Conditional nodule-level sanctions provide long-term stability to the Pea-*Rhizobium* symbiosis

Thomas J. Underwood[1], Beatriz Jorrin[1], Lindsay A. Turnbull[1], Philip S. Poole[1]

[1]Department of Biology, University of Oxford, Oxford, United Kingdom

Author for correspondence:

Philip Poole

Email: philip.poole@biology.ox.ac.uk

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1 table providing a list and description of strains used

Supporting information

The following Supporting Information is available for this article:

Table. S1 Data sheet containing counts from flow cytometer of undifferentiated bacteria and bacteroids from nodules (single occupancy and mixed nodules).

Doc.S2 R markdown file of statistical analysis.

Fig. S3 Representative iBright image of pea inoculated with two strains of differing effectiveness.

Fig. S4 Representative confocal images of nodule sections of both nodule types from all three co-inoculation combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int}, Fix^{int} vs Fix^-) at 28-, 35- and 42-days post inoculation.

Summary

- Legumes sanction root nodules containing rhizobial strains with low nitrogen fixation rates (ineffective fixers). Pea (*Pisum sativum*) nodules contain both undifferentiated bacteria and terminally differentiated nitrogen-fixing bacteroids. It is critical to understand whether sanctions act on both bacteria and bacteroids. As only undifferentiated bacteria are able to regrow from senescent nodules, if sanctioning only acts on bacteroids then ineffective strains might evade sanctioning. In addition, ineffective strains could potentially evade sanctioning by entering the same nodule as an effective strain i.e., piggybacking. *P. sativum* was co-inoculated with pairwise combinations of three strains of rhizobia with different effectiveness, to test whether ineffective strains can evade sanctions in this way.
- We assessed the effect of sanctions on nodule populations of bacteria and bacteroids using flow cytometry and confocal microscopy.
- We show that sanctioning affects bacteroids and bacteria, altering survival of ineffective strains by reducing their numbers and hastening nodule senescence. In nodules containing two strains differing in their nitrogen-fixing ability, both were sanctioned equally. Thus, peas sanction whole nodules based on their nitrogen output, but do not sanction at the cellular level.
- Our results demonstrate that conditional sanctioning in peas provides long-term stability to symbiosis by effectively reducing fitness of ineffective strains.

Key words: Conditional sanctioning, Symbiosis, Mixed nodules, Nitrogen fixation, legumes, *Rhizobium*

Introduction

Legumes have overcome nitrogen limitation by establishing a mutually beneficial interaction with nitrogen-fixing bacteria, hosted within nodules along their root systems (Poole et al., 2018). Certain species of legumes, like peas, form indeterminate nodules, in which some bacteria undergo terminal differentiation to become large swollen cells called bacteroids, which carry out the costly process of nitrogen fixation, but cannot resume their free-living existence (Mergaert et al., 2006). The plant provides carbon compounds in the form of photosynthetically-derived dicarboxylates in return for ammonia provided by the bacteroids, and this relationship continues until the plant's nitrogen requirements have been met (Udvardi & Poole, 2013). At this point nodule senescence ensues, and while the bacteroids then die, any undifferentiated bacteria are released into the soil, which can dramatically increase the concentration of the strain in question, potentially providing a significant fitness advantage (Timmers et al., 2000).

However, this interaction presents an evolutionary dilemma. A 'cheating' strain that reduces investment in the costly process of nitrogen fixation, might be able to produce more reproductive bacteria within the nodules, potentially giving it a fitness advantage. Legumes are known to have evolved sanctions to punish cheating bacteria by cutting off their supply of carbon compounds (Kiers et al., 2003; Oono et al., 2011; West et al., 2002; Westhoek et al., 2017) and these sanctions can be applied conditionally, with pea plants known to sanction nodules that contain an intermediate-fixing strain, only when a better strain is available (Westhoek et al., 2021). But such sanctions could potentially be evaded, allowing intermediate and poor fixers to prosper.

Even in legumes that form indeterminate nodules, there are two clear routes by which cheating might persist in the face of plant sanctions. First, natural selection only concerns itself with short-term fitness; therefore, sanctions targeted only at bacteroids (which are the major consumers of plant-derived carbon) might be selected for, even if they did not affect the undifferentiated bacteria within the nodule. If those undifferentiated bacteria were then released back into the soil in large numbers, this would enhance the populations of

87 cheating strains and so undermine the long-term stability of the symbiosis. Second, poor
88 fixers may persist by 'piggybacking' on a more effective strain, which can occur because
89 multiple strains sometimes occupy a single nodule (Mendoza-Suárez et al., 2021). This route
90 could be avoided if the plant were able to distinguish between different strains within a
91 nodule and sanction the less effective one, but despite some evidence for so-called cell
92 autonomous sanctioning, in the form of premature senescence of cells containing an
93 ineffective strain (Daubech et al., 2017; Regus et al., 2014), metabolic evidence is missing
94 (Agtuca et al., 2020), so it remains contentious.
95
96 To determine whether either route would allow the persistence of poor fixers, we built on
97 previous work carried out by Westhoek et al. (2021) using pea plants and a set of otherwise
98 isogenic rhizobia strains that differ in their ability to fix nitrogen. Specifically, we used (1)
99 flow cytometry to establish whether sanctioned nodules occupied by a less effective strain
100 contain fewer undifferentiated bacteria; and (2) fluorescence microscopy to establish how
101 sanctions influence the timing of nodule senescence. Finally, we used the same techniques
102 on mixed nodules to determine whether or not pea plants carry out cell-autonomous
103 sanctioning within nodules. Our results show that plant sanctions are effective at reducing
104 the fitness of poorly fixing strains. These sanctions operate at the level of whole nodules but
105 not at the cellular level.
106
107 **Methods**
108 We co-inoculated plants with two rhizobial strains selected from three otherwise isogenic
109 strains differing only in their ability to fix nitrogen. They were characterised as a good fixer
110 (Fix^+), an intermediate fixer (Fix^{int}) and a non-fixer (Fix^-, see *Bacterial strains and culture*
111 *conditions* for more details) This gives three pairwise combinations of effective (E) and
112 ineffective (I) strains; Fix^+ (E) vs Fix^- (I), Fix^+ (E) vs Fix^{int} (I) and Fix^{int} (E) vs Fix^- (I). i.e., Fix^+ is
113 always effective and Fix^- always ineffective, but Fix^{int} can be either effective or ineffective,
114 depending on the co-inoculated strain. To measure conditional sanctioning at the whole-
115 nodule level, we sampled nodules occupied by single strains only (identified by a fluorescent
116 marker), and passed them through a flow cytometer to quantify the bacteroid and
117 undifferentiated bacterial populations. Sections of nodules were also examined using
118 confocal microscopy to assess cell health and morphology at a range of time points. Both of

119 these techniques allowed us to assess the effectiveness of plant sanctions on bacterial
120 populations.
121
122 Mixed nodules also form at low frequency on plants co-inoculated with two or more strains.
123 To test the theory of cell-autonomous sanctioning, bacteroids and bacteria from mixed
124 nodules were isolated, quantified by flow cytometry, and imaged by confocal microscopy.
125 We set up three to four plants of each of our three strain combinations (Fix^+ & Fix^-, Fix^+ &
126 Fix^{int} and Fix^{int} & Fix^-) and passed between two and six nodules from each plant through the
127 flow cytometer. Further nodules were also picked for confocal microscopy to inspect the
128 health of different cell types. These techniques allow us to see whether the plant can
129 discriminate between strains within a single nodule and apply sanctions accordingly.
130
131 **Bacterial Strains and Culture Conditions**
132
133 Rhizobial strains used in this study are all derivatives of a highly effective nitrogen-fixing
134 strain (Rlv3841) of *Rhizobium leguminosarum* bv. *viciae* (Johnston & Beringer, 1975), that
135 infects pea (*Pisum sativum* L. cv. Avola). We used mutant strains that differ in their nitrogen-
136 fixation ability but are otherwise genetically identical (Table 1). They are labelled with a
137 fluorescent marker (mCherry or GFP) in order to distinguish the nodules formed by each
138 strain (Table 1). Strains were maintained on tryptone-yeast (TY) agar (Beringer, 1974) with
139 the appropriate concentrations of antibiotics (Table 1). For long-term storage, strains were
140 kept at −80 °C in TY with 15 to 20% glycerol. Rhizobial inoculant was grown on TY agar
141 before dilution to $1x10^8$ cells ml^{-1}.
142
143 **Plant Growth**
144
145 Before sowing, all pea seeds were surface sterilized (1 minute in 95% ethanol followed by 5
146 minutes in 2% NaClO), rinsed, and left to germinate on 1% w/v agar plates at room
147 temperature in the dark. Seedlings were transplanted after five days by transferring them to
148 sterilized 1 litre Azlon beakers containing a 1:1 mixture of silver sand and fine vermiculite,
149 150ml sterilized nitrogen-free nutrient solution, and 5×10^7 cells of each of the two rhizobial
150 strains (see also Westhoek et al., (2017)). Beakers were covered with clingfilm to reduce

151 aerial contamination, which was slit after a few days to allow seedlings to grow through.
152 Plants were grown in a growth room (21 °C, 16 h photoperiod) for 28 to 42 days and
153 watered as necessary from 7 days onwards.

154

155 Table 1: Rhizobial strains and their fixation abilities. All strains derived from Rlv3841 and provided with a strain code, resistance markers, short description and reference.

156

157

158

Name	Strain	Antibiotic resistance	Description	Reference
Fix^{+} mCherry	OPS1341	Streptomycin, Gentamicin	Rlv3841 Tn*7*-Gm-mCherry False colour: orange	(Westhoek et al., 2021)
Fix^{-} GFP	OPS2270	Streptomycin, Spectinomycin, Gentamicin	Ω*nifH* Tn*7*-Gm-sfGFP (Rlv3841 mutant, ΩSpc cassette in *nifH*)	(Westhoek et al., 2021)
Fix^{int} GFP	OPS2268	Streptomycin, Spectinomycin, Gentamicin	Fix^{int} Tn*7*-Gm-sfGFP (Rlv3841 mutant, ΩSpc cassette in promoter region of *nifA*)	(Westhoek et al., 2021)
Fix^{int} mCherry	OPS2269	Streptomycin, Spectinomycin, Gentamicin	Fix^{int} Tn*7*-Gm-mCherry False colour: orange	(Westhoek et al., 2021)

159 **Harvesting**

160 Plants were harvested at 28-, 35- and 42-days post-inoculation by removing the plant from
161 the sand/vermiculite mixture and washing the roots carefully. Nodules were then imaged

162 using a LEICA M165 FC fluorescent stereo microscope and an iBright FL1500 imaging system.
163 Nodule occupants were identified based on their fluorescence (red for Fix^+, green for Fix^-,
164 green or red for Fix^{int}). When selecting single occupancy nodules, the largest of each nodule
165 type was selected.

166 **Confocal imaging**

167 To assess the health of cells containing undifferentiated bacteria and bacteroids within
168 nodules, we imaged sections using confocal microscopy, which allows the visualisation of
169 fluorescently tagged strains and discrimination of the two strains within a mixed nodule. We
170 placed selected nodules in 8 % w/v agar and cut 100 µm sections using a Leica VT1200S
171 vibratome. Nodule slices were then imaged with a Zeiss LSM 880 Airy Scan confocal
172 microscope and analysed with ZEN Black software. To visualise fluorescent tags, mCherry
173 was excited using a 561 nm wavelength laser and emissions detected between 598 and 649
174 nm, while GFP was excited using a 488 nm wavelength laser and emissions detected
175 between 498 and 562 nm.

176 **Flow Cytometry**

177 To identify and quantify the population sizes of undifferentiated bacteria and bacteroids
178 within nodules, we carried out flow cytometry. This was done initially with selected single
179 occupant nodules from the three co-inoculant combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int} and
180 Fix^{int} vs Fix^-). We then carried out flow cytometry on the three types of mixed nodules (Fix^+
181 & Fix^-, Fix^+ & Fix^{int} and Fix^{int} & Fix^-). All nodules were prepared by placing them in a 1.5ml
182 Eppendorf tube with 300µl of harvest solution (0.9% NaCl, 0.02% SILWET L-77) and crushing
183 them with an autoclaved microcentrifuge pestle. This solution was passed through a 40µm
184 filter and diluted tenfold to increase the accuracy of the flow cytometry. Flow cytometry
185 was conducted with a Amnis® CellStream® flow cytometer (Luminex) equipped with a 488
186 and 561nm lasers, which were used for excitation of GFP and mCherry, respectively. Flow
187 rates were set to low speed and high sensitivity (3.66 µL/min). Bacteria events were defined
188 based on custom gating parameters. Singlets and doublets were gated with a threshold of
189 0.400 forward scatter (FSC) aspect ratio. Bacterial singlets with emission at 611/631nm,
190 when excited at 561 nm, above 6,000 arbitrary units were defined as red. Bacterial singlets

191 with emission at 528/546nm, when excited at 488nm, above 4,000 arbitrary units were
192 defined as green. Bacteroids were defined as those events with FSC values of over 10,000
193 arbitrary units, while undifferentiated bacteria were defined as events with values below
194 10,000. Flow cytometry data available at http://flowrepository.org with experiment codes
195 FR-FCM-Z6XN and FR-FCM-Z6XX for the single occupancy experiments and the mixed
196 nodule, experiments respectively.

197 **Statistical Analysis**

198 We first analysed population sizes for undifferentiated bacteria and bacteroids within
199 single-strain nodules (Table S1). In total, we had bacterial counts from sixty-nine single-
200 strain nodules collected from nine plants with different pairwise combinations of strains,
201 which we treated as independent data points. We compared population sizes in effective vs
202 ineffective nodules, based on the idea that in each pairwise combination we can identify an
203 effective and an ineffective strain based on known fixation rates from Westhoek et al.,
204 (2021). Specifically, Fix^+ nodules can always be classed as the effective partner, as can Fix^{int}
205 nodules when co-inoculated with a Fix^- strain. In contrast, Fix^- nodules can always be classed
206 as the ineffective partner, as can Fix^{int} nodules when co-inoculated with a Fix^+ strain. To test
207 for conditional sanctioning at the whole-nodule level, we binned the data into the three
208 different nodule types: Fix^+, Fix^- and Fix^{int}, and tested whether population sizes were
209 dependent on the identity of the co-inoculated strain.

210 To test for the presence of cell autonomous sanctioning within mixed nodules, we used a
211 one-sample t-test to determine whether the proportion of the two strains differed
212 significantly from 50% for both undifferentiated bacteria and bacteroid populations. To
213 compare the absolute numbers of undifferentiated bacteria and bacteroids within mixed
214 nodules, we used paired t-tests. To test whether the total number of undifferentiated
215 bacteria and bacteroids in a mixed nodule differs from that in their respective single-strain
216 nodules, we directly compared all three nodule types using one-way ANOVA followed by a t-
217 test comparing effective and ineffective nodule data with mixed nodule data.

218 All statistical tests were carried out using R version 4.2.1 (2022-06-23) and R studio
219 2022.07.2 and graphs were produced using Graph Pad version 9. An R markdown document

is provided (Doc. S2). In figures, data is presented log10 transformed. However, statistical analysis was performed on the untransformed data to avoid back-transformations, which can distort estimates (Lee, 2020). The analysis was repeated on the log10 transformed data and is available in Doc.S2. There were no qualitative differences in the outcome of transformed vs untransformed data.

Results

Sanctioning of less effective nodules

As found by Westhoek et al., (2017 & 2021), we observed, on all plants, clear phenotypic differences between nodules containing an effective strain (i.e., either Fix^{+} nodules (vs Fix^{-} (I) or vs Fix^{int} (I)) or Fix^{int} nodules (vs Fix^{-} (I)) and those containing an ineffective strain (i.e., either Fix^{-} nodules (vs Fix^{+} (E) or vs Fix^{int} (E)) or Fix^{int} nodules (vs Fix^{+} (E)) at 28 dpi. Notably, nodules containing the effective strain were larger and more tubular, while nodules containing the ineffective strain were smaller and more spherical (Figure S2). These differences were observed, regardless of whether the ineffective strain was the non-fixer (Fix^{-}) or the intermediate fixer (Fix^{int}). In tandem, there was a significant reduction in the number of undifferentiated bacteria in nodules containing the ineffective strain (mean difference = -119622, SE = 23424, t = -5.085, p = 3.17×10^{-6}, n = 69) (Figure 1A) and a reduction in the number of bacteroids (mean difference = -602492, SE = 127765, t = -4.716, p = 1.26×10^{-5}, n = 69) (Figure 1B).

Conditional sanctioning on whole nodules determines bacterial survival

If plants impose conditional sanctions, then we expect the number of undifferentiated bacteria and bacteroids within nodules to depend on the identity of the co-inoculated strain for the intermediate fixer only. In support of this, the number of bacteroids does not depend on the identity of the co-inoculated strain for either Fix^{+} nodules (mean difference = 97172, SE = 338503, t = 0.284, p = 0.779, n = 24) or Fix^{-} nodules (mean difference = -14164, SE = 43638 t = -0.325, p = 0.750, n = 16). However, nodules occupied by the intermediate-

249 fixing strain contained significantly fewer bacteroids when the co-inoculated strain was Fix^+
250 rather than Fix^- (mean difference = -143208, SE = 53448, t = -2.679, p = 0.0124, n = 29)
251 (Figure 1C). We found the same pattern for undifferentiated bacteria. Their numbers did not
252 depend on the identity of the co-inoculated strain for either Fix^+ nodules (mean difference =
253 18176, SE = 57560, t = 0.316, p = 0.755, n = 24), or Fix^- nodules (mean difference = -13185,
254 SE = 31632, t = -0.417, p = 0.683, n = 16). However, for the intermediate fixing nodules,
255 there were significantly fewer undifferentiated bacteria when the co-inoculated strain was
256 Fix^+ rather than Fix^- (mean difference = -68824, SE = 19082, t = -3.607, p = 0.00124, n = 29)
257 (Figure 1D). This demonstrates that conditional sanctioning affects the undifferentiated
258 bacteria (and hence bacterial fitness) and not just the terminally differentiated bacteroids.
259 However, the absolute and relative differences between Fix^+, Fix^{int} and Fix^- are greater for
260 bacteroids than for undifferentiated bacteria, implying that sanctioning has more impact on
261 bacteroid populations at 28 days (compare Figures 1C&D).
262
263 **Conditional sanctioning precipitates premature nodule senescence**
264
265 At 28 days post-inoculation, there was no visible difference in the nodule sections between
266 those containing the Fix^- strain (Figure 2A) and those containing the Fix^+ strain (Figure 2B).
267 However, at 35 days post-inoculation, there was a clear difference between these nodule
268 types. Nodules containing the Fix^- strain had begun to senesce and the cells within nodules
269 were undergoing cell death (Figure 2C), while in contrast, the nodules containing the Fix^+
270 strain showed no evidence of senescence and looked indistinguishable from Fix^+ nodules
271 after 28 days (compare Figures 2B&2D). At 42 days post-inoculation, nodules containing the
272 Fix^+ strain had also begun to senesce (Figure 2F), while cells within nodules containing the
273 Fix^- strains were fully senesced (Figure 2E).
274
275 Similar to the Fix^+ strain, after 28 days post-inoculation, sections from nodules containing
276 the Fix^{int} strain looked the same, regardless of whether the co-inoculated strain was Fix^+
277 (Figure 3A) or Fix^- (Figure 3B). However, at 35 days post-inoculation, the status of nodules
278 containing the Fix^{int} strain was visibly affected by the identity of the co-inoculated strain,
279 consistent with conditional sanctioning. In particular, if the co-inoculated strain was Fix^+,
280 then senescence had begun and cell death was visible (Figure 3C), whereas, if the co-

281 inoculated strain was Fix^-, then there was no sign of senescence, and nodules were not
282 visibly different from Fix^{int} nodules at 28 days post-inoculation (compare Figures 3B&3D).
283 After 42 days, all nodules containing a Fix^{int} strain had begun to senesce. In those where the
284 co-inoculated strain was Fix^+ (Figure 3E), senescence had progressed to a more advanced
285 state than in those co-inoculated with a Fix^- strain (Figure 3F).
286
287 Example nodules from co-inoculated plants of all three combinations (Fix^+ vs Fix^-, Fix^+ vs
288 Fix^{int}, Fix^{int} vs Fix^-) were imaged at 28-, 35- and 42-days post-inoculation and examined for
289 evidence of senescence and changes to cell morphology (Figure S3).
290
291
292 **Conditional sanctioning is not cell-autonomous in peas**
293
294 In mixed nodules, the proportion of bacteroids that belonged to the less effective strain was
295 not significantly different from 50% (mean = 49.78%, 95%CI = 41.80 & 57.76%, t = -0.058; p
296 >0.05, n = 18) (Figure 4A); hence, we did not find evidence for cell-autonomous sanctioning.
297 Indeed, contrary to the predictions of cell-autonomous sanctioning, the proportion of
298 undifferentiated bacteria that belonged to the effective strain was significantly less than
299 50% (mean = 37.78%, 95%CI = 47.6 & 27.94%, t = -2.621, p = 0.0178, n = 18) (Figure 4A),
300 indicating that the less effective strain could even have an advantage within mixed nodules.
301 The same pattern emerged when we compared absolute numbers. The number of
302 bacteroids belonging to the effective strain within a mixed nodule was not significantly
303 different from the number belonging to the ineffective strain (mean difference = -10312,
304 95%CI = 172698 & 193321, t = -0.119, p >0.05, n = 18). However, the number of
305 undifferentiated bacteria belonging to the effective strain was significantly lower than that
306 belonging to the ineffective strain (mean difference = -616752, 95%CI = -156712 & -
307 1076790, t = -2.8285, p = 0.0116, n = 18).
308
309 **Mixed nodules are treated as ineffective nodules by the plant**
310
311 There were significant differences in the total numbers of bacteroids found in nodules of
312 different types (mixed vs single-strain effective vs single-strain ineffective; $F_{2,84}$ = 15.89, p =

313 1.40×10^{-6}; Figure 1A & B) and the same was true for the total number of undifferentiated
314 bacteria ($F_{2,84}$ = 11.62, p = 3.50×10^{-5}). There were significantly more bacteroids in effective
315 nodules compared with mixed nodules (mean difference = -89474, SE = 140734, t = 4.189, p
316 = 6.89×10^{-5}; Figure 4B), but no significant difference between mixed and ineffective nodules
317 (mean difference = -13018, SE = 141435, t = -0.092, p >0.5; Figure 4B). The same pattern
318 was seen for the undifferentiated bacterial populations. There were more undifferentiated
319 bacteria in effective nodules compared with mixed nodules (mean difference = 73709, SE =
320 30076, t = 2.451, p = 0.0163; Figure 4C), but no significant difference between mixed and
321 ineffective nodules (mean difference = -45913, SE = 30226, t = -1.519, p > 0.05; Figure 4C).
322 This would suggest that mixed nodules are treated as ineffective nodules and sanctioned
323 accordingly.
324
325 **Timing of senescence is not cell-autonomous in pea**
326
327 Within mixed nodules at 28 days post-inoculation (n = 13 nodules), there was no evidence
328 of any difference in cell morphology or health between the two strains, as all cells appeared
329 healthy (Figure 5A:C). However, at 35 days post-inoculation, we observed that a few cells
330 within some mixed nodules appear to be undergoing apoptosis (Figure 5F). However, cells
331 belonging to both strains (whether the more or less effective) appeared to be undergoing
332 this process with equal probability (Figure 5). As such, within nodules at 35 days post-
333 inoculation (n = 7 nodules), there was no evidence of cell-autonomous sanctioning (Figure
334 5D:F). At 42 days post-inoculation, all nodules (n = 9 nodules) showed evidence of apoptosis
335 and programmed cell death with very few intact cells remaining (Figure 5G:I).
336
337 ## Discussion
338
339 We show by quantification of undifferentiated bacteria (Figure 1A) and bacteroids (Figure
340 1B) that peas sanction nodules containing ineffectively fixing strains, which is consistent
341 with previous studies (Kiers et al., 2003; Oono et al., 2011; West et al., 2002; Westhoek et
342 al., 2017). It is also consistent with conditional sanctioning, as the fate of nodules infected
343 by a Fix^{int} strain depends on whether the co-inoculated strain is Fix^{+} or Fix^{-} (Westhoek et al.,
344 2021). Sanctions affect both bacteroids (Figure 1C) and undifferentiated bacteria (Figure 1D)

345 within nodules, although for undifferentiated bacteria the magnitude of effects was smaller
346 than for bacteroids. This is consistent with the findings of Westhoek et al., (2021) that the
347 number of viable bacteria has not begun to drop significantly in sanctioned nodules prior to
348 52 days post inoculation.
349
350 We also show that the timing of nodule senescence is influenced by sanctioning but they are
351 separate events and do not occur simultaneously (Figure 2 & 3). While sanctioning is
352 discernible after 16 days (Westhoek et al., 2017) and very marked by 28 days post-
353 inoculation (Figure 1), there is no evidence of cellular senescence in nodules infected by any
354 strain at either time point. It was only after 35 days that nodules containing an ineffective
355 strain began to show signs of senescence, while those containing an effective strain only
356 senesced after 42 days (Figure 5). Thus, while the timing of cellular senescence is influenced
357 by the relative effectiveness of the strain within a nodule, sanctioning at the whole nodule
358 level occurs well before cells start to break down.
359
360 It has been proposed that cell-autonomous sanctions might occur within mixed nodules,
361 allowing plants to combat ineffective "cheating" strains that enter nodules alongside a more
362 effective strain. Evidence for such sanctions would include a bias in the proportion of the
363 effective strain within mixed nodules. However, in our study, the ratio of effective to
364 ineffective bacteroids within mixed nodules did not significantly differ from 50% (Figure 4A).
365 The ratio of effective to ineffective undifferentiated bacteria did significantly differ from
366 50%, but this is because the number of ineffective bacteria was actually higher – the
367 opposite of what we would expect if cell-autonomous sanctions operated. These results
368 therefore show that peas are incapable of cell-autonomous sanctions. When comparing
369 total mixed nodule populations of bacteroids (Figure 4B) and undifferentiated bacteria
370 (Figure 4C) with both single occupancy effective and ineffective nodules, we see that the
371 mixed nodule populations are significantly lower than the effective nodules and not
372 significantly different from those of ineffective nodules. This demonstrates that mixed
373 nodules are treated as ineffective nodules by pea plants.
374
375 Cells within mixed nodules underwent senescence simultaneously, regardless of variation in
376 effectiveness (Figure 5). Consistent with the timing of cell senescence not occurring in a cell-

377 autonomous manner. Although the timing of senescence varied among mixed nodules,
378 where senescence occurred, it affected both strains equally. It may therefore be
379 hypothesised that the timing of senescence within mixed nodules is determined by the ratio
380 of effective to ineffective strains within the nodule. This would be challenging to test,
381 because sectioning nodules does not give an accurate measure of strain ratio due to the
382 irregular 3D distribution of strains within nodules.

383 Our results align with the findings of Agtuca et al., (2020) who found that the metabolic
384 profile of sectors of mixed nodules containing different strains did not show significant
385 differences. However, our results contrast with studies which have found evidence for cell
386 autonomous sanctions based on variation in the timing of senescence, such as Regus et al.,
387 (2017) and Daubech et al., (2017). The differences may be explained by variation in the
388 legume-rhizobium system used: Daubech et al. (2017) used *Cupriavidus* taiwanensis –
389 *Mimosa pudica*; the Regus et al. (2017) study used both *Acmispon strigosus* –
390 *Bradyrhizobium* and *Lotus japonicus* – *Mesorhizobium*; and the Agtuca et al. (2020) study
391 used *Glycine max* – *Bradyrhizobium japonicum.* A comprehensive understanding of
392 sanctions will therefore require the continued use of multiple experimental systems.

393 We have shown that the sanctioning of ineffective nodules within the pea-rhizobium
394 symbioses is conditional in nature and occurs at the nodule level. In Fix^{+}/Fix^{int} coinfected
395 plants, nodules containing Fix^{int} but not Fix^{+} strains are sanctioned, suggesting conditional
396 sanctioning requires comparison between nodule-specific and global nitrogen signals. This
397 might be achieved through the interaction between a nitrate receptor such as the Nitrate
398 transreceptor NRT1.1, which both transports nitrate as well as detecting nitrate levels
399 (Zhang et al., 2019) and the NIN-like Proteins (NLPs) 1 and 4, which are essential for the
400 nitrate-based regulation of nodule maturation (Lin et al., 2021). In high nitrate conditions
401 NLP1/4 inhibit cytokinin biosynthesis. Cytokinin biosynthesis drives nodule maturation
402 through the activation of a signal cascade through the Cytokinin responsive element CRE1
403 (Lin et al., 2021), which acts to promote expression of the *cep* and *cle* genes (Laffont et al.,
404 2020). However, how a global signalling mechanism is able to detect the nitrogen output of
405 individual nodules and compare it to global nitrogen status remains unclear. The
406 mechanism of sanctions on individual nodules may be achieved through nodule-specific

proteins responding to nitrate levels. One possibility is SnRK1 which, when phosphorylated by the DMI2 kinase in response to Nod factor, phosphorylates malate dehydrogenase 1 and 2, leading to increased malate production within the nodule to supply bacteroids (Guo et al., 2023). This is therefore a prime candidate for a pathway in which the legume would be able to reward effective strain-containing nodules, as well as to punish nodules containing ineffectively fixing strains by cessation of malate production and supply. However, these remain speculations about signalling pathways whose elucidation may aid future efforts to engineer symbioses in the selection and use of more effective nitrogen-fixing bacteria.

Having identified two potential routes for persistence of a poor nitrogen-fixing and/or 'cheating' strain, we now have clear evidence that plants can deal with both. First, while sanctions, at least initially, affect nitrogen-fixing bacteroids more severely, undifferentiated bacteria are also greatly reduced in number inside ineffective nodules. Second, while the plant is unable to discriminate between effective and ineffective strains within a mixed nodule, 'piggybacking' on an effective strain does not provide a viable route by which to evade sanctions. Any potential benefit is limited not only by the scarcity of mixed nodules, but also because the plant views mixed nodules as ineffective, and sanctions them accordingly.

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Competing interests

The authors declare no competing interests

Author contributions

All authors listed have made a substantial direct and intellectual contribution to the work and approved it for publication. TJU, BJ and PSP conceived this study and designed the experiments. TJU and BJ performed the experiments. TJU analysed the data and prepared the manuscript. TJU, BJ, LAT and PSP critically reviewed the manuscript.

Data Availability

535 Flow cytometry: Data is available at http://flowrepository.org with experiment codes FR-
536 FCM-Z6XN and FR-FCM-Z6XX for the single occupancy experiments and the mixed nodule
537 experiments respectively.

538 **Supporting information**

539 The following Supporting Information is available for this article:

540 **Table. S1** Data sheet containing counts from flow cytometer of undifferentiated bacteria
541 and bacteroids from nodules (single occupancy and mixed nodules).

542 **Doc.S2** R markdown file of statistical analysis.

543 **Fig. S3** Representative iBright image of pea inoculated with two strains of differing
544 effectiveness.

545 **Fig. S4** Representative confocal images of nodule sections of both nodule types from all
546 three co-inoculation combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int}, Fix^{int} vs Fix^-) at 28-, 35- and 42-
547 days post inoculation.

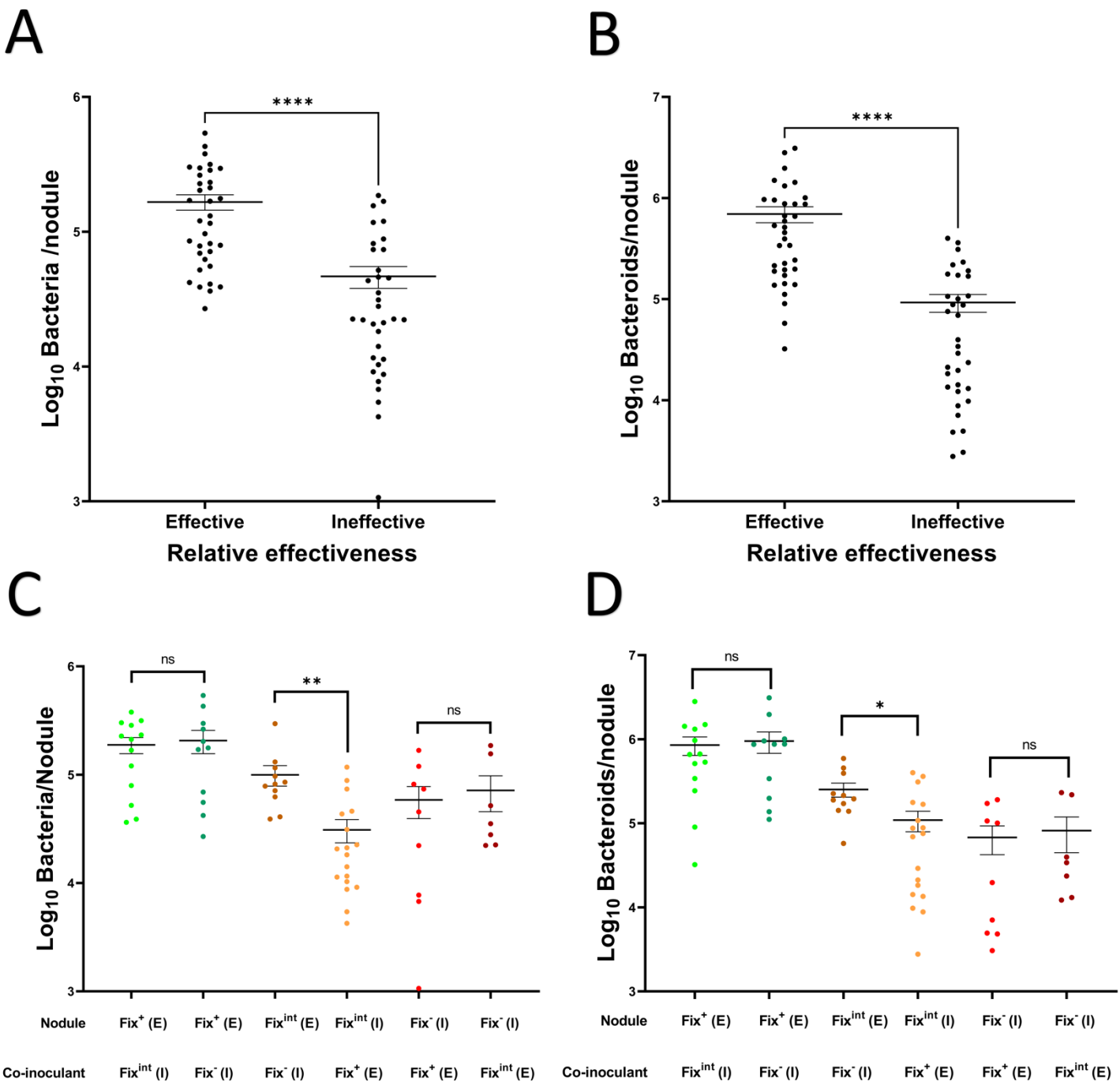

Fig. 1. Nodules containing the ineffective strain have fewer bacteria and bacteroids.

Numbers of (A) undifferentiated bacteria and (B) bacteroids in nodules from pea plants co-inoculated with two strains of rhizobia, one effective (either Fix^{+} or Fix^{int} co-inoculated with Fix^{-}) and the other ineffective (either Fix^{-} or Fix^{int} co-inoculated with Fix^{+}). (C) and (D) show the same data broken down into different nodule types (Fix^{+}, Fix^{int} or Fix^{-}). The identity of the co-inoculated strain is also shown. In each case, one strain is denoted effective (E) and the other ineffective (I) as determined by their relative ability to fix nitrogen. Significance levels from unpaired t-tests: ****< 0.0001; *** < 0.001; ** < 0.01; * <0 .05; ns, not significant. Nodules harvested at 28 days post-inoculation (*n* = 69).

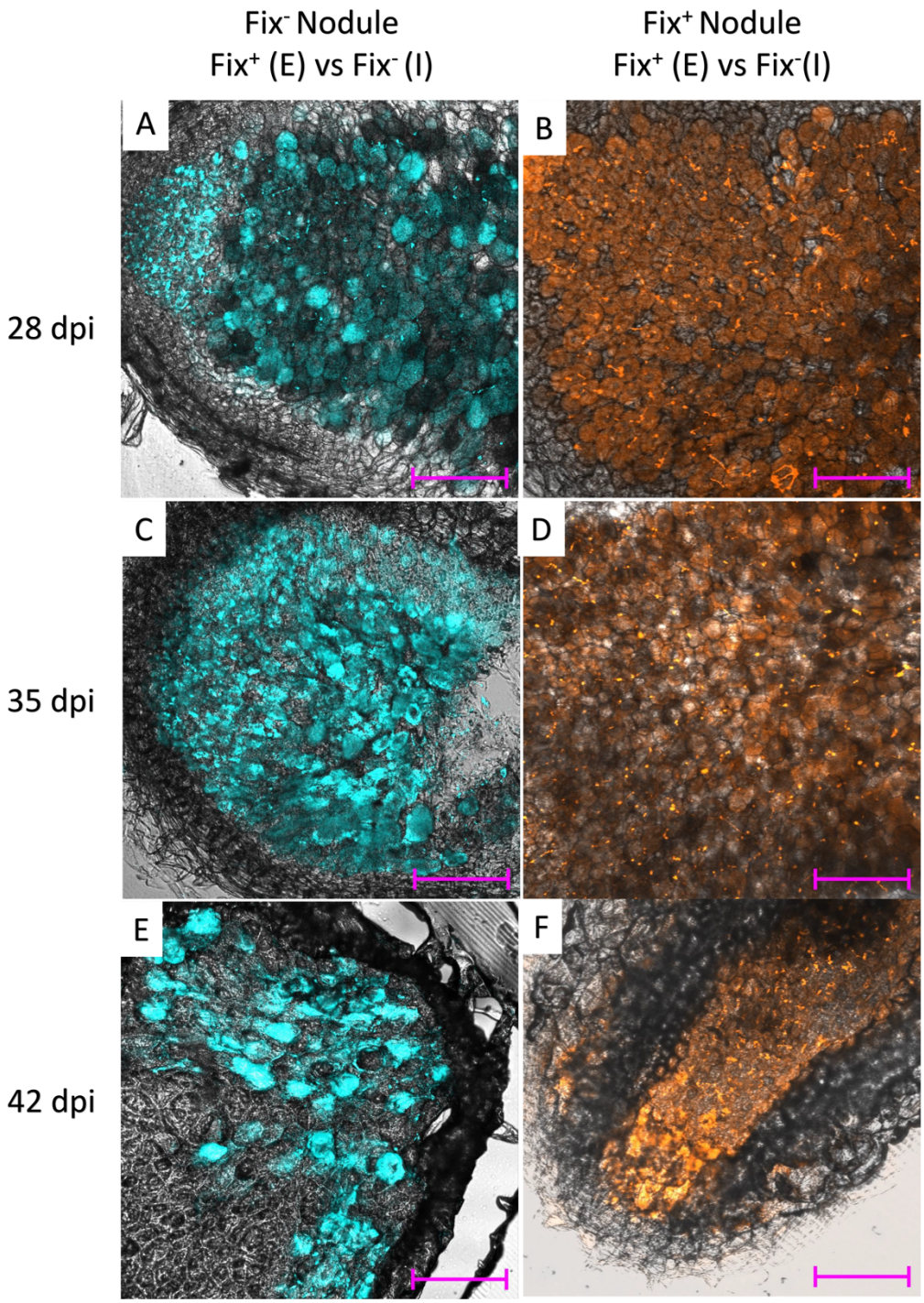

Fig. 2. Nodules containing a Fix⁻ strain senesce earlier then nodules containing a Fix⁺ strain.
(A), (C) and (E) Fix⁻ nodule labelled with GFP (blue) and (B), (D) and (F) Fix⁺ nodule labelled with mCherry (orange) at 28 dpi, 35 dpi and 42 dpi, respectively. Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm.

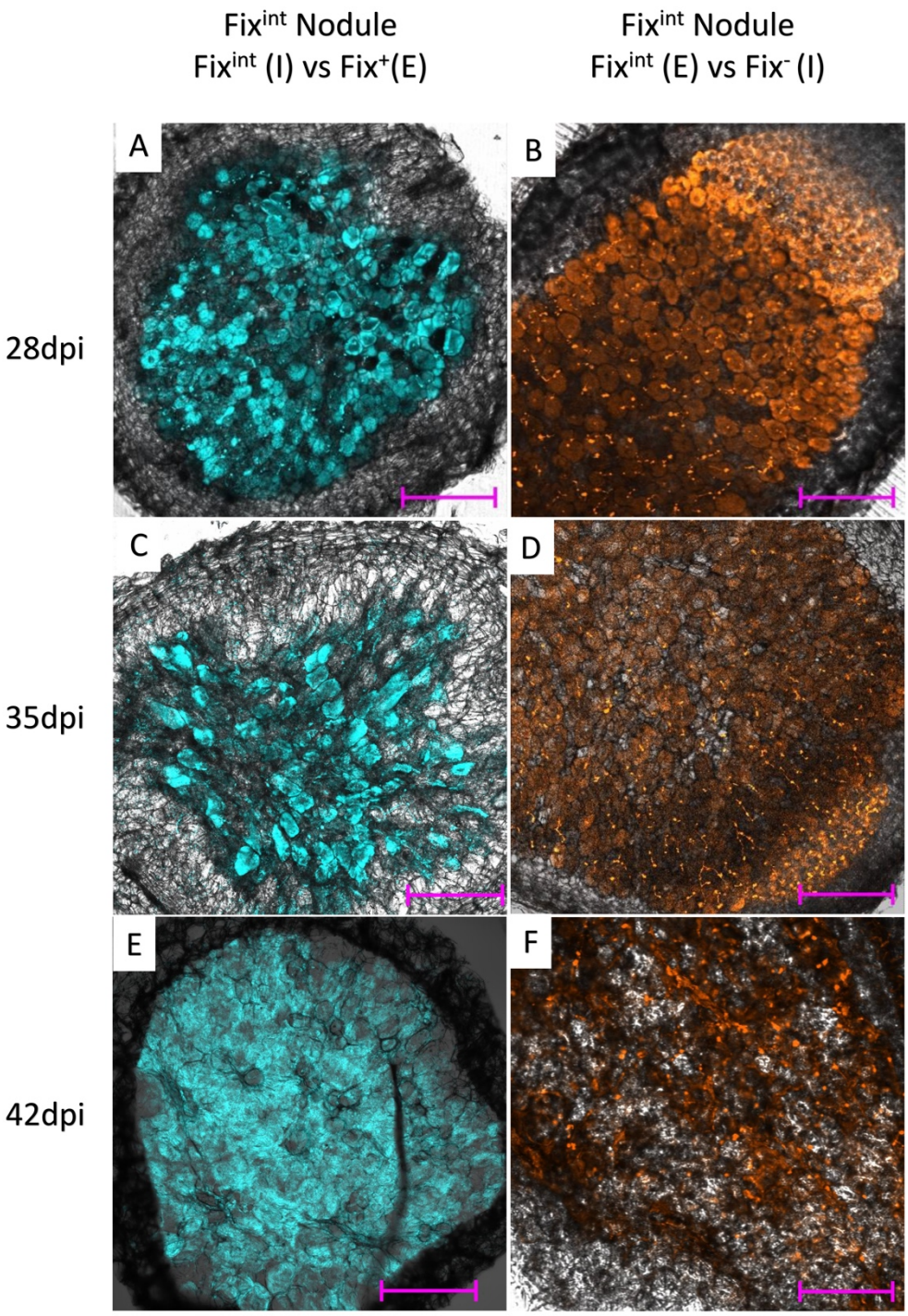

Fig. 3. Fix^{int} nodules co-inoculated with Fix^{+} strain senesce earlier than those co-inoculated with Fix^{-} strain
(A), (C) and (E) Fix^{int} nodule labelled with GFP (blue), co-inoculated with Fix^{+}. (B), (D) and (F) Fix^{int} nodule labelled with mCherry (orange), co-inoculated with Fix^{-}. Nodules were harvested at 28 dpi, 35 dpi and 42 dpi, respectively. Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm.

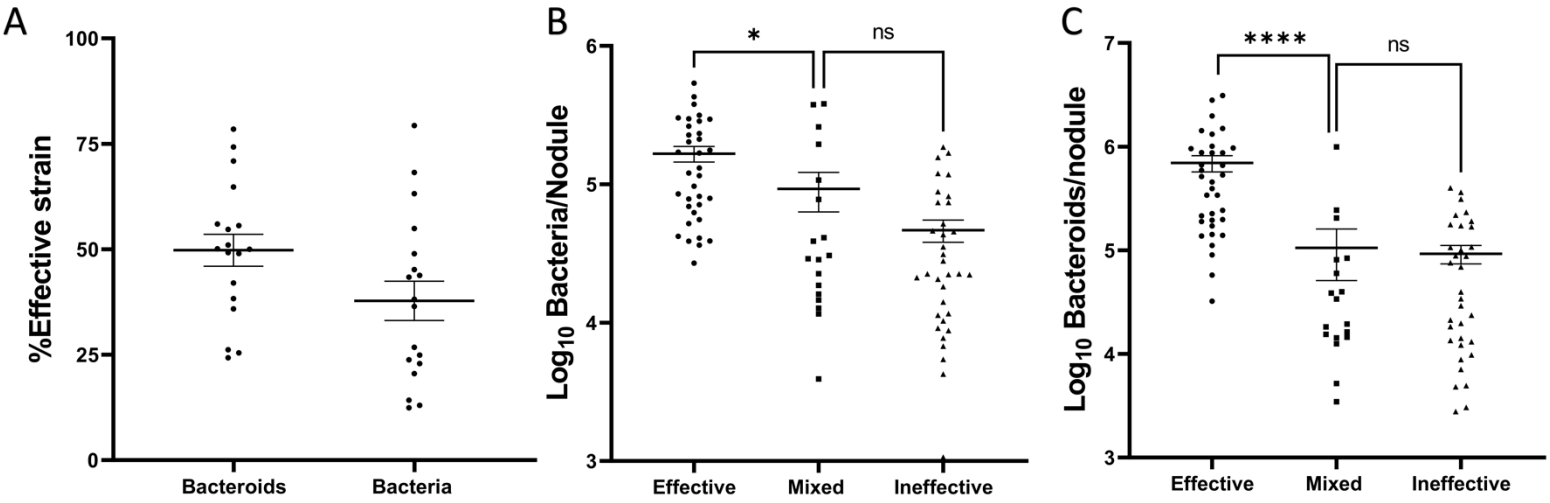

Fig. 4. The percentage of bacteria and bacteroids of the effective strain within a mixed nodule do not exceed 50%, and the combined population size is lower than that of an effective nodule.

(A) Percentage of bacteroids and undifferentiated bacteria within a mixed nodule that belonged to the effective strain (either Fix^{+} or Fix^{int} co-inoculated with Fix^{-}). The total number of (B) bacteria and (C) bacteroids within single-strain effective nodules, mixed nodules (both populations combined) and single-strain ineffective nodules. ns, not significant. Significance levels in (B) and (C) assessed from pairwise comparisons using t-tests: ****< 0.0001 *** < 0.001, ** < 0.01, * <0 .05. Nodules harvested at 28 dpi.

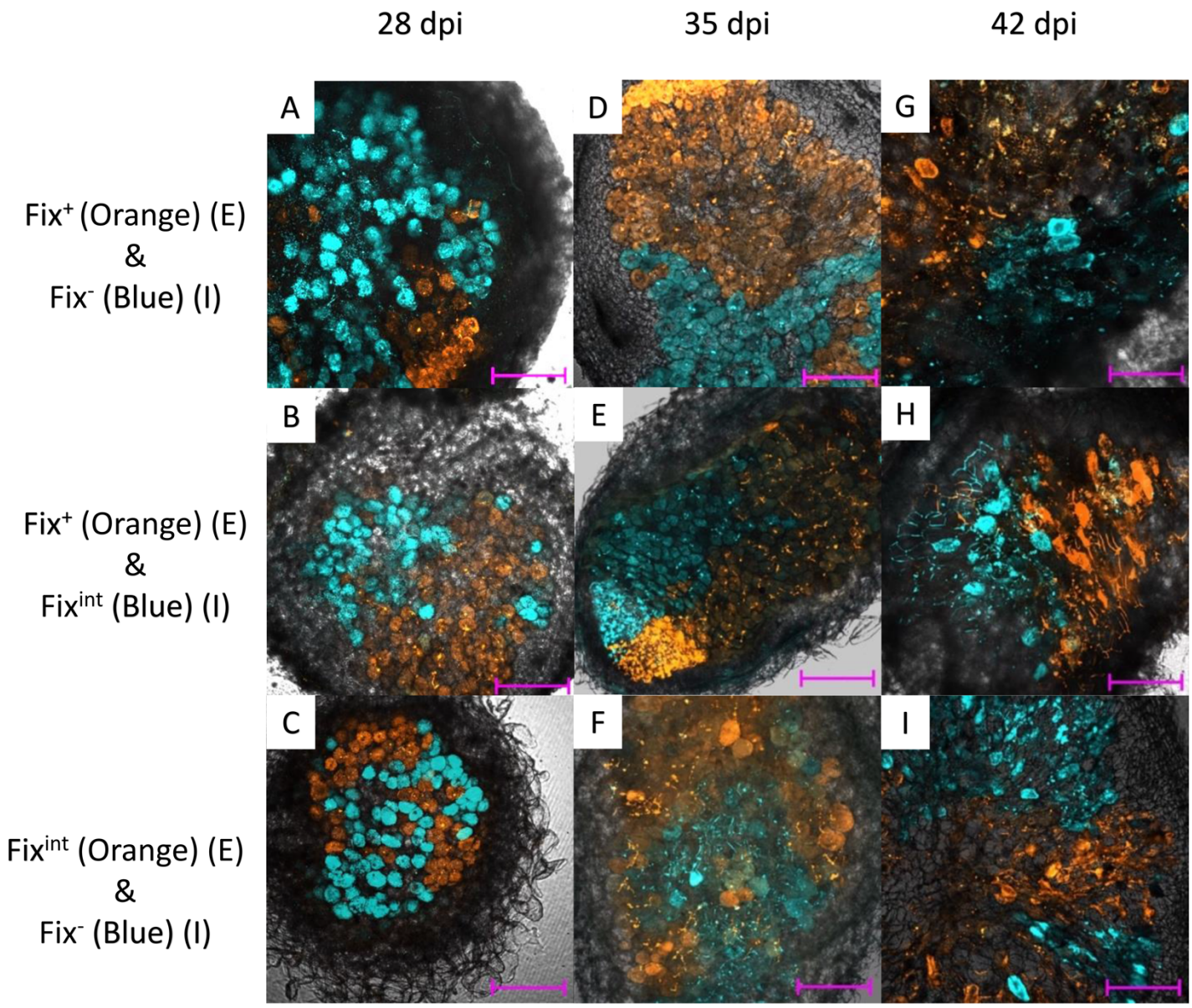

Fig. 5. The two strains within a mixed nodule do not senesce at different times despite varying in relative effectiveness.

(A), (D) and (G) mixed nodule of Fix^+ (orange) and Fix^- (blue). (B), (E) and (H) mixed nodule of Fix^+ (orange) and Fix^{int} (Blue). (C), (F) and (I) mixed nodule of Fix^{int} (orange) and Fix^- (blue). Nodules were harvested at 28 dpi, 35 dpi and 42 dpi, as indicated. Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm.