant ROS generated by RbohD and RbohF stimulate biofilm formation on
115 the root. This idea is consistent with the ability of ROS to enhance biofilm in vitro (Figure 2B).
116 Consistently, the colonization of *ΔcsgD* cells isn't reduced when colonizing *rbohd rbohf* plants
117 (Figure 2C). On the contrary, *ΔcsgD* cells exhibited a significant increase in the colonization of
118 *rbohd rbohf* and bbc plants (Figure 2C), as well as *ΔbcsA* and *ΔbcsA ΔcsgB* cells (Figure S2A).
119 Further, treatment of the roots with Diphenyliodonium (DPI), an inhibitor of the NADPH
120 oxidase enzymes rbohD and rbohF[23] reduced the growth of WT cells while increase the
121 growth of *ΔcsgD* cells (Figure S2C-S2D). These results indicate that the immune system can
122 inhibit the growth of *Salmonella*. However, the ability to form biofilm protected the WT
123 bacteria from the ROS.

124 The results so far suggested that ROS positively affect salmonella colonization. Thus,
125 we wondered if chemical ROS scavengers will inhibit colonization. Indeed, vitamin C and
126 potassium iodide (KI) inhibited *Salmonella* colonization in a concentration dependent manner
127 (Figure 2D and figure S3A-S3B). We further concentrated on vitamin C, which is a common
128 antioxidant and is considered safe for human consumption even at high doses. Of note, the

129 effect of vitamin C was independent of its acidity as we titrated the pH back to normal.
130 Vitamin C did not affect biofilm formation *in vitro* (Figure 2B), but it can at least partially
131 abolish the increase observed upon H_2O_2 addition (Figure 2B).

132 **Salicylic acid and vitamin C synergistically inhibited *Salmonella* colonization of Arabidopsis**
133 **and edible plants.**

134 The results so far indicate that *Salmonella* exploits plant immunity to enhance biofilm
135 formation. Nevertheless, excessive stimulation of the plant immunity using the stress
136 hormone salicylic acid (SA) significantly inhibited *salmonella* colonization (Figure 3A-3B), [see
137 also[6]]. Of note, SA did not affect the colonization of pCsgD, suggesting that robust biofilm
138 matrix can protect the cells even from SA activity (Figure 3B). We next examined the
139 application of vitamin C and SA together. Applying both chemicals together (50µM SA and
140 1Mm vitamin C) exhibited synergistic inhibition of salmonella colonization (Figure 3C) (The
141 observed reduction is significantly lower than the theoretical combination of vitamin C x SA,
142 $P<0.05$). Neither the compounds nor their combination affects bacterial growth in vitro
143 (Figure 3D), indicating that their effect is plant specific.

144 The genus *Salmonella* harbors more than two thousand different serovars, with varying
145 abilities cause disease in humans. The combination of vitamin C and SA significantly inhibited
146 the colonization of 11 different serovars isolated from human patients we tested (Figure 4A),
147 with inhibition efficiency ranging from 60% (*S. virginia*) to 95%-99% in most other serovars.
148 We next examine if SA and vitamin C can inhibit plant colonization of other human enteric
149 pathogens associated with plant food contamination. Examination root colonized by
150 enteropathogenic *E.coli* (EPEC serotype O127:H6) [29] and the gram-positive pathogen *Listeria*
151 *monocytogenes* (strain 10403s) revealed the efficiency of vitamin C and SA and the synergistic
152 effect of their combination for inhibiting these bacteria as well (Figure 4B-4C).

153 Next, we explored the effect of vitamin C and SA on *salmonella* colonization of edible plants.
154 Using sterile alfalfa sprouts and Basil leaves (Ocimum basilicum), we confirmed the effect of
155 vitamin C and SA on *salmonella* contamination of these economically important foods (Figure
156 5A-5B). Thus, the effect is extended beyond Arabidopsis to edible plants and beyond the root
157 to plant leaves, highlighting its general effect. Notably, neither vitamin C nor SA inhibits leaf
158 colonization alone, but the combination efficiently inhibited colonization (Figure 5B).

159 The experiments so far have been done on sterile plants grown in axenic conditions without
160 resident microbiota. We further examined the effect of vitamin C and SA on Salmonella while

161 colonizing non-sterile alfalfa sprouts. The colonization of nontreated plants by *salmonella* was
162 reduced by two orders of magnitude compared to sterile plants [compare Figure 5A to Figure
163 5C], suggesting that competition with the resident microbiota interferes with the pathogen's
164 colonization. Nevertheless, vitamin C and SA further inhibited the colonization (Figure 5C).
165 Similar inhibition was observed for *Listeria monocytogenes* (Figure 5D). Thus, our data suggest
166 that SA and vitamin C or the combination of both is effective way to prevent plant
167 contamination by enteric pathogens.
168 We finally wondered if other bacteria may exploit the plant immune system to enhance their
169 colonization the same way we found in Salmonella. To examine this possibility, we monitored
170 root colonization by a collection of bacteria from the *Pseudomonadota* phylum isolated from
171 diverse environments. We found isolates that exploit the plant immune system, as suggested
172 by the reduction in colonization on bbc triple mutant roots (Figure S4). Interestingly, all the 3
173 isolates that seem to exploit the plant immune system were isolated from the leaf niche and
174 may evolve to adapt to the plant environment. However, a larger sample size and a careful
175 comparison are necessary to support such a conclusion. Anyway, our result suggest that other
176 bacteria can also exploit the plant immune system to enhance their colonization.
177 **Discussion**
178 *Salmonella E.coli* and *Listeria* are the three main bacteria causing contamination of fresh
179 produce, leading to enormous health and economic burdens. In order to develop sustainable
180 and environmentally friendly methods to control fresh produce contamination by enteric
181 pathogens, we need a mechanistic understanding of their interaction with plants. Our
182 research revealed that *salmonella* exploits the plant immune system activation, using plant-
183 produced ROS to stimulate biofilm formation and root adhesion. Interestingly, biofilm
184 protects the bacteria from the plant ROS, suggesting a feedback loop where 1. colonization
185 stimulates immune system activation (Figure 1A). 2. The immune system produces ROS to
186 inhibit the bacteria. 3. Bacteria can utilize ROS to enhance biofilm formation (Figure 2B) 4.
187 Biofilm protects bacteria from the immune system (Figure 2C and Figure 3A) and further
188 enhances colonization [see model in (Figure 6)].
189 Previous research claimed that *Salmonella* can infiltrate the plant endophytic compartment
190 and exhibit plant pathogenicity phenotype[11,25]. Our results suggest that pathogenic
191 phenotypes only manifested at high bacterial concentrations, while low concentration exhibit
192 beneficial effects (Figure S2A). The difference in the number of inoculated bacteria may also

193 explain why we failed to observe endophytic infiltration in our system. Remarkably, it was
194 recently demonstrated that non-pathogenic bacteria may elicit pathogenic like effects in
195 Arabidopsis leaves when inoculated at high concentrations[30]. Our data suggest that
196 Salmonella face extensive competition from the resident plant microbiota (compare figure 5A
197 to figure 5D) and the number of *Salmonella* is reduced to ~100 bacteria per sprout when
198 colonized non-sterile sprouts suggesting that 10^5 CFU is an unlikely scenario in the real world.
199 Work on *Salmonella* infection in human and mice also found indications that
200 salmonella might exploit the ROS produced by the immune system[31]. However, such a
201 phenomenon has not been described in plant. We have previously demonstrated that
202 immune system activation enhances *Bacillus veleznesis* root adhesion[23]. The current work
203 further highlights the complex interaction between bacteria and the immune system, beyond
204 merely bacterial killing or growth inhibition.
205 We further found that vitamin C and salicylic acid synergistically inhibited salmonella
206 colonization, we hypothesize that vitamin C inhibit biofilm formation exposing the bacteria to
207 the immune system activity stimulated by SA like proteolytic enzymes, antimicrobial
208 molecules etc [32]. Our work demonstrates how mechanistic understanding of the complex
209 interaction between bacterial colonization and plant immunity opens a new avenue for
210 dealing with food contamination. We discovered that safe-to-use antioxidants like vitamin C
211 can inhibit plant colonization by enteric pathogens and synergize with immune system
212 activation by salicylic acid. The results from the root of the model plant arabidopsis were also
213 applied to basil leaves and alfalfa sprouts, even under non-sterile conditions in the presence
214 of resident microbiota. Vitamin C and low doses of salicylic acid are safe for human
215 consumption and have other health benefits on their own[33,34]. Further exploring the
216 utilization of this combination on fresh produce in real world settings is a promising avenue
217 for sustainable food safety.
218
219 **Figure legends**
220 **Figure 1. Immune system activation enhances root colonization by *Salmonella***
221 **(A)** 7 days old Arabidopsis seedlings (pFrk1::3xmVenus-NLS) were inoculated with bacteria on
222 MS agar plates. mVenus fluorescence was monitored after 18 hrs using confocal microscopy.

223 Shown are representative maximal projection x20 fluorescent images out of 4 roots from each
224 treatment. Red arrow mark nuclear localized mVenus expression, scale bar = 25µm.
225 **(B)** Seedlings of the indicated genotypes were inoculated with *Salmonella*, and the number of
226 colonizing bacteria was determined after 48 hrs. Shown are averages and standard deviations
227 (SD) of bacterial colonization from at least 2 independent experiments with 4 seedlings in
228 each. *** = P < 0.005.
229 **(C)** *Salmonella* constitutively expressing GFP were inoculated into seedlings of the indicated
230 genotypes for 48 hrs. Root colonization was monitored by confocal microscopy. Shown are
231 representative maximal projection x20 fluorescent images and the complementary brightfield
232 images of the same roots, out of 4 roots from each treatment. Bacteria colonizing WT roots
233 (Col0) appeared in long stripes, while on roots mutant in ROS production (*rbohd;rbohf*) they
234 did not form these strips ,scale bar = 50µm.
235 **Figure 2. ROS enhances biofilm formation on the root**
236 **(A)** 7 days old seedlings were inoculated with the indicated *Salmonella* strains on MS agar
237 plates, and the number of colonizing bacteria was determined after 48 hrs. Shown are
238 averages and standard deviations (SD) of bacterial colonization from 3 seedlings.
239 **(B)** Bacteria inoculated into LB with no NaCl with the addition of 100µM H_2O_2, 1mM vitamin
240 C, a combination of both or no addition, in a multi-well plate. The plate was kept with no
241 shaking at 23° for 5 days, and adhesion to the well was monitored by crystal violet staining as
242 an indicator of biofilm matrix formation. Shown are average and SD of at least 6 wells, * = P
243 < 0.05.
244 **(C)** Seedling of WT and the indicated immune mutant plants were inoculated with ΔcsgD
245 bacteria, and the number of colonizing bacteria was determined after 48 hrs. Shown are
246 averages and standard deviations (SD) of bacterial colonization from at least 2 independent
247 experiments with 4 seedlings in each. * = P < 0.05, ** = P <0.01. ΔcsgD bacteria exhibited a
248 significant increase in colonization on immune system mutants, in contrast to the decrease
249 exhibited by WT bacteria.
250 **(D)** Seedlings were inoculated with bacteria on control plates or plates with the indicated of
251 vitamin C and the number of colonizing bacteria was determined after 48 hrs. Shown are
252 averages and standard deviations (SD) of bacterial colonization from at least 2 experiments
253 with 4 seedlings in each. All the treatments lead to a significant reduction in root colonization
254 P < 0.05.

255 **Figure 3. Salicylic acid and vitamin C synergistically inhibit root colonization**

256 **(A)** 7 days old seedlings were inoculated with *Salmonella* on MS agar plates with or without
257 the indicated concentration of salicylic acid, and the number of colonizing bacteria was
258 determined after 48 hrs. Shown are averages and standard deviations (SD) of bacterial
259 colonization from 2 independent experiments with 4 seedlings in each, all the treatments
260 significantly reduced bacterial colonization P<0.005.

261 **(B)** 7 days old seedlings were inoculated with the indicated *Salmonella* strains on MS agar
262 plates with or without salicylic acid 50µM, and the number of colonizing bacteria was
263 determined after 48 hrs. Shown are averages and standard deviations (SD) of bacterial
264 colonization from 3 independent experiments with 4 seedlings in each, ***=P<0.005, N.S=
265 non-significant.

266 **(C)** seedlings were inoculated bacteria on MS agar plates with the indicated chemicals: 50µM
267 salicylic acid, 1mM vitamin C and a combination of both, and the number of colonizing
268 bacteria was determined after 48 hrs. Shown are averages and standard deviations (SD) of
269 bacterial colonization from 3 independent experiments with 3 seedlings in each.
270 ***=P<0.005.

271 **(C)** Bacteria were grown in LB with the addition of the indicated chemicals and OD_{600}
272 measured. Shown are averages and SD of 5 independent cultures for each treatment.

273 **Figure 4. SA and VC affect root colonization by diverse human pathogens**

274 **(A)** 7 days old seedlings were inoculated with the indicated *Salmonella* serovars on MS agar
275 plates with or without a combination of 50µM salicylic acid and 1mM vitamin C. The number
276 of colonizing bacteria was determined after 48 hrs. Shown are averages and standard
277 deviations (SD) of bacterial colonization from at least 4 seedlings for each serovar. For all the
278 serovars, the P<0.005, except for *S. Bareilly* and *S.* Havana, where P<0.05.

279 (**B-C**) Seedlings were inoculated with either enteropathogenic *E. coli* (O127:H6) (B) or *Listeria*
280 *monocytogenes* (10403s) with the indicated chemicals: 50µM salicylic acid, 1mM vitamin C
281 and a combination of both, and the number of colonizing bacteria determined after 48hrs.
282 Shown are averages and standard deviations (SD) of bacterial colonization from 3
283 independent experiments with 3 seedlings in each. ***=P<0.005.

284 **Figure 5. SA and VC affect basil leaves, and alfalfa sprout colonization**

285 **(A)** 5 days old alfalfa seedlings were inoculated bacteria on MS agar plates with the indicated
286 chemicals: 50µM salicylic acid, 1mM vitamin C and a combination of both, and the number of

287 colonizing bacteria was determined after 48 hrs. Shown are averages and standard deviations
288 (SD) of bacterial colonization from 3 independent experiments with 3 seedlings in each.
289 ***=P<0.005.
290 **(B)** Leaves excised from 5 sterile grown 5 weeks old basil and placed on MS agar plates with
291 the indicated chemicals as described in (A) and the number of colonizing bacteria determined
292 after 48hrs. Shown are averages and standard deviations (SD) of bacterial colonization from
293 3 independent experiments with 3 seedlings in each. **=P<0.01, ***=P<0.005.
294 (**C-D**) nonsterile alfalfa sprouts were inoculated with GFP expressing *Salmonella* (C) and
295 *Listeria* (D) with the indicated chemicals as described in (A), and the number of colonizing
296 bacteria was determined after 48 hrs after plating on semi-selective plates and examination
297 under the microscope. Shown are averages and standard deviations (SD) of bacterial
298 colonization from 3 independent experiments with 3 seedlings in each. *=P<0.05, **=P<0.01,
299 ***=P<0.005.
300 **Figure 6. A model describing how *Salmonella* exploits the plant immune system and how**
301 **we can outsmart it**
302 **(A)** The plant recognizes the *Salmonella* and activates an immune response and ROS
303 production (1) to inhibit bacterial colonization (2). *Salmonella* exploits the ROS for enhancing
304 biofilm formation and promoting root colonization (3). The biofilm formation further protects
305 the bacteria from the immune system activity and ROS toxicity (4). Reducing ROS by
306 antioxidants like VC inhibits colonization by interfering with biofilm formation (5) and, in turn,
307 makes the bacteria vulnerable to the strong immune system activated by SA (6). Thus, VC and
308 SA exhibit synergistic activity in preventing plant contamination by *Salmonella* and other
309 pathogens.
310
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317
318

319 **Material and methods**

320 **Plant and bacterial strains** are listed in Table S1

321 **Plant growth**

322 Arabidopsis seedlings and sterile alfalfa seedlings were grown on 0.5 MS media containing 1.1
323 gr Murashige and Skoog basal salts (in 500 ml ddH2O), 1% sucrose, 1% agar and 5 ml (in 500
324 ml ddH2O) MES (50 gr/l, pH=5.8 with NaOH). Arabidopsis seeds were stratified for 2 d in a 4C
325 dark room and grown vertically for 7 days under long-day light conditions. Sterile alfalfa
326 seedlings were grown vertically for 5 days under long-day light conditions. Non-sterile sprouts
327 were bought in the local market. Basil plants were grown in a sterile box on 0.5 MS media for
328 5 weeks under long-day light conditions and then leaves were excised for the bacterial
329 inoculation experiments.

330 **Monitoring bacterial growth and plant colonization**

331 Bacterial inoculation and CFU measurement was done as described previously [35]. Briefly, fresh
332 bacterial colonies were grown at 250 RPM, shaking overnight in LB media at 23°. The next
333 day, the culture was diluted 1:500 in fresh LB and grown at 37° for OD_{600}=1. We washed the
334 bacteria in 10mM $MgCl_2$ buffer and then diluted the culture 1:100 in 10mM $MgCl_2$. 2µL of the
335 diluted culture was gently applied on the tip of the root/stem (alfalfa)/cut leaf surface (basil)
336 and placed on an ½ MS agar plate with no sucrose. For figure S1A (2) bacteria were added to
337 the MS media before pouring the plates to the concentration denoted. The square plates
338 were kept in a vertical position during the incubation time at 23° under long-day light
339 conditions (16 h light/8 h darkness) in a plant growth chamber. For bacterial CFU counting
340 and microscopy, plants were incubated with bacteria for 48 hrs. Then, the inoculated plant
341 roots were cut and washed three times in sterile water. The seedlings were transferred to a
342 tube with 1 ml of $MgCl_2$ and vortexed vigorously for 20 seconds, then serial dilutions were
343 plated on LB plates for CFU counting or observed under confocal microscope Zeiss LSM980.
344 The whole root and stem were taken from Arabidopsis and alfalfa, while a circle cut of 5mm
345 in diameter was taken from the basil leaves. For colonization of non-sterile sprouts, we used
346 GFP *Salmonella* and GFP *Listeria* and plated the samples on Kanamycin plates for the
347 Salmonella and Listeria selective enrichment supplement containing plates (Sigma) for the
348 Listeria. The identity of the bacteria was validated by monitoring the GFP expression under a
349 binocular (Leica). When indicated, the following chemicals were added to the MS media:
350 salicylic acid, KI, Diphenyl iodonium (DPI) all the chemicals purchased from Sigma Aldrich. The

351 pH of the MS was titrated back to normal after the addition of salicylic acid and ascorbic acid
352 with NaOH. For measuring endophytic colonization seedlings were grown with *Salmonella* as
353 described above, after 48 hrs the seedlings were surface sterilized for 5 minutes in 10%
354 hypochlorite + 1% Tween 20. Then the roots were washed 3 times in sterilized water, the root
355 were crushed in 10mM $MgCl_2$ and plated on LB agar plates.
356 Bacterial growth in the presence SA and vitamin C was measured in 48 well plates. Overnight
357 cultures were diluted into fresh media with or without the chemicals, and OD_{600} was
358 measured every 10 minutes for 12 hours in Tecan spark 10M.

359 **Biofilm formation assay**

360 Quantification of biofilm formation was done as described previously [36]. Briefly, the overnight
361 culture of bacteria was diluted into LB with no NaCl in a multi-well plate. The plates were
362 incubated at 23° for 5 days without shaking. After 5 days the media was poured, and a solution
363 of 0.1% crystal violet was added and incubated for 10 min. Next, the wells were washed 3
364 times with tape water and dried on the bench. A solution of 33% acetic acid was added to
365 dissolve the crystal violet bound to the wall, and the OD_{570} was measured.

366 **Pseudomonas isolation**

367 We isolated *pseudomonases* from the local area (Kiryat Shmona, north of Israel) from 3
368 different niches: Local soil, local stream, and mint leaves. The soil was vigorously shaken in
369 LB. The leaf was ground in LB. The samples were plated on Pseudomonas isolation agar
370 (Sigma) for 48 hours at 30°. Colonies were re-streaked on Pseudomonas isolation agar to get
371 pure colonies, and the bacterial identity was determined by 16S sequencing (Table S1).

372 **Data analysis**

373 Root length was manually measured in imageJ. Statistical analysis (student T-tset) was done
374 in Excel. Data representation in graphs was done in Excel.

375

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467

Figure 1

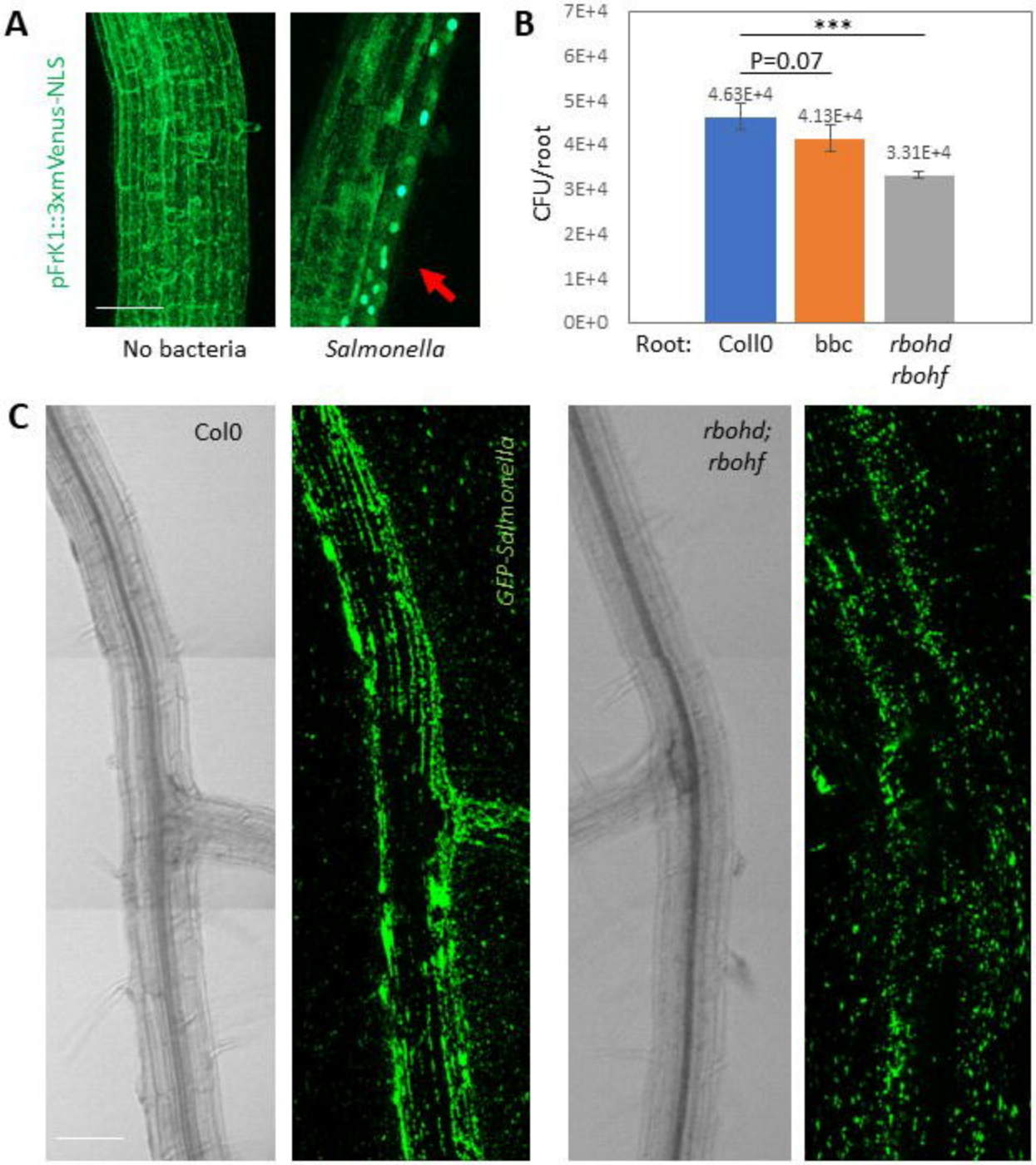

A
pFrK1::3xmVenus-NLS
No bacteria
Salmonella
B
7E+4
6E+4
5E+4
4E+4
3E+4
2E+4
1E+4
0E+0
CFU/root
***
P=0.07
4.63E+4
4.13E+4
3.31E+4
Root:
Col0
bbc
rbohd
rbohf
C
Col0
GFP-Salmonella
rbohd;
rbohf

Figure 2

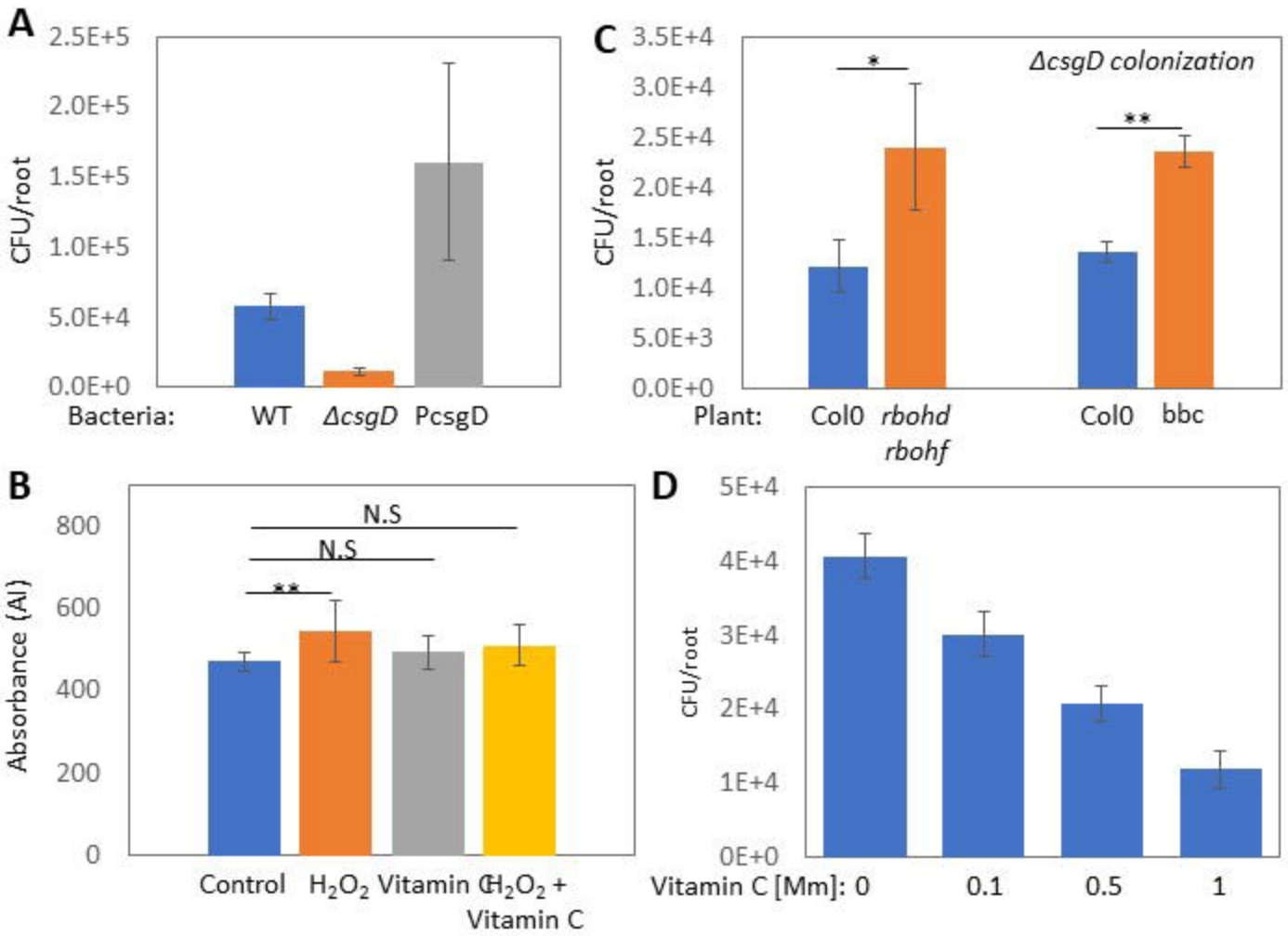

A
2.5E+5
2.0E+5
1.5E+5
1.0E+5
5.0E+4
0.0E+0
CFU/root
Bacteria:
WT
ΔcsgD
PcsgD
C
3.5E+4
3.0E+4
2.5E+4
2.0E+4
1.5E+4
1.0E+4
5.0E+3
0.0E+0
CFU/root
ΔcsgD colonization
*
**
Plant:
Col0
rbohd
rbohf
Col0
bbc
B
800
600
400
200
0
Absorbance (Al)
N.S
N.S
**
Control
H2O2
Vitamin C
H2O2 + Vitamin C
D
5E+4
4E+4
3E+4
2E+4
1E+4
0E+0
CFU/root
Vitamin C [Mm]: 0
0.1
0.5
1

Figure 3

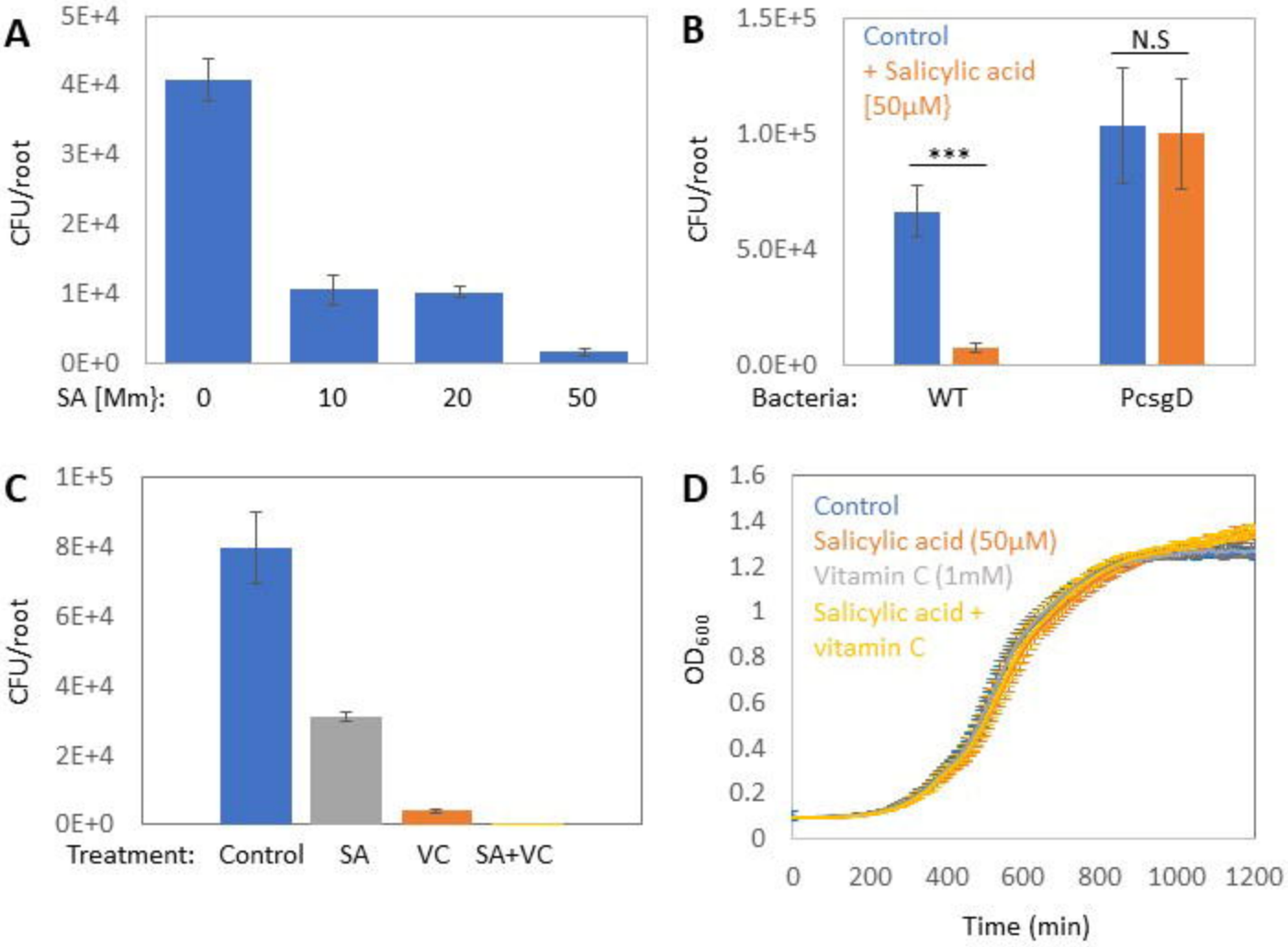

A
5E+4
4E+4
3E+4
2E+4
1E+4
0E+0
CFU/root
SA [Mm}: 0 10 20 50
B
1.5E+5
1.0E+5
5.0E+4
0.0E+0
CFU/root
Control
+ Salicylic acid
[50µM}
N.S
***
Bacteria: WT PcsgD
C
1E+5
8E+4
6E+4
4E+4
2E+4
0E+0
CFU/root
Treatment: Control SA VC SA+VC
D
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
OD600
Control
Salicylic acid (50µM)
Vitamin C (1mM)
Salicylic acid +
vitamin C
0 200 400 600 800 1000 1200
Time (min)

Figure 4

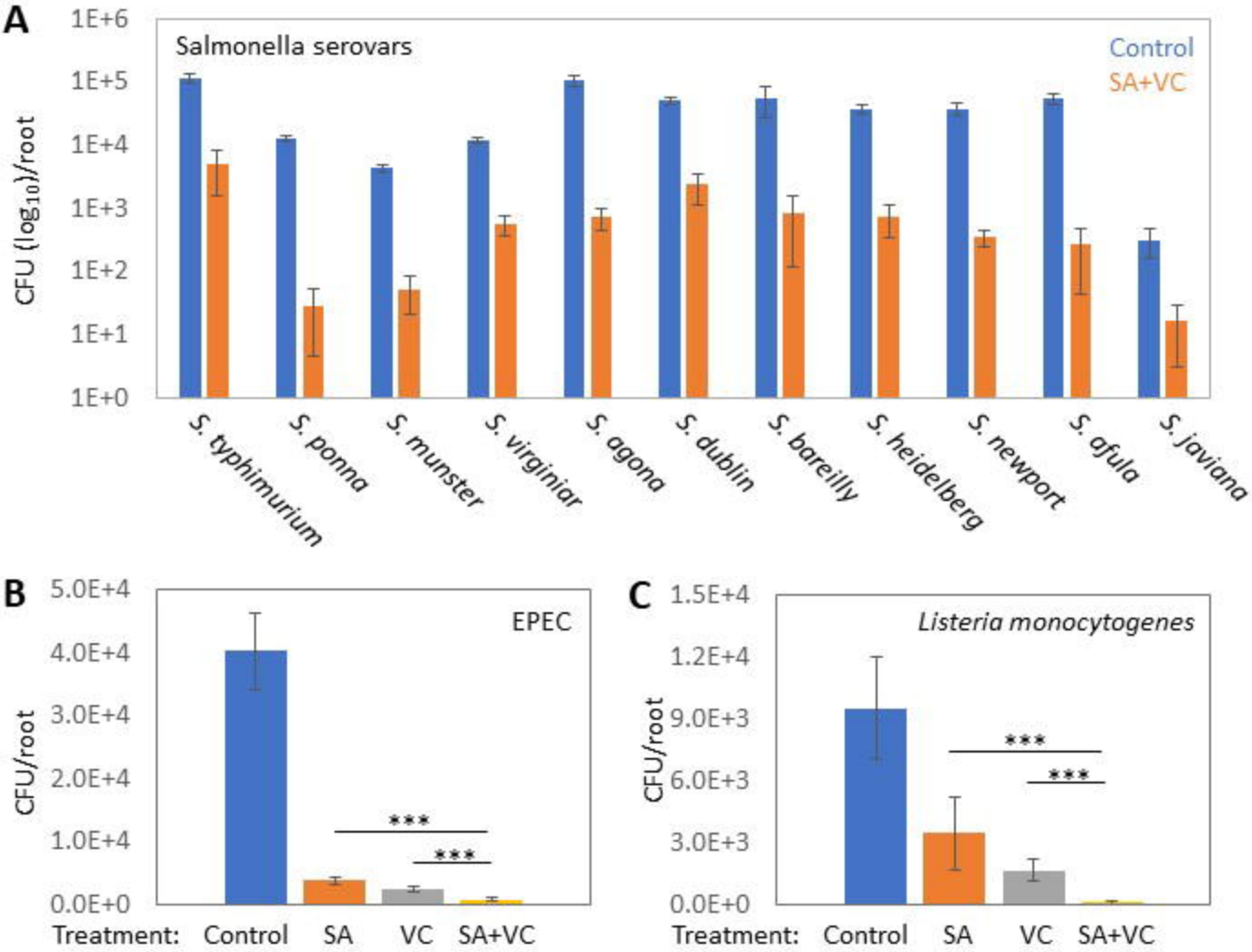

A
Salmonella serovars
Control
SA+VC
CFU (log10)/root
1E+6
1E+5
1E+4
1E+3
1E+2
1E+1
1E+0
S. typhimurium
S. ponna
S. munster
S. virginiar
S. agona
S. dublin
S. bareilly
S. heidelberg
S. newport
S. ofula
S. javiana
B
EPEC
CFU/root
5.0E+4
4.0E+4
3.0E+4
2.0E+4
1.0E+4
0.0E+0
***
***
Treatment: Control SA VC SA+VC
C
Listeria monocytogenes
CFU/root
1.5E+4
1.2E+4
9.0E+3
6.0E+3
3.0E+3
0.0E+0
***
***
Treatment: Control SA VC SA+VC

Figure 5

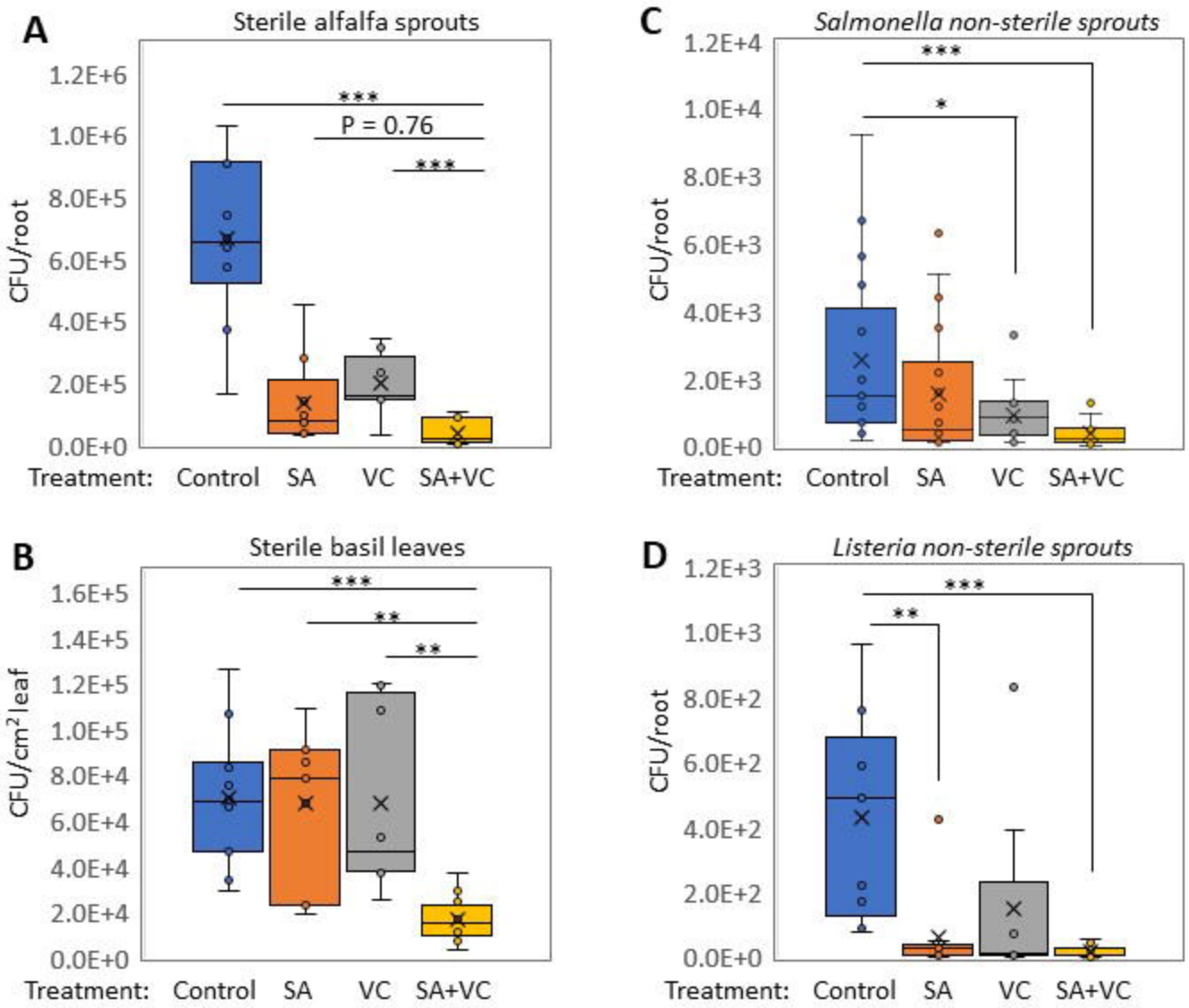
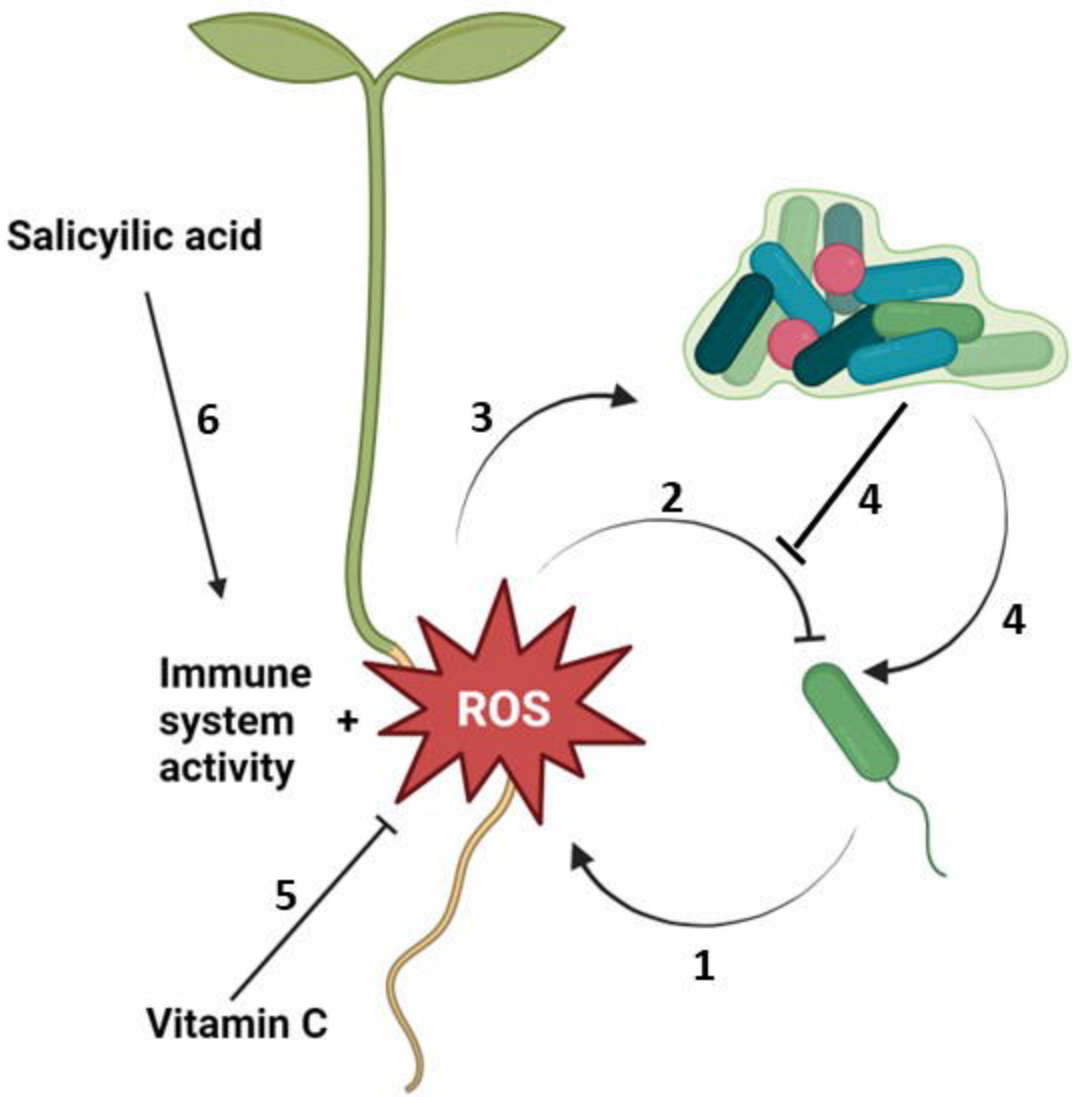

A
Sterile alfalfa sprouts
CFU/root
1.2E+6
1.0E+6
8.0E+5
6.0E+5
4.0E+5
2.0E+5
0.0E+0
***
P = 0.76
***
Treatment: Control SA VC SA+VC
B
Sterile basil leaves
CFU/cm² leaf
1.6E+5
1.4E+5
1.2E+5
1.0E+5
8.0E+4
6.0E+4
4.0E+4
2.0E+4
0.0E+0
***
**
**
Treatment: Control SA VC SA+VC
C
Salmonella non-sterile sprouts
CFU/root
1.2E+4
1.0E+4
8.0E+3
6.0E+3
4.0E+3
2.0E+3
0.0E+0
***
*
Treatment: Control SA VC SA+VC
D
Listeria non-sterile sprouts
CFU/root
1.2E+3
1.0E+3
8.0E+2
6.0E+2
4.0E+2
2.0E+2
0.0E+0
***
**
Treatment: Control SA VC SA+VC

Figure 6

A


Salicyilic acid
6
3
2
4
4
Immune
system
activity
+
ROS
5
1
Vitamin C