Pisum sativum **applies sanctions to less effective strains at the nodule level, but not within mixed nodules**

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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:

Fig. S1 Image of flow cytometry output showing the two populations differentiated by size based on Forward Scatter (FSC)

Table. S2 Flow cytometry data, analysed using custom gating to calculate the number of undifferentiated bacteria and bacteroids in single occupancy and mixed nodules, and acetylene reduction assay and nodulation results from fluorescent tag control experiments.

Doc.S3 R markdown file of statistical analysis.

Fig. S4 Representative iBright image of pea inoculated with two strains of differing effectiveness.

Fig. S5 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.

Fig. S6 Representative images of the three types of mixed nodules at 28, 35 and 42 days post inoculation

Summary

- Legumes sanction root nodules containing rhizobial strains with low nitrogen fixation rates (less effective fixers). Pea (*Pisum sativum*) nodules contain both undifferentiated bacteria and terminally differentiated nitrogen-fixing bacteroids. It is critical to understand how sanctions act on both bacteria and bacteroids and how they differ. In addition, less effective 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 the effects on nodule ultra-structure using confocal microscopy.
- We show that sanctioning lowered the bacteroid population and caused a reduction in the size of bacteria. Sanctions also precipitated an early change in nodule cell morphology. In nodules containing two strains that differed in their nitrogen-fixation 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 whole-nodule conditional sanctioning in peas provides long-term stability to the symbiosis by effectively reducing the 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 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 (Timmers et al., 2000). The presence of a host leads to an increase in the soil population of rhizobia (Beringer et al., 1979); therefore, by engaging in the symbiosis, legumes potentially provide a significant fitness advantage to rhizobia.

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 (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). Intriguingly, while Westhoek et al (2021) did see a significant drop in carbon transport and nodule size by twenty-eight days post inoculation, they did not see a significant decrease in the number of viable bacteria within the sanctioned nodule (although the number of viable bacteria had plummeted dramatically by fifty-six days post-inoculation). As such, the cause of the

reduction in nodule size at twenty-eight days post-inoculation, while hypothesised to be due to a drop in the bacteroid population, has yet to be proven.

Poor fixers may be able to evade plant sanctions by 'piggybacking' on a more effective strain, which can occur because multiple strains can occupy a single nodule, with mixed nodules sometimes at frequencies of over 20%, (Mendoza-Suárez et al., 2020),. This route could be avoided if the plant were able to distinguish between different strains within a nodule and sanction the less effective one, but despite some evidence for so-called cell-autonomous sanctioning, in the form of premature senescence of cells containing an ineffective strain (Daubech et al., 2017; Regus et al., 2014), metabolic evidence has suggested that this does not occur (Agtuca et al., 2020), so it remains contentious.

This study builds on previous work carried out by Westhoek et al. (2021) using pea plants and a set of otherwise isogenic rhizobia strains that differ in their ability to fix nitrogen. First, we used flow cytometry to measure fitness characteristics of both undifferentiated bacteria and nitrogen-fixing bacteroids within nodules infected with a single rhizobial strain and fluorescence microscopy to establish how sanctions influenced the health and morphology of the infected plant cells within those nodules. Second, we used the same techniques on mixed nodules to determine whether or not pea plants carry out cell-autonomous sanctioning within nodules. Our results show that sanctions reduce the number of bacteroids within whole nodules as well as reducing the size of the undifferentiated bacteria. Fluorescence microscopy showed that the breakdown of cells within the nodule occurred prematurely in sanctioned nodules. Our results also show that these sanctions operate at the level of the whole nodule but not at the cellular level. As such, a piggybacking strain cannot be punished independently of the non-cheater within a mixed nodule; however, mixed nodules were treated as ineffective and sanctioned at the nodule level. As such, piggybacking is not an effective route by which cheaters can succeed.

Methods

We co-inoculated plants with two rhizobial strains selected from three otherwise isogenic strains differing only in their ability to fix nitrogen. They were characterised as a good fixer (Fix^{+}), an intermediate fixer (Fix^{int}) and a non-fixer (Fix^{-}, see *Bacterial strains and culture*

118 *conditions* for more details) This gives three pairwise combinations of a more effective (E)
119 and less effective (L) strains; Fix^+ (E) vs Fix^- (L), Fix^+ (E) vs Fix^{int} (L) and Fix^{int} (E) vs Fix^- (L). i.e.,
120 Fix^+ is always effective and Fix^- always less effective, but Fix^{int} can be either effective or less
121 effective, depending on the co-inoculated strain. To measure conditional sanctioning at the
122 whole-nodule level, we sampled nodules occupied by single strains only (identified by a
123 fluorescent marker), and passed them through a flow cytometer to count and measure the
124 bacteroid and undifferentiated bacterial populations. To confirm that the fluorescent
125 marker had no effect on the symbiotic characteristics of Rlv3841 the fixation rate and
126 nodulation efficiency of a strain containing each of our fluorescent tags was compared
127 against each other and a non-fluorescing wild-type strain. Sections of nodules were also
128 examined using confocal microscopy to assess cell health and morphology at a range of time
129 points. Both of these techniques allowed us to assess the effectiveness of plant sanctions on
130 bacterial populations.
131
132 Mixed nodules also form on plants co-inoculated with two or more strains. To test the
133 theory of cell-autonomous sanctioning, the size and number of bacteroids and bacteria from
134 within mixed nodules were quantified using flow cytometry, and mixed nodules were
135 imaged using confocal microscopy. We set up five or six plants of each of our three strain
136 combinations (Fix^+ & Fix^-, Fix^+ & Fix^{int} and Fix^{int} & Fix^-) and passed five nodules from each
137 plant through the flow cytometer. Further nodules were also picked for confocal microscopy
138 to inspect the health of different cell types. These techniques allow us to see whether the
139 plant can discriminate between strains within a single nodule and apply sanctions
140 accordingly.
141
142 **Bacterial Strains and Culture Conditions**
143
144 Rhizobial strains used in this study are all derivatives of a highly effective nitrogen-fixing
145 strain, *Rhizobium leguminosarum* bv. (Rlv) 3841, that infects pea (*Pisum sativum* L. cv.
146 Avola) (Johnston & Beringer, 1975). The mutant strains thus differ in their nitrogen-fixation
147 ability but are otherwise genetically identical (Table 1). They are labelled with a fluorescent
148 marker (mCherry or GFP) in order to distinguish the nodules formed by each strain (Table 1).
149 Strains were maintained on tryptone-yeast (TY) agar (Beringer, 1974) with the appropriate

150 concentrations of antibiotics (Table 1). For long-term storage, strains were kept at −80 °C in
151 TY with 15 to 20% glycerol. Rhizobial inoculant was grown on TY agar and the number of
152 bacteria on the slope was determined by measuring the OD600 of the washed slope using a

Name	Strain	Antibiotic resistance	Description	Reference

153 Genesys 150 UV-Visible spectrophotometer. A dilution of $1x10^8$ cells ml^{-1} was then
154 produced.
155
156 **Plant Growth**
157
158 Before sowing, all pea seeds were surface sterilized (1 minute in 95% ethanol followed by 5
159 minutes in 2% NaClO), rinsed, and left to germinate on 1% w/v agar plates at room
160 temperature in the dark. Seedlings were transplanted after five days by transferring them to
161 sterilized 1 litre Azlon beakers containing a 1:1 mixture of silver sand and fine vermiculite,
162 150 ml sterilized nitrogen-free nutrient solution, and 5 x 10^7 cells of each of the two
163 rhizobial strains (see also Westhoek et al., (2017)). Beakers were covered with clingfilm to
164 reduce aerial contamination, which was slit after a few days to allow seedlings to grow
165 through. Plants were grown in a growth room (21 °C, 16 h photoperiod) for 28 to 42 days
166 and watered as necessary from 7 days onwards.

167

168

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.

Fix mCherry	OPS1341	Streptomycin, Gentamicin	Rlv3841 Tn7-Gm-mCherry	(Westhoek et al., 2021)
Fix^{+} GFP	OPS1339	Streptomycin, Genatmicin	Rlv3841 Tn7-Gm-GFP	(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)

Harvesting

177 Plants were harvested at 28-, 35- and 42-days post-inoculation by removing the plant from
178 the sand/vermiculite mixture and washing the roots carefully. Nodules were then imaged
179 using a LEICA M165 FC fluorescent stereo microscope and an iBright FL1500 imaging system.
180 Nodule occupants were identified based on their fluorescence. When selecting single
181 occupancy nodules, the five largest of each nodule type was selected.

182 **Acetylene reduction assay**

183 Fixation rates were measured via an acetylene reduction assay using the method described
184 in Westhoek et al (2021).

185 **Confocal imaging**

186 To assess the health of cells containing undifferentiated bacteria and bacteroids within
187 nodules, we imaged sections using confocal microscopy, which allows the visualisation of
188 fluorescently tagged strains and discrimination of the two strains within a mixed nodule. We
189 placed selected nodules in 8 % w/v agar and cut 100 µm longitudinal sections through the
190 centre of the nodule using a Leica VT1200S vibratome. Nodule slices were then imaged with
191 a Zeiss LSM 880 Airy Scan confocal microscope and analysed with ZEN Black software. To
192 visualise fluorescent tags, mCherry was excited using a 561 nm wavelength laser and
193 emissions detected between 598 and 649 nm, while GFP was excited using a 488 nm
194 wavelength laser and emissions detected between 498 and 562 nm.

195 **Flow Cytometry**

196 To identify and quantify the population sizes of undifferentiated bacteria and bacteroids
197 within nodules, we carried out flow cytometry. This was done initially with selected single
198 occupant nodules from the three co-inoculant combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int} and
199 Fix^{int} vs Fix^-). We then carried out flow cytometry on the three types of mixed nodules (Fix^+
200 & Fix^-, Fix^+ & Fix^{int} and Fix^{int} & Fix^-). All nodules were prepared by placing them in a 1.5ml
201 Eppendorf tube with 300µl of harvest solution (0.9% NaCl, 0.02% SILWET L-77) and crushing
202 them with an autoclaved microcentrifuge pestle. This solution was passed through a 40µm
203 filter and diluted tenfold to increase the accuracy of the flow cytometry. Flow cytometry
204 was conducted with a Amnis® CellStream® flow cytometer (Luminex) equipped with a 488

205 and 561nm lasers, which were used for excitation of GFP and mCherry, respectively. Flow
206 rates were set to low speed and high sensitivity (3.66 µL/min). Analysis of flow cytometry
207 data was carried out using the CellStream® Analysis software (Version 1.5.17). Bacterial
208 events were defined based on custom gating parameters. Singlets and doublets were gated
209 with a threshold of 0.400 forward scatter (FSC) aspect ratio. Bacterial singlets with emission
210 at 611/631nm, when excited at 561 nm, above 6,000 arbitrary units were defined as red.
211 Bacterial singlets with emission at 528/546nm, when excited at 488nm, above 4,000
212 arbitrary units were defined as green. Bacteroids and bacteria were defined based on size,
213 as in Mergaert et al., (2006), using custom gating of the FSC detection to separate the two
214 populations (Figure S1). Flow cytometry data available at http://flowrepository.org with
215 experiment codes (FR-FCM-Z8AA &) for the single occupancy experiments and the mixed
216 nodule, experiments respectively.

217 **Statistical Analysis**

218 To test the effect of the fluorescent markers on the symbiotic characteristics of Rlv3841, we
219 compared the fixation rates of Rlv3841, OPS1341 and OPS1399 (wild-type strains with
220 different fluorescent markers) using one-way ANOVA. We compared the nodulation
221 competitiveness of the same three strains using paired t-tests.

222 To test for conditional sanctioning at the whole-nodule level, we subsetted the data into the
223 three different nodule types: Fix^{+}, Fix^{-} and Fix^{int}, and tested whether three relevant
224 measures of bacterial fitness and plant sanctioning behaviour were dependent on the
225 identity of the co-inoculated strain. The chosen measures were: (1) the size of the
226 undifferentiated bacterial population (a key measure of bacterial fitness), (2) the size of the
227 bacteroid population and (3) the size of individual undifferentiated bacteria. We did not
228 measure the size of individual bacteroids because the numbers of bacteroids within some
229 sanctioned nodules was so small as to make this measure unreliable. Both population size
230 and size of individuals were analysed using mixed-effects models in which individual plant ID
231 was the random effect and the identity of the co-inoculant was the fixed effect.

232 To test for the presence of cell autonomous sanctioning within mixed nodules, we used a
233 one-sample t-test to determine whether the proportion of the two strains differed

significantly from 50% for both undifferentiated bacteria and bacteroid populations. To compare the absolute numbers of undifferentiated bacteria and bacteroids within mixed nodules, we used paired t-tests.

All statistical tests were carried out using R version 4.2.1 (2022-06-23) and R studio 2022.07.2 and graphs were produced using Graph Pad version 9. An R markdown document is provided (Doc. S3). In figures, bacteria and bacteroid population data is presented log10 transformed. Where a log transformation was carried out for statistical analysis to create a better model this has been noted in the results.

Results

Fluorescently tagging strains does not affect symbiotic characteristics of Rlv3841

We found that there was no significant difference in the nodulation competitiveness of the two fluorescently tagged strains when co-inoculated against the untagged wild-type Rlv3841 (Fix^+), nor did the two tagged strains differ from each other when co-inoculated together (Fix^+ vs Fix^+ mCherry: estimate = 22.750, SE = 13.288, t = 1.712, $p > 0.05$, df = 6; Fix^+ vs Fix^+ GFP: estimate = -5.75, SE = 20.84, t = -0.276, $p>0.05$, df =6; Fix^+ GFP vs Fix^+ mCherry: estimate = -12.250, SE = 11.265, t = -1.087, $p>0.05$, df = 6). In addition, single inoculated peas with each of the three strains did not differ in fixation rates per nodules ($F = 1.387$, $p > 0.05$, n = 4, df = 2) so no further pairwise comparisons were made.

Conditional sanctioning on whole nodules determines bacteroid population size

The number of bacteroids does not depend on the identity of the co-inoculated strain for either Fix^+ nodules (estimate = 970769, SE = 2039541, t value = 0.476, p =0.644, n =60) or Fix^- nodules (Log transformed data, estimate = -0.540, SE = 0.299, t value = -1.806, p = 0.104, n = 55) . However, nodules occupied by the intermediate-fixing strain contained significantly fewer bacteroids when the co-inoculated strain was Fix^+ rather than Fix^- (Log transformed data, estimate = -2.209, SE = 0.324, t value = -6.818, $p = 7.74 \times 10^{-5}$, n = 55). (Figure 1A)

262 **Conditional sanctioning on whole nodules determines the size of bacteria**

263

264 The average size of the undifferentiated bacteria did not depend on the identity of the co-
265 inoculant for Fix^+ nodules (estimate = 561.3, SE = 301.6, t = 1.861, p = 0.0923, n = 60).
266 However, undifferentiated Fix^- bacteria were significantly smaller when co-inoculated with a
267 Fix^+ strain (Log transformed data, estimate.= -0.112, SE = 0.043, t value = -2.566, p value =
268 0.03, n = 55), and undifferentiated Fix^{int} bacteria were significantly smaller when co-
269 inoculated with a Fix^+ strain (estimate -1417.6, SE = 405.9, t value = -3.492, p value = 0.00, n
270 = 55; Figure 1B)

271

272 **Conditional sanctioning on whole nodules does not impact bacterial survival after 28 days**

273

274 The number of undifferentiated bacteria within a nodule did not depend on the identity of
275 the co-inoculant for Fix^+ nodules (estimate = -212437, SE = 472483, t = -0.450, p = 0.663, n =
276 60), Fix^- nodules (estimate = 2794843, SE = 2008643, t = 1.391, p = 0.19753, n = 55) or Fix^{int}
277 nodules (Log transformed data, estimate = 0.500, SE = 0.5855, t = 0.855, p = 0.415, n = 55;
278 Figure 1C).

279

280 **Conditional sanctioning causes changes in cell morphology**

281

282 There is a clear change to the morphology of the whole nodule of sanctioned nodules after
283 twenty-eight days (Figure S4), as seen in Westhoek et al., (2017). Nodules from co-
284 inoculated plants of all three combinations (Fix^+ vs Fix^-, Fix^+ vs Fix^{int}, Fix^{int} vs Fix^-) were
285 imaged at 28-, 35- and 42-days post-inoculation and examined for evidence of changes to
286 cell morphology (Figure S5 for all pictures).

287

288 At twenty-eight days post-inoculation, all sections taken from nodules containing the Fix^{int}
289 strain looked similar, regardless of whether the co-inoculated strain was Fix^- (Figure 2A) or
290 Fix^+ (Figure 2B). However, at 35 days post-inoculation, the status of nodules containing the
291 Fix^{int} strain was visibly affected by the identity of the co-inoculated strain, consistent with
292 conditional sanctioning. In particular, if the co-inoculated strain was Fix^+, then the infected
293 cells within Fix^{int} nodules had irregular shapes and the active infected region had retracted

294 away from the nodule edge (Figure 2D). However, if the co-inoculated strain was Fix^-, then
295 most cells retained a round morphology; indeed these nodules were not visibly different
296 from Fix^{int} nodules at 28 days post-inoculation (compare Figures 2A&2B). At 42 days post-
297 inoculation, all nodules containing the Fix^{int} strain showed clear changes to cell morphology;
298 however, when co-inoculated with Fix^+ (Figure 2F), these changes were more pronounced
299 than when co-inoculated with Fix^- (Figure 2E).
300
301
302
303 **Conditional sanctioning is not cell-autonomous in peas**
304
305 In mixed nodules, the proportion of bacteroids that belonged to the less effective strain was
306 not significantly different from 50% (mean = 50.57%, 95%CI = 39.92 & 61.22%, t = 0.11; p
307 >0.05, n = 24) (Figure 3A) and the same was true for the undifferentiated bacteria (mean =
308 59.62%, 95%CI = 49.72 - 69.51%, t = 2.01, p >0.05, n = 24) (Figure 3C); hence, we did not find
309 evidence for cell-autonomous sanctioning (although we note the number of
310 undifferentiated bacteria was not an indicator of sanctioning in our single strain nodule
311 data). There was also no significant difference in the size of the undifferentiated bacteria
312 within mixed nodules (95%CI = -139.18 & 897.55, t = 1.513, p >0.05, n = 24) (Figure 3B); The
313 same pattern emerged when we compared absolute numbers rather than proportions. The
314 number of bacteroids belonging to the effective strain within a mixed nodule was not
315 significantly different from the number belonging to the less effective strain (95%CI = -
316 236167 - 1834023, t = 0.124, p >0.05, n = 24) and the same was true for undifferentiated
317 bacteria (95%CI = -148145 & 192398, t = 0.269, p > 0.05, n = 24). These results therefore
318 show no evidence for cell-autonomous sanctioning.
319
320 **Mixed nodules are moderately sanctioned**
321
322 When we consider the total population within mixed nodules, we see that the total number
323 of bacteroids and the average size of undifferentiated bacteria within mixed nodules
324 occupies an intermediate value between nodules occupied by the two single strains (Figure
325 4A&B). This would suggest that mixed nodules are treated as less effective nodules and

326 sanctioned accordingly. This may imply that mixed nodules are treated as a third strain
327 which is supplying nitrogen at an intermediate value between the two other strains present.
328 We did not see a clear pattern in the data for the number of undifferentiated bacteria from
329 the same set of nodules (Figure 4C). However, given that we did not see evidence for
330 sanctioning within this data set for the single occupant nodules this result neither supports
331 nor refutes our conclusions.
332
333 **Changes to cell morphology are not cell-autonomous in pea**
334
335 Within mixed nodules there was no evidence of any differences in cell morphology between
336 the more and less effective strain. If the nodule was unsanctioned (Figure 5A) then all of the
337 cells within the nodule were healthy, regardless of the strain occupying the cell. In contrast,
338 if a nodule was sanctioned (Figure 5B) then – as for a single occupant nodule (Figure 2D) –
339 infected cells within the nodule lost their typical spherical morphology and some burst
340 open. However, this was equally likely to affect cells containing the more or the less
341 effective strain. Therefore, we have seen no evidence to support the cell-autonomous
342 application of sanctions. This was true regardless of the strain combination within the
343 nodule or the time point at which they were harvested (Figure S6)
344
345
346 ## Discussion
347
348 We show that after twenty-eight days the size of undifferentiated bacteria (Figure 1B) and
349 the number of bacteroids (Figure 1A) but not the number of undifferentiated bacteria
350 (Figure 1C) within a sanctioned nodule have significantly decreased. That peas sanction
351 nodules containing less effectively fixing strains after twenty-eight days is consistent with
352 previous studies (Kiers et al., 2003; Oono et al., 2011; West et al., 2002; Westhoek et al.,
353 2017). Our undifferentiated bacteria data was consistent with the findings of Westhoek et
354 al., 2021, the number of undifferentiated bacteria did not drop significantly in sanctioned
355 nodules after twenty-eight days, post-inoculation. Our data is also consistent with
356 conditional sanctioning, as the fate of nodules infected by a Fix^{int} strain depends on whether
357 the co-inoculated strain is Fix^{+} or Fix^{-} (Westhoek et al., 2021). As such we propose that the
358 sanctioned nodule phenotype, particularly their reduced size, is driven by this significant
359 drop in bacteroid population.

Within sanctioned nodules there was also a reduction in the size of the undifferentiated bacteria. This would suggest that the cessation of the carbon supply to the nodule has resulted in the starvation of these bacteria. As such, while their numbers remain high, they are much smaller (Figure 1B), pre-dating the collapse in their population at a later time point. As with the bacteroid data, the intermediate-fixing bacteria significantly differed in size depending on the identity of the co-inoculating strain. Although we also found that the size of undifferentiated Fix^-bacteria in single-strain nodules changed with the identity of the co-inoculant, the estimated size difference was smaller, and close to the significance threshold, so could be spurious.

Not only did the number of undifferentiated bacteria within sanctioned nodules not decrease but the number of undifferentiated bacteria in our Fix^- nodules appeared to be higher than in the unsanctioned nodules (Figure 1C). This result is particularly noteworthy as only the undifferentiated bacteria are capable of replication once released into the soil. As such the ability of sanctions to impact the fitness of a less effective strain is reliant on their ability to reduce the number of undifferentiated bacteria. However, we know from previous work that the population of undifferentiated bacteria within less effective nodules collapses at a later time point. Westhoek et al., (2021) showed that while the population is the same or slightly higher in sanctioned nodules after twenty-eight days it has reduced dramatically by fifty-six. Crucially therefore, the population has been reduced by the time of nodule release and the fitness of the sanctioned strain has been reduced.

Our results also cast some doubt over the use of non-fixing strains in studies investigating sanctioning. Given that the process of conditionally sanctioning an intermediate fixer must be an active process, unlike in the case of a non-fixing strain where the plant can simply respond to the presence or absence of fixed nitrogen, as they must compare nitrogen outputs. The use of non-fixing strains, which may be sanctioned passively through an alternate system, in studies examining sanctions does not necessarily allow for the investigation of the more nuanced elements of the mechanisms behind sanctioning. By comparing our Fix^- and Fix^{int} data in figure 1 we see that non fixing and less effective strains

392 are treated differently. Particularly, the number of the undifferentiated bacteria which is
393 much higher in Fix^{-} nodules then in a sanctioned Fix^{int} nodule. This result adds weight to the
394 argument that future studies of sanctioning should be focussed on using strains of varying
395 fixation effectiveness and should not utilise non-fixing strains.
396
397 We also show that there is a change in the morphology of the ultra-structure within
398 sanctioned nodules. Previous studies (Regus et al., 2017) have made the link between these
399 morphological changes and the process of nodule senescence, which seems reasonable to
400 us. By tracking this change as the nodule develops, we have now established an
401 understanding of the sequence of events that take place throughout sanctioning. The effect
402 of sanctions on whole nodule morphology, nodules are smaller and spherical, is discernible
403 by 16 days (Westhoek et al., 2017). This initial nodule change is present at the same time as
404 a drop in the number of bacteroids and the size of undifferentiated bacteria after 28 days
405 (Figure 1). However, there is no visible change in the nodule ultra-structure of sanctioned
406 nodules before 35 days (Figure 2). All of these changes appear to precipitate the collapse in
407 undifferentiated bacteria population which is observed by 56 days.
408
409 It has been proposed that cell-autonomous sanctions might occur within mixed nodules
410 (Daubech et al., 2017; Regus et al., 2017), allowing plants to combat less effective
411 "cheating" strains that enter nodules alongside a more effective strain. If a plant can
412 differentiate among strains in a mixed nodule and target sanctions accordingly, then for two
413 equally competitive strains, we expect the average proportion of the more effective strain
414 to be greater than 50%. Therefore, evidence for cell autonomous sanctions would include a
415 bias in the proportion of the effective strain within mixed nodules. However, in our study,
416 the ratio of effective to less effective bacteroids within mixed nodules did not significantly
417 differ from 50% (Figure 3A). As shown in this study (Figure 1B) plant sanctions also reduce
418 the average size of individual undifferentiated bacteria within single-strain nodules;
419 therefore we would expect to see the same size reduction within the mixed nodule, if cell-
420 autonomous sanctions occur. No such difference was found (Figure 3B).
421
422 We also found no difference in the numbers of undifferentiated bacteria within mixed
423 nodules belonging to the two strains. However, as sanctions did not change the numbers of

424 undifferentiated bacteria in single-strain nodules after twenty-eight days, this seems to be
425 of limited relevance to the presence, or lack of, cell autonomous sanctions. As such we
426 found no evidence within our flow cytometry data to support the presence of cell-
427 autonomous sanctions in pea plants.
428
429 By examining the treatment of mixed nodules at the whole nodule level (Figure 4) we have
430 shown that mixed nodules are sanctioned on the whole nodule level. This sanctioning is less
431 severe than for single strain nodules containing the less effective strain on the plant. As
432 seen in Figure 4, therefore, the mixed nodules are moderately sanctioned with their
433 bacteroid number and bacteria sizes fitting between the values for the single occupant
434 sanctioned and unsanctioned nodules. As such we have shown that while mixed nodules
435 are not sanctioned in a cell-autonomous fashion they are sanctioned at the whole nodule
436 level.
437
438 Cells within mixed nodules underwent senescence simultaneously, regardless of variation in
439 effectiveness (Figure 5), consistent with the timing of cell senescence not occurring in a cell-
440 autonomous manner. Although the timing of senescence varied among mixed nodules,
441 where senescence occurred, it affected both strains equally. It may therefore be
442 hypothesised that the timing of senescence within mixed nodules is determined by the ratio
443 of effective to less effective strains within the nodule. This would be challenging to test,
444 because sectioning nodules does not give an accurate measure of strain ratio due to the
445 irregular 3D distribution of strains within nodules.

446 Our results align with the findings of Agtuca et al., (2020) who found that the metabolic
447 profile of sectors of mixed nodules containing different strains did not show significant
448 differences. However, our results contrast with studies which have found evidence for cell
449 autonomous sanctions based on variation in the timing of senescence, such as Regus et al.,
450 (2017) and Daubech et al., (2017). The differences may be explained by variation in the
451 legume-rhizobium system used: Daubech et al. (2017) used *Cupriavidus* taiwanensis –
452 *Mimosa pudica*; the (Regus et al., 2017) study used both *Acmispon strigosus* –
453 *Bradyrhizobium* and *Lotus japonicus* – *Mesorhizobium*; and the Agtuca et al. (2020) study

454 used *Glycine max – Bradyrhizobium japonicum.* A comprehensive understanding of
455 sanctions will therefore require the continued use of multiple experimental systems.

456 We have shown that the sanctioning of less effective nodules within the pea-rhizobium
457 symbioses is conditional in nature and occurs at the nodule level. In Fix^{+}/Fix^{int} coinfected
458 plants, nodules containing Fix^{int} but not Fix^{+} strains are sanctioned, suggesting conditional
459 sanctioning requires comparison between nodule-specific and global nitrogen signals. This
460 might be achieved through the interaction between a nitrate receptor such as the Nitrate
461 transreceptor NRT1.1, which both transports nitrate as well as detecting nitrate levels
462 (Zhang et al., 2019) and the NIN-like Proteins (NLPs) 1 and 4, which are essential for the
463 nitrate-based regulation of nodule maturation (Lin et al., 2021). In high nitrate conditions
464 NLP1/4 inhibit cytokinin biosynthesis. Cytokinin biosynthesis drives nodule maturation
465 through the activation of a signal cascade through the Cytokinin responsive element CRE1
466 (Lin et al., 2021), which acts to promote expression of the *cep* and *cle* genes (Laffont et al.,
467 2020). However, how a global signalling mechanism is able to detect the nitrogen output of
468 individual nodules and compare it to global nitrogen status remains unclear. The
469 mechanism of sanctions on individual nodules may be achieved through nodule-specific
470 proteins responding to nitrate levels. One possibility is SnRK1 which, when phosphorylated
471 by the DMI2 kinase in response to Nod factor, phosphorylates malate dehydrogenase 1 and
472 2, leading to increased malate production within the nodule to supply bacteroids (Guo et al.,
473 2023). This is therefore a prime candidate for a pathway in which the legume would be able
474 to reward effective strain-containing nodules, as well as to punish nodules containing less
475 effectively fixing strains by cessation of malate production and supply. However, these
476 remain speculations about signalling pathways whose elucidation may aid future efforts to
477 engineer symbioses in the selection and use of more effective nitrogen-fixing bacteria.
478

479 We now have clear evidence that the change in nodule morphology shown by Westheok et
480 al., (2017) is driven by a reduction in the number of bacteroids and the size of the
481 undifferentiated bacteria. However, the number of undifferentiated bacteria does not drop
482 until a later timepoint. These changes precipitate the changes to the morphological
483 structure of infected plant cells within sanctioned nodules. We have also shown that, while
484 the plant is unable to discriminate between effective and less effective 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, because the plant views mixed nodules as less effective, and sanctions them accordingly.

References

Agtuca, B. J., Stopka, S. A., Evans, S., Samarah, L., Liu, Y., Xu, D., Stacey, M. G., Koppenaal, D. W., Paša-Tolić, L., Anderton, C. R., Vertes, A., & Stacey, G. (2020). Metabolomic profiling of wild-type and mutant soybean root nodules using laser-ablation electrospray ionization mass spectrometry reveals altered metabolism. *Plant Journal*, *103*(5), 1937–1958. https://doi.org/10.1111/tpj.14815

Beringer, J. E. (1974). R Factor Transfer in Rhizobium leguminosarum. *Journal of General Microbiology*, *84*(1g74), 188–198.

Beringer, J. E., Brewin, N., Johnston, A. W., Schulmanf, H. M., & Hopwood, D. A. (1979). The Rhizobium-legume symbiosis. *Proc. R' Soc.Lond' B*, *204*, 219–233. https://royalsocietypublishing.org/

Daubech, B., Remigi, P., Doin de Moura, G., Marchetti, M., cile Pouzet, C., Auriac, M.-C., Gokhale, C. S., Masson-Boivin, C., & Capela, D. (2017). Spatio-temporal control of mutualism in legumes helps spread symbiotic nitrogen fixation. *ELIFE*, 1–21. https://doi.org/10.7554/eLife.28683.001

Guo, D., Liu, P., Liu, Q., Zheng, L., Liu, S., Shen, C., Liu, L., Fan, S., Li, N., Dong, J., & Wang, T. (2023). Legume-specific SnRK1 promotes malate supply to bacteroids for symbiotic nitrogen fixation. *Molecular Plant*, *16*(9), 1396–1412. https://doi.org/10.1016/j.molp.2023.08.009

Johnston, A. W. B., & Beringer, J. E. (1975). Identification of the Rhizobium Strains in Pea Root Nodules Using Genetic Markers. *Journal of General Microbiology*, *87*, 343–350.

Kiers, E. T., Rousseau, R. A., West, S. A., & Denlson, R. F. (2003). Host sanctions and the legume-rhizobium mutualism. *Nature*, *425*(6953), 78–81. https://doi.org/10.1038/nature01931

Laffont, C., Ivanovici, A., Gautrat, P., Brault, M., Djordjevic, M. A., & Frugier, F. (2020). The NIN transcription factor coordinates CEP and CLE signaling peptides that regulate nodulation antagonistically. *Nature Communications*, *11*(1). https://doi.org/10.1038/s41467-020-16968-1

Lee, D. K. (2020). Data transformation: A focus on the interpretation. *Korean Journal of Anesthesiology*, *73*(6), 503–508. https://doi.org/10.4097/kja.20137

Lin, J., Roswanjaya, Y. P., Kohlen, W., Stougaard, J., & Reid, D. (2021). Nitrate restricts nodule organogenesis through inhibition of cytokinin biosynthesis in Lotus japonicus. *Nature Communications*, *12*(1). https://doi.org/10.1038/s41467-021-26820-9

Mendoza-Suárez, M., Andersen, S. U., Poole, P. S., & Sánchez-Cañizares, C. (2021). Competition, Nodule Occupancy, and Persistence of Inoculant Strains: Key Factors in the Rhizobium-Legume Symbioses. In *Frontiers in Plant Science* (Vol. 12). Frontiers Media S.A. https://doi.org/10.3389/fpls.2021.690567

Mendoza-Suárez, M., Barney Geddes, B., Sánchez-Cañizares, C., Ramírez-González, R., Kirchelle, C., Jorrin, B., & Poole, P. (2020). Optimizing Rhizobium-legume symbioses by

530 simultaneous measurement of rhizobial competitiveness and N2 fixation in nodules.
531 *PNAS*, *117*(18).
532 Mergaert, P., Uchiumi, T., Alunni, B., Naë Lle Evanno, G., Lique Cheron, A., Catrice, O.,
533 Mausset, A.-E., Dé Rique Barloy-Hubler, F., Galibert, F., Kondorosi, A., & Kondorosi, E.
534 (2006). *Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium-*
535 *legume symbiosis*. https://www.pnas.org
536 Oono, R., Anderson, C. G., & Denison, R. F. (2011). Failure to fix nitrogen by non-
537 reproductive symbiotic rhizobia triggers host sanctions that reduce fitness of their
538 reproductive clonemates. *Proceedings of the Royal Society B: Biological Sciences*,
539 *278*(1718), 2698–2703. https://doi.org/10.1098/rspb.2010.2193
540 Poole, P., Ramachandran, V., & Terpolilli, J. (2018). Rhizobia: From saprophytes to
541 endosymbionts. *Nature Reviews Microbiology*, *16*(5), 291–303.
542 https://doi.org/10.1038/nrmicro.2017.171
543 Regus, J. U., Gano, K. A., Hollowell, A. C., & Sachs, J. L. (2014). Efficiency of partner choice
544 and sanctions in Lotus is not altered by nitrogen fertilization. *Proceedings of the Royal*
545 *Society B: Biological Sciences*, *281*(1781). https://doi.org/10.1098/rspb.2013.2587
546 Regus, J. U., Quides, K. W., O'Neill, M. R., Suzuki, R., Savory, E. A., Chang, J. H., & Sachs, J. L.
547 (2017). Cell autonomous sanctions in legumes target ineffective rhizobia in nodules
548 with mixed infections. *American Journal of Botany*, *104*(9), 1299–1312.
549 https://doi.org/10.3732/ajb.1700165
550 Timmers, A. C. J., Soupène, E., Auriac, M.-C., De Billy, F., Vasse, J., Boistard, P., & Truchet, G.
551 (2000). Publication no. M-2000-0914-03R.. 1996; Long 1996) and takes place through a
552 series of developmental stages. In *Schultze and Kondorosi* (Vol. 13, Issue 11).
553 Udvardi, M., & Poole, P. S. (2013). Transport and metabolism in legume-rhizobia symbioses.
554 In *Annual Review of Plant Biology* (Vol. 64, pp. 781–805).
555 https://doi.org/10.1146/annurev-arplant-050312-120235
556 West, S. A., Kiers, E. T., Simms, E. L., & Denison, R. F. (2002). Sanctions and mutualism
557 stability: Why do rhizobia fix nitrogen? *Proceedings of the Royal Society B: Biological*
558 *Sciences*, *269*(1492), 685–694. https://doi.org/10.1098/rspb.2001.1878
559 Westhoek, A., Clark, L. J., Culbert, M., Dalchau, N., Griffiths, M., Jorrin, B., Karunakaran, R.,
560 Ledermann, R., Tkacz, A., Webb, I., James, E. K., Poole, P. S., & Turnbull, L. A. (2021).
561 Conditional sanctioning in a legume-Rhizobium mutualism. *Proceedings of the National*
562 *Academy of Sciences of the United States of America*, *118*(19), 1–8.
563 https://doi.org/10.1073/pnas.2025760118
564 Westhoek, A., Field, E., Rehling, F., Mulley, G., Webb, I., Poole, P. S., & Turnbull, L. A. (2017).
565 Policing the legume-Rhizobium symbiosis: A critical test of partner choice. *Scientific*
566 *Reports*, *7*(1). https://doi.org/10.1038/s41598-017-01634-2
567 Zhang, X., Cui, Y., Yu, M., Su, B., Gong, W., Baluška, F., Komis, G., Šamaj, J., Shan, X., & Lin, J.
568 (2019). Phosphorylation-mediated dynamics of nitrate transceptor NRT1.1 regulate
569 auxin flux and nitrate signaling in lateral root growth. *Plant Physiology*, *181*(2), 480–
570 498. https://doi.org/10.1104/pp.19.00346
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576

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

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

Supporting information

Fig. S1 Image of flow cytometry output showing the two populations differentiated by size based on Forward Scatter (FSC)

Table. S2 Flow cytometry data, analysed using custom gating to calculate the number of undifferentiated bacteria and bacteroids in single occupancy and mixed nodules, and acetylene reduction assay and nodulation results from fluorescent tag control experiments.

Doc.S3 R markdown file of statistical analysis.

Fig. S4 Representative iBright image of pea inoculated with two strains of differing effectiveness.

Fig. S5 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.

Fig. S6 Representative images of the three types of mixed nodules at 28, 35 and 42 days post inoculation

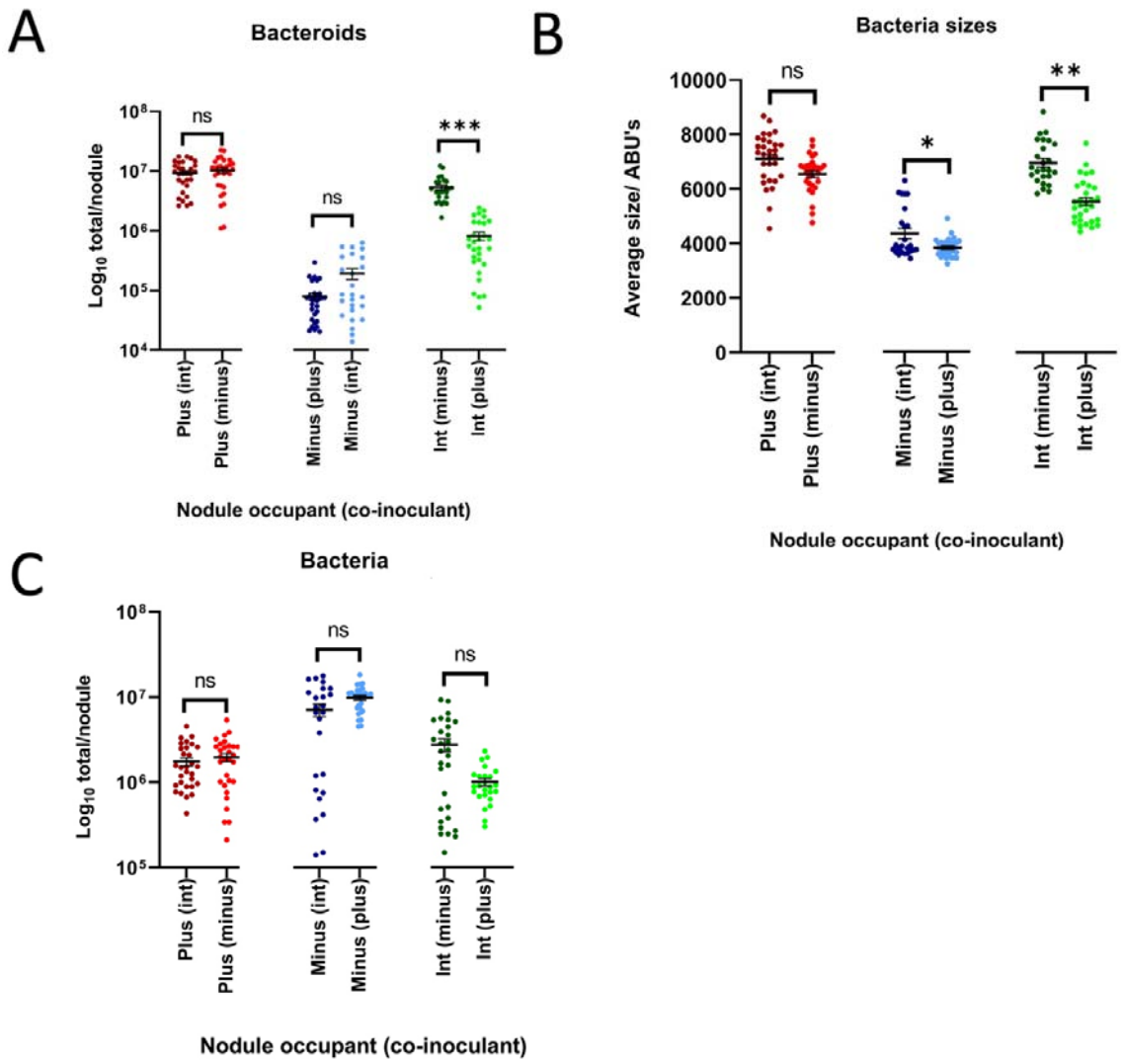
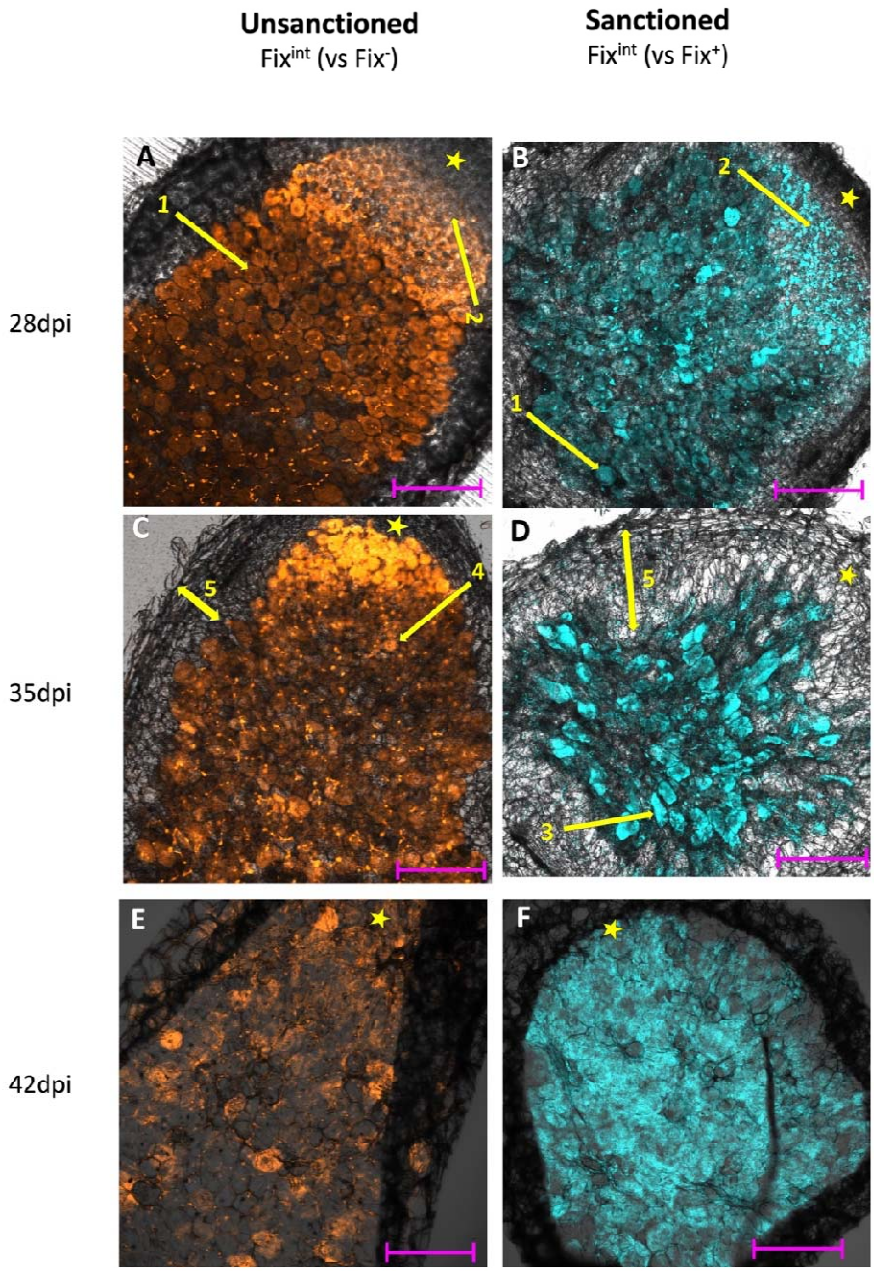


Fig 1. After 28 days sanctions reduce the number of bacteroids and bacterial size, nut not the number of bacteria.

The number of bacteroids (A) and the size of bacteria (B) in nodules from pea plants co-inoculated with two strains of rhizobia. Data is split into different nodule types (Fix^{+}, Fix^{int} or Fix^{-}). The identity of the co-inoculant is given in brackets. Black lines are the mean value with error bars showing the standard error of the mean. Significance level from paired t-test *** < 0.001; ** < 0.01; * <0 .05; ns, not significant. Nodules harvested at 28 days post-inoculation.


Unsanctioned
Fix^int (vs Fix^-)
Sanctioned
Fix^int (vs Fix^+)
28dpi
35dpi
42dpi
A
B
C
D
E
F
1
2
3
4
5

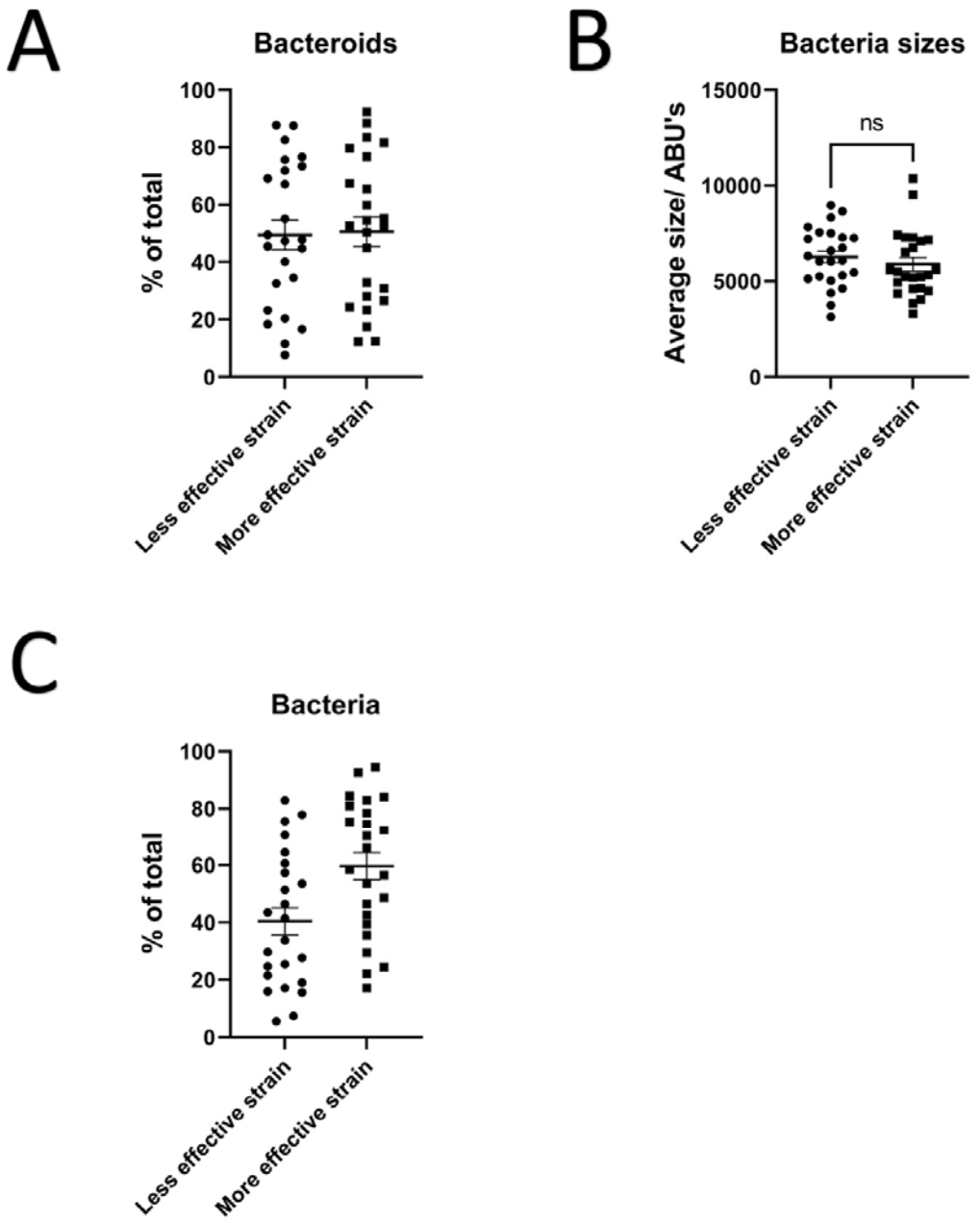


Fig. 3. The two strains within a mixed nodule are not sanctioned independently of one another

The proportion of the bacteroid (A), average size of the bacteria (B) and the proportion of bacteria (C) within a mixed nodule belonging to the more or less effective of the two strains.

Nodules harvested twenty-eight days post inoculation.

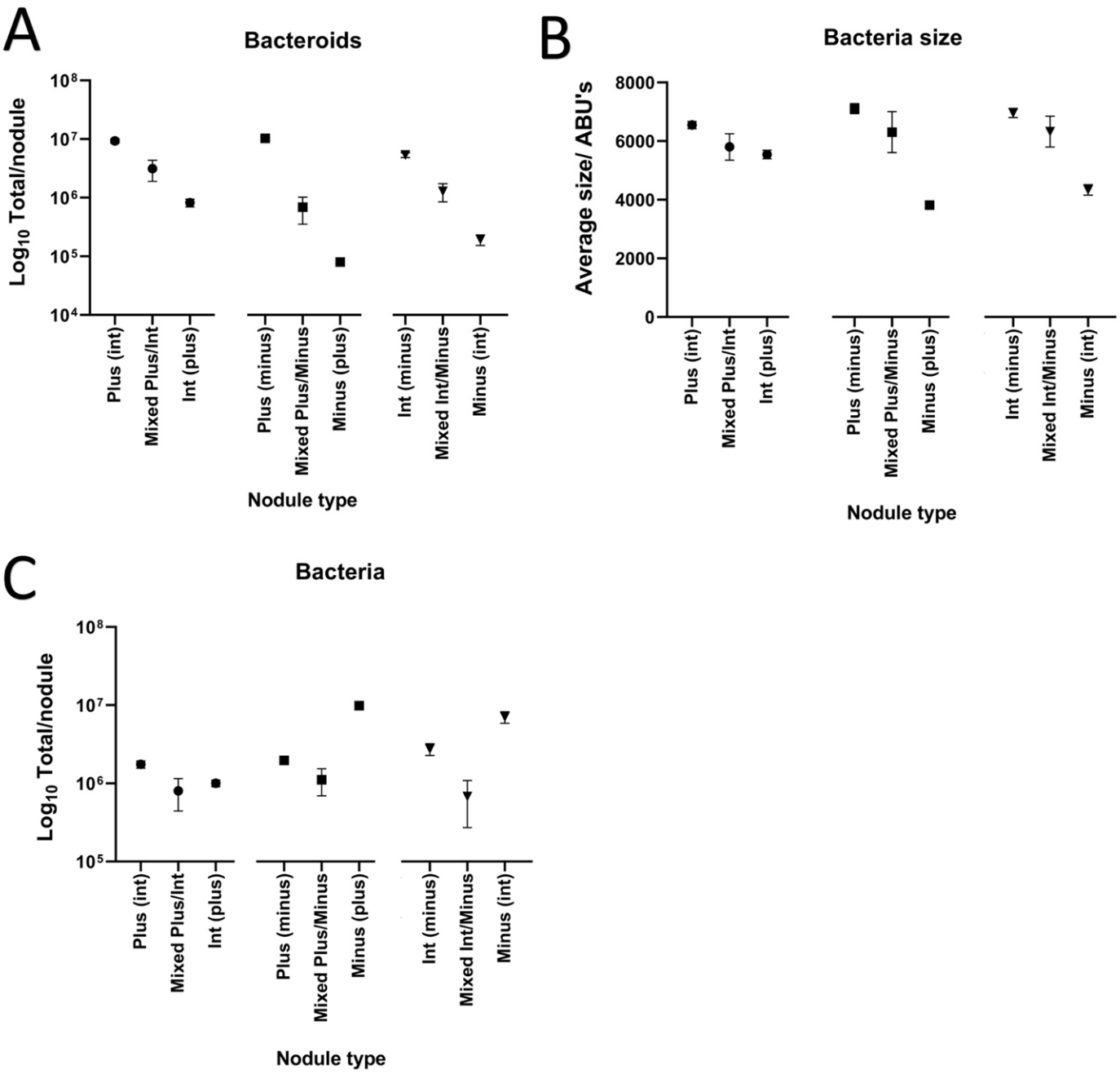


Fig. 4. Mixed nodules are sanctioned, but less severely than single strain nodules containing the less effective strain.

The number of bacteroids (A) and the average size of the undifferentiated bacteria (B) and the number of undifferentiated bacteria (C) within pea nodules. Data is split by co-inoculant combination (Fix^+ vs Fix^{int}, Fix^+ vs Fix^- and Fix^{int} vs Fix^-). The mean value for the single strain nodules of each of the two strains in each combination and the mean value for mixed nodules containing the two strains is given with error bars showing the standard error of the mean. Nodules were harvested twenty-eight days post inoculation.

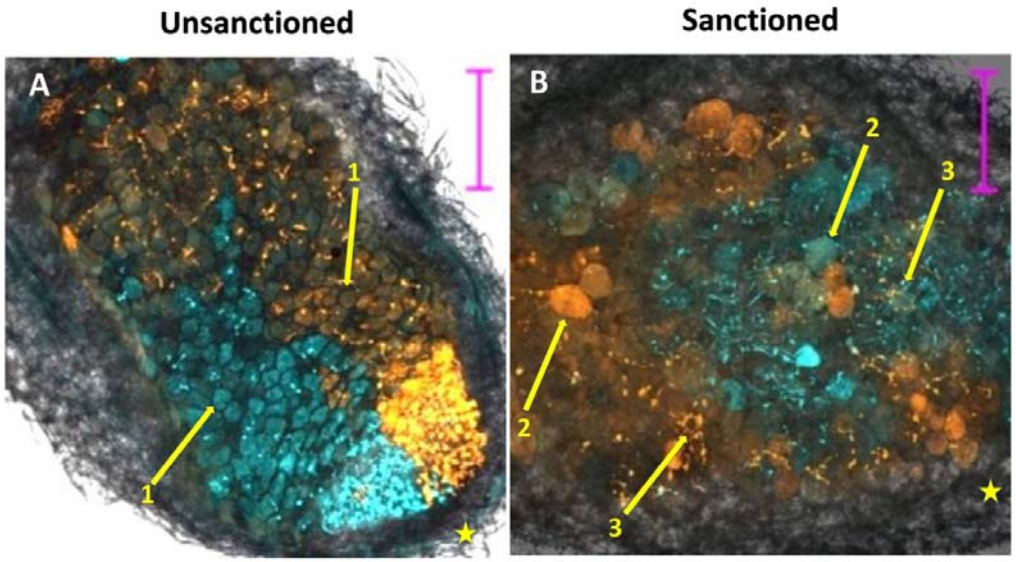


Fig. 5. The two strains within a mixed nodule do not senesce at different times despite varying in relative effectiveness.

Thirty-five day post-inoculation unsanctioned (A) and sanctioned (B) mixed nodules containing two strains of differing fixation effectiveness. When unsanctioned (A), all cells of both strains remain spherical and intact (1). When sanctioned (B), some cells of both strains remain intact (2) but they are no longer spherical and other cells of both strains have burst open (3).
Longitudinal nodule slices (100µm) were imaged by confocal microscopy. Scale bar = 200µm.