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Roger Arditi
Editor-in-Chief
Acta Oecologica

Dear Dr Arditi,

I am resubmitting the revised Ms. Ref. No.: ACTOEC-D-09-00082
Title: A mechanistic molecular test of the plant-sanction hypothesis in legume-rhizobia mutualism. Diana Marco et al.

We have deeply appreciated the comments from the reviewers, and tried to follow them as close as possible. To do this, we made several changes in the text. All the changes are detailed in the Responses to Reviewers.

We think that after the revision the paper has been greatly improved and we are very obliged to reviewers.

Yours sincerely,

Diana Marco

* Manuscript

A mechanistic molecular test of the plant-sanction hypothesis in legume-rhizobia mutualism

Running Title: Mechanistic test of plant-sanction hypothesis

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Abstract

The origin and persistence of mutualism is difficult to explain because of the widespread occurrence of exploitative, 'cheating' partners. As a policing strategy stabilising intraspecific cooperation, host sanctions against non N_2 fixing, cheating symbionts have been proposed to stabilise mutualism in legume-rhizobium symbiosis. Mechanism of penalisations would include decreased nodular rhizobial viability and/or early nodule senescence. We tested these potential mechanisms of penalisations in split-root experiments using two soybean varieties and two rhizobial strains, a cooperative, normal N_2 fixing strain and an isogenic non-fixing derivative. We found no differences in the number of viable rhizobia recovered from nodules and no differential expression of a nodular senescence molecular marker. Thus, our results do not support the hypothesis of plant sanctions acting against cheating rhizobia in our experimental conditions.

Keywords: legume-rhizobia mutualim; plant-host sanctions; mechanistic molecular test

Introduction

The existence of defective, cheating partners in mutualistic associations (Bronstein 2001) has raised theoretical interest for long, since it directly challenges the evolutionary stability of mutualisms (Axelrod and Hamilton 1981). The main question is, how can cooperation be maintained if partners seek only self-benefit? Different mechanisms have been proposed that could protect mutualisms against cheating (Bull and Rice 1991, Sachs et al. 2004), however, cheating and exploitative strategies appear to be ubiquitously extended in nature (Machado et al. 1996, Pellmyr et al. 1996), including legume-rhizobia mutualism (Bronstein 2001). In this interaction, bacteria (commonly known as rhizobia) from the soil infect the plant´s meristem cells of the root through a fine tuned signalling mechanism between both partners and a new organ is formed, the nodule, where the bacteria reproduce and differentiate into bacteroids able to fix atmospheric N_2 for plant utilization. In exchange, rhizobia inside nodules receive carbon fixed by the plant as carbohydrated compounds. After nodule senescence, surviving bacteroids or undifferentiated bacteria are released into the soil as free-living rhizobia, where they may compete with resident rhizobia populations (Hirsch 1996). Apparently, the benefits that should be obtained by the two partners, the plant host and the microsymbiont, are clear. However, the occurrence of low N_2-fixing or even ineffective rhizobia cheating strains has been recognized for long in agricultural practices (Amarger 1981, Singleton and Tavares 1986).

Plant host sanctions have been proposed as a stabilizing force (Frank 1998) defending mutualism from cheating rhizobia (Denison 2000, West et al. 2002, Kiers et al. 2003, Simms et al. 2006). The plant would penalize cheating rhizobia by reducing their survival and fitness and/or accelerating nodule senescence (Denison 2000, West et al. 2002). A decrease in viability of rhizobia recovered from nodules was reported when N_2-fixing rhizobia were ‘forced’ to cheat soybean plants by replacing normal, N_2 containing atmosphere by a gas mixture ($Ar:O_2$) (Kiers et all. 2003, 2006). Here, We

tested the two proposed mechanisms for potential sanctions, that the plant would reduce viability of non-fixing rhizobia inside nodules, performing viable rhizobia counts from nodules, and that the plant would cause early senescence of nodules occupied by the non-fixing strain, by measuring the relative expression of gene markers for nodule senescence and maturity (Alessandrini et al. 2003), in split-root soybean plants of Williams and Osumi cultivars. Split-roots were respectively inoculated with two strains of *Bradyrhizobium japonicum*, a highly efficient nitrogen fixing wild-type strain USDA110, and its non-fixing, nifH mutant derivative H1 (Hahn et al. 1984) at different times after root inoculation. H1 lacks nitrogenase activity but shows similar infection and nodule formation levels respect to the wild-type (Hahn et al. 1984, Hahn and Studer 1986). This experimental approach allowed us to test the potential mechanisms suggested for plant host sanction using non-fixing and fixing rhizobia sharing the same plant.

Methods

Plant split-root experimental setting. Seeds of soybean (*Glycine max*) cultivars Williams and Osumi were surface sterilized and germinated. Tip root was removed to generate regrowth of two equally sized half-roots, each placed in a glass tube containing sterilized N_2 free liquid Fahraeus nutrient solution (Vincent 1970). Each tube was inoculated and sealed to prevent cross-contamination, with the appropriate strain of *Bradyrhizobium japonicum*, either the wild type, normally N2 fixing USDA 110 ($5x10^5$ cells/ml) or the Nod+ Fix-, nifH:: Tn5 mutant H1 derived from the wild type (Hahn et al. 1984) ($5x10^5$ cells/ml) in the following treatments: half roots of the same plant (USDA110-1/H1-1), or in both roots of the same plant (USDA110-2 or H1-3) (Fig. S1). We checked that the H1 rhizobial strain showed similar infection and nodule formation levels and temporal patterns respect to the wild-type (Fig. S2). Each tube was carefully filled with nutrient solution as needed, while maintaining the other tube sealed. Plants

were placed in a growth chamber with 16 h and 600 μEm-2 s-1 photosynthetically active radiation at 25 °C, and 8 h darkness at 18 °C. Control uninoculated plants showed no nodulation. Nodule numbers were counted in each half root every three days until nodule production reached a plateau (Fig. S2). Total number of nodules produced per half-root (inoculated with either USDA 110 or H1) was about 40 and 30 for Williams and Osumi cultivars respectively. Three, four and five weeks after inoculation nodules of each half root of five plants/treatment were collected. Two well developed nodules of same size per half root were independently weighted and used immediately for rhizobia viable counts (weeks 3 and 5). Groups of the remaining nodules were weighted and immediately stored at -80 °C for further determination of nodule gene marker expression.

Viable rhizobial counts. From 5 (occasionally 3) plants for each treatment (USDA110-1/H1-1, USDA110-2 and H1-3) in each date (3 and 5 weeks after inoculation), we collected two nodules of similar size and root location from each half-root. Nodules were individually surface sterilized using Cl2Hg (2.5%), manually crushed, homogeneized and resuspended in a buffer containing 0.05M Tris-HCL and 0.25 manitol. Appropriate serial dilutions were plated (two replicates per dilution) in yeast extract-mannitol (YEM, Vincent 1970) supplemented with selective antibiotics depending on the strain (Spc for USDA 110 and Spc + Kan for H1). Plates were incubated at 28 °C for a week or until no further growth was detected, and colony-forming units (c.f.u.) were counted. As nodules produced by USDA 110 inoculated roots were slightly heavier than those produced by H1 (5.67 ± 1.62, 5.02 ± 1.02 respectively for Williams cultivar, and 5.37 ± 0.82 and 4.73 ± 0.904 respectively for Osumi cultivar, n = 6 for each cultivar), and since soybean plants may compensate against ineffective nodulations by increasing effective nodule mass (Singleton and

Stockinger 1983), c.f.u. numbers from individual nodules were compared using per nodule mass with paired t-test analysis on original, untransformed data (n: number of nodules compared in each date for each treatment was between 10 to 6 depending on plant number). We checked statistical assumptions for using the *t* test, and they were fairly met in most cases. In a few cases where there was a small departure from normal distribution assumptions we performed non parametric tests (Mann-Whitney *U*-test), and we found that results were the same as using the *t*-test.

Nodule gene expression. cDNA markers differentially expressed in mature (DD10) and senescent (DD15) soybean nodules (Alessandrini et al 2003) were used to assess the developmental stage of nodules and to detect any early senescence in the different treatments. DD10 expression increases with nodule development reaching a peak with nodule maturity and then decreases slowly with nodule age, while DD15 expresses only in senescent nodules (SI2). Total RNA was extracted using the RNeasy Kit, Qiagen. To check for RNA quality, we performed an ethidium bromide stained denaturing formaldehyde gel electrophoresis. To avoid DNA contamination, RNA extraction was performed using DNAse I (Quiagen). RNA was extracted from two nodule groups from each half-root of two plants of each treatment for weeks 3, 4 and 5, previously weighted and frozen (individual nodules did not yield enough RNA). Expression of the nodule markers of senescence DD15 and maturity DD1022 was assessed using quantitative real-time PCR (RT-qPCR), with the soybean 18S ribosomal subunit as internal control, using three dilutions. Appropriate controls, including a DNA contamination control reaction (one without RT mix), were performed. 20-mer primers were designed with a G/C content of 50-60 %, and a Tm of about 60 °C. Length of PCR products ranged between 152-180 bp. Primer design software (Primer3) was used to select primer

sequences. Secondary structures and dimer formation were checked (Oligo Analyzer 3.0 software). Designed DD15 primers 5′- TGGTTTTCTCCTCCTGCTGATT-3′ and 5-GGCAGCATACTCACTTTCACTT-3′, DD10 primers 5′-AGAAGAAGCTGGTGGTATTGGT-3′and 5′-GGAGTTGCTGAGATTGGATTGA-3′, and 18S primers 5′-TACAACGCGCAAAACCTTACCA-3′and 5′-GTTTCGCTCGTTATAGGACTTG-3′ were purchased from Roche. RT-qPCR was performed with a iCycler iQ real-time PCR detection system from Bio-Rad. Primer efficiencies were between 85 an 100%. RT-qPCR was performed with a iCycler iQ real-time PCR detection system from Bio-Rad, using Reverse Transcriptase SuperScript II and Platinum Taq DNA polymerase (Invitrogen). The cycling program was 1 cycle: 5 min at 94 °C, 30 cycles: 1 min at 94 °C, 1 min at 60 °C and 30 s at 72 °C, and 1 cycle: 10 min at 72 °C. Transcript expression levels of DD15 and DD10 were related to the expression levels of the soybean 18S gene that served as an internal standard. We therefore expressed the standardized transcript expression ct levels as DD15/18S and DD10/18S ratios. ct ratio values were compared using paired t-test analysis (n= 12).

Results

Viability of the non-fixing strain was not significantly lower comparing half roots of the same plant separately inoculated with each strain for the two soybean varieties (Fig. 1 and Table S1). Comparing treatments where both half roots of each plant were inoculated with the same strain, non-fixing rhizobia viability was significantly lower, except for Osumi at 3 weeks after inoculation (Fig. 1). In addition, we found no evidence of early nodule senescence in nodules occupied by non-fixing rhizobia when compared with half roots inoculated with the N_2-fixing strain in the same plant (Fig. 2

and Table S2). Plants with both roots inoculated with the non-fixing strain showed decreased expression of the senescence marker compared with plants inoculated only with the N_2-fixing strain (Fig. 2). This correlates with the expression of the molecular marker for nodule maturity, showing increased expression in plants with both half roots inoculated with the non-fixing strain (Fig. 3 and Table S3).

Discussion

Results from the rhizobial viability experiments show that nodules occupied by non-fixing rhizobia do not differ in bacteroid viability and nodule senescence, at least when the plant can get some amount of fixed N_2 from the effectively mutualistic rhizobia occupying some nodules, in this case half of total plant nodules. As expected, plants with all nodules occupied by non-fixing rhizobia are not able of maintaining good vegetative conditions and high rhizobia populations as plants partially or exclusively associated with fixing rhizobia (Fig. S3a, b), and ultimately they die due to N starvation about 6 weeks after inoculation (Fig. S3c). The finding of no greater senescence in nodules occupied by non-fixing rhizobia in plants associated with both strains is in agreement with the rhizobial viability. Besides, higher nodule maturation and lower senescence in the extreme case of entirely cheated plants may suggest that non-fixing rhizobia are exerting some control over the plant to accelerate nodule development and counteract nodule senescence to get ready early viable populations in face of premature host death by starvation, acting in a true parasitic way (Law et al. 2001). It is known that some rhizobia can overcome the plant controlled nodule initiation (Ma et al. 2002). However, to our knowledge this is the first work providing evidence on a possible control of nodule maturation and senescence by normally nodulating but non-fixing

rhizobial strains. This proposed control and possible mechanisms behind it deserve to be further tested.

The two main assumptions behind the sanction hypothesis in mutualisms, that it is costly for the host to be associated with the exploiter, and that mutualism would break unless cheaters are punished, seem not to hold for the majority of mutualistic associations known (Bronstein 2001). Moreover, for the rhizobia-legume mutualism, costs of being cheated may not be as high as assumed if the host is still able of obtaining benefits from other mutualistic partners, for example in coinfected plants which is a common situation in field (Dowling and Broughton 1986, Singleton and Tavares 1986). More conclusive evidence supporting the host plant sanction hypothesis is needed from experiments designed to allow fixing and non-fixing rhizobia coexistence in the same plant. In a recent experiment, Kiers et al. (2007) found not significant differences among cultivars inoculated with rhizobia strains of different grade of effectiveness in N_2-fixation in the ratio of effective: ineffective rhizobia released from their nodules. In another experimental work involving several genetic lines of Medicago truncatula and different rhizobia strains, Heath and Tiffin (2009) did not find evidence for plant host sanctions towards less efficient rhizobia strains.

Although our experiment aimed to test the proposed mechanisms of plant sanctions and more tests would be necessary to be conclusive in an evolutionary context, our results point in the direction that cheating does not necessarily menace rhizobia-legume mutualism. There is increasing empirical evidence that punishment is not always applied to defective mutualistic partners (Ferriere et al. 2002). For example, in a palm-pollinator mutualistic association, female plants inhibit the development of a weevil pollinator eggs and larvae, benefiting from pollination services but not

reciprocating, thus cheating their partner (Dufay and Anstett 2004). It was expected that the weevils would suspend pollination visits to female plants. However, no evidence of sanctions against female plants was found, and apparently the mutualism persistence is not compromised. Coexistence of cheaters and true mutualistic partners is also theoretically possible (Ferriere et al. 2002).

Supporting Information is included.

Acknowledgements

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Figure Legends

Fig. 1. Rhizobia viability per nodule mass in the split-root experiments for two soybean plant varieties, A, Williams, B, Osumi.

Rhizobia inside nodules infected by the N2-fixing USDA110 strain or the non-fixing strain H1, either in half roots of the same plant (USDA110-1/H1-1), or in both roots of the same plant (USDA110-2 or H1-3) were counted as colony forming units (c.f.u.) three and five weeks after inoculation. H1-3 value at week 5 for Williams was too low to be shown (675.5 $\pm$ 368.4). *P < 0.05, ** P < 0.01 significant differences by paired t-tests performed on untransformed data. Bars are means $\pm$ 1 s.d.

Fig. 2. Relative expression of the DD15 gene marker of nodule senescence in nodules from two soybean plant varieties, A, Williams, B, Osumi.

*P < 0.05 significant differences by paired t-tests at three, four and five weeks after inoculation. Bars are means $\pm$ 1 s.d. Treatments as in Fig. 1.

Fig. 3. Relative expression of the DD10 gene marker of nodule maturation in nodules from two soybean plant varieties, A, Williams, B, Osumi.

*P < 0.05, ** P < 0.01 significant differences by paired t-tests at three, four and five weeks after inoculation. Bars are means ± 1 s.d.

Supporting Information (Tables S1, S2, S3; Figs. S1, S2, S3)

Table S1. t-values and associated *p*-values () for rhizobia viability per nodule mass in the split-root experiments for two soybean plant varieties, Williams and Osumi, showed in **Fig. 1.**

	Williams		Osumi	
Weeks after inoculation	USDA110-1/H1-1	USDA110-2/H1-3	USDA110-1/H1-1	USDA110-2/H1-3
3	1.929 (0.072)	7.985 (0)	0.358 (0.726)	-1.156 (0.26)
5	0.915 (0.384)	2.075 (0.077)	1.727 (0.122)	6.496 (0)

Table S2. t-values and associated *p*-values () for the relative expression of the DD15 gene marker of nodule senescence in nodules from two soybean plant varieties, Williams, and Osumi, showed in **Fig. 2.**

	Williams		Osumi	
Weeks after inoculation	USDA110-1/H1-1	USDA110-2/H1-3	USDA110-1/H1-1	USDA110-2/H1-3
3	1.595 (0.125)	0.580 (0.567)	0.951 (0.352)	-0.457 (0.652)
4	0.811 (0.423)	1.185 (0.244)	0.319 (0.752)	0.703 (0.490)
5	0.541 (0.591)	2.171 (0.038)	2.109 (0.054)	10.916 (3.12 x 10^{-8})

Table S3. t-values and associated *p*-values () for the relative expression of the DD10 gene marker of nodule senescence in nodules from two soybean plant varieties, Williams, and Osumi, showed in **Fig. 2.**

	Williams		Osumi	
Weeks after inoculation	USDA110-1/H1-1	USDA110-2/H1-3	USDA110-1/H1-1	USDA110-2/H1-3
3	-0.537 (0.599)	-0.831 (0.424)	-0.576 (0.571)	0.254 (0.802)
4	-1.635 (0.115)	-1.003 (0.326)	-0.284 (0.778)	-0.06 (0.942)
5	-6.261 (6.7 $x10^{-7}$)	-5.152 (1.51 $x10^{-5}$)	-1.732 (0.104)	-11.981 (9.55 $x10^{-9}$)

Figure Legends

Fig. S1. Schematic representation of the split-root plant experiment to test the plant sanction hypothesis.

Split roots in each plant were inoculated with *B. japonicum*, either the N_2 fixing strain (USDA 110, fix+), or the non-fixing strain (H1, fix-), in three treatments, USDA 110 / H1-1 (a), USDA 110-2 (b) or H1-3 (c). At weeks 3, 4 and 5 after inoculation, nodules (represented by circles in roots) were harvested to count viable rhizobia, and to determine expression of senescence and maturity nodule molecular markers.

Fig. S2. Temporal pattern of nodule production (mean ± 1 s.d.) in the split-root experiments for two soybean plant varieties, A, Williams, B, Osumi.

Nodule numbers were counted in each half root every three days until nodule production reached a plateau, in half roots of the same plant inoculated with the fixing USDA110 strain or the non-fixing strain H1 (USDA110-1/H1-1).

Fig. S3. Plants of Williams soybean cultivar inoculated with the N_2-fixing USDA110 strain or the non-fixing strain H1, either in half roots of the same plant (USDA110-1/H1-1, a), or in both roots of the same plant (USDA110-2, b, or H1-3, c).
After 6 weeks of inoculation, plants a, b showed no evidence of stress, but plant c, with both roots inoculated with the non-fixing strain H1, showed extreme N starvation.

Figure

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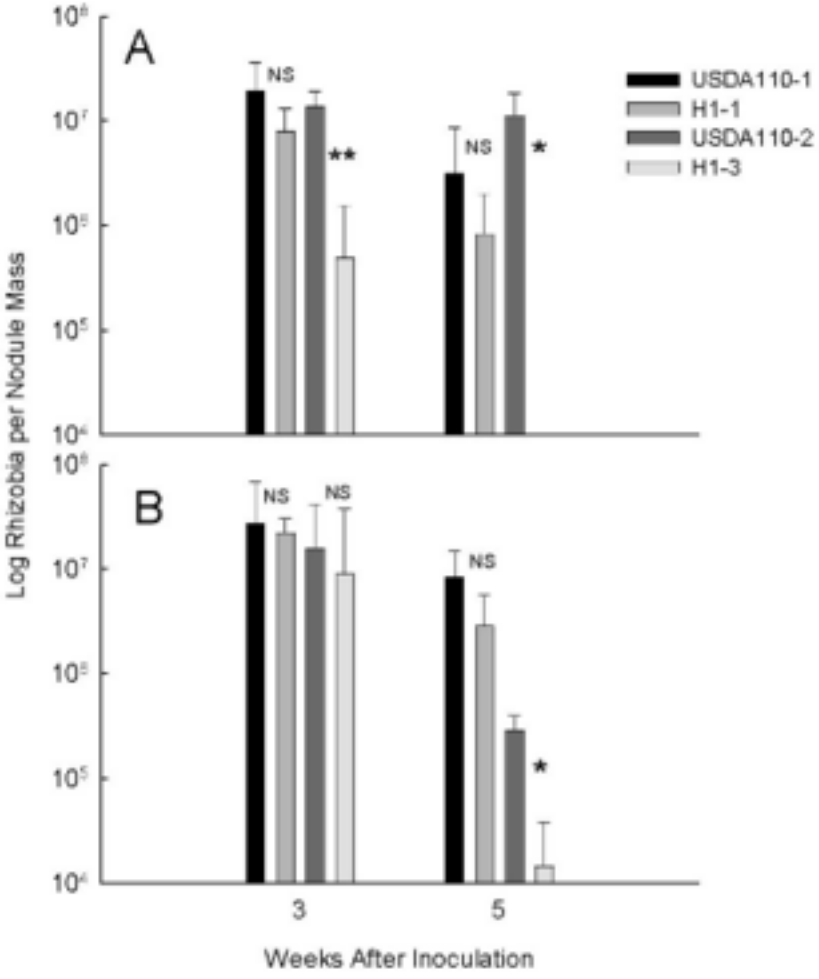

Fig. 1

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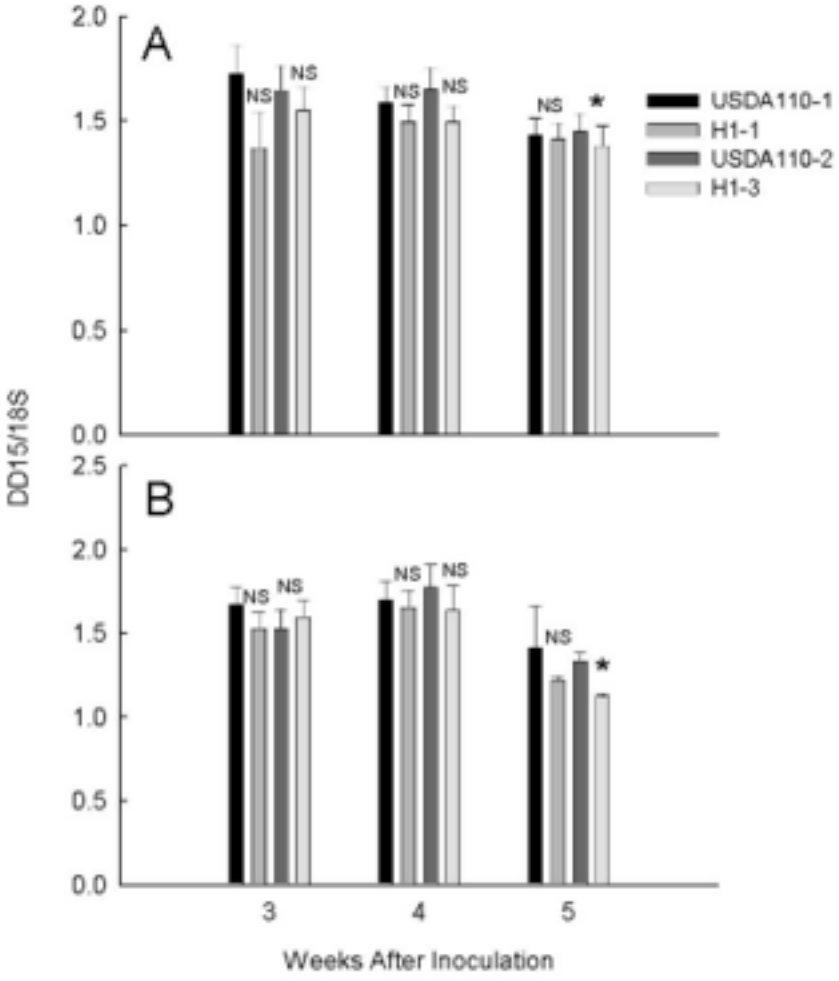

Fig. 2

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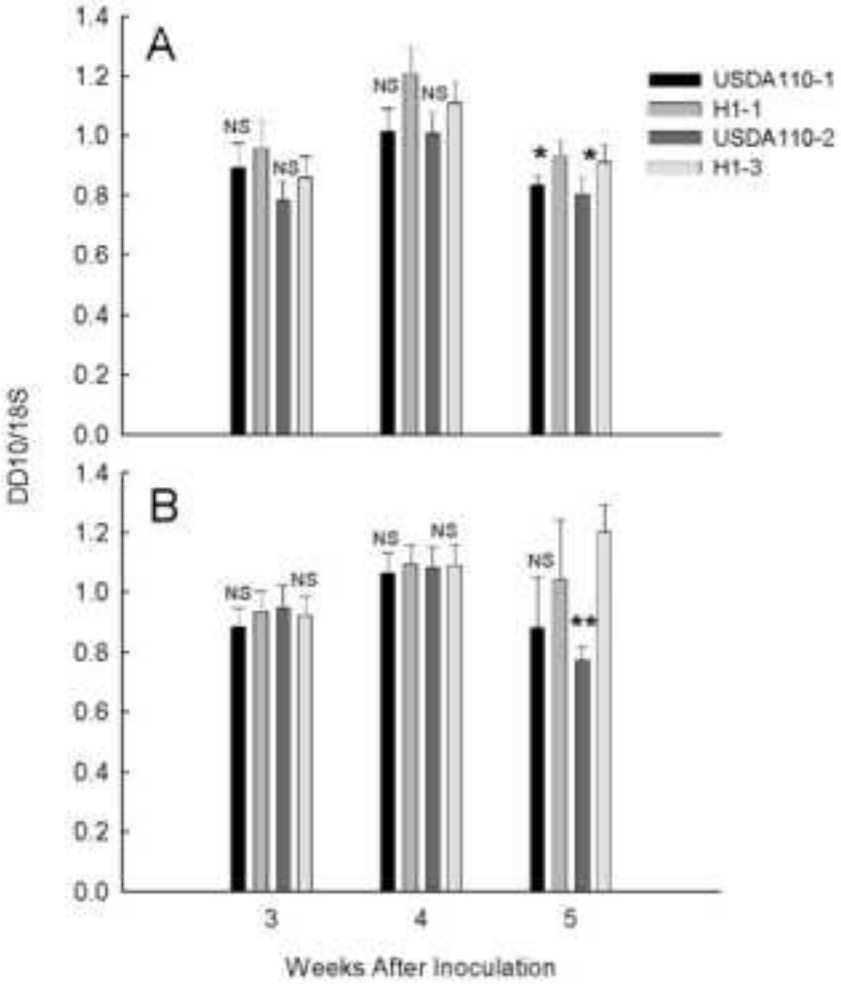

Fig. 3

Figure

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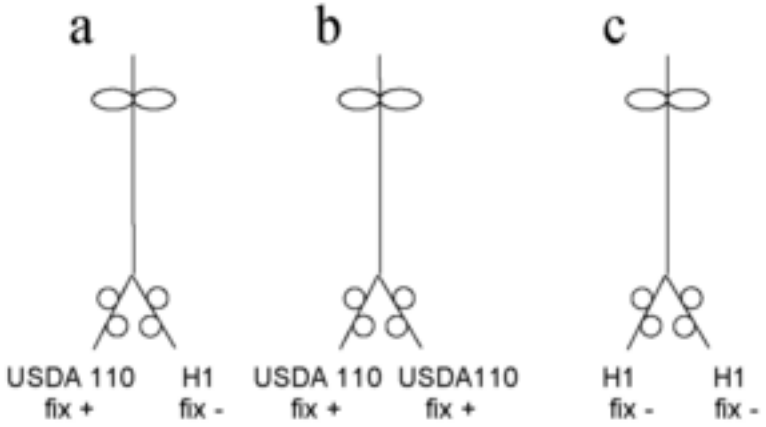

Fig. S1

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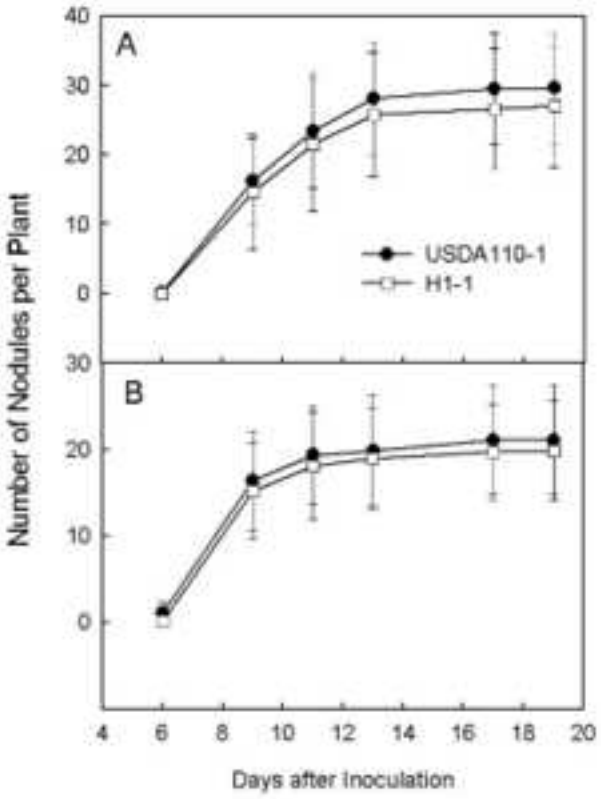

Fig. S2

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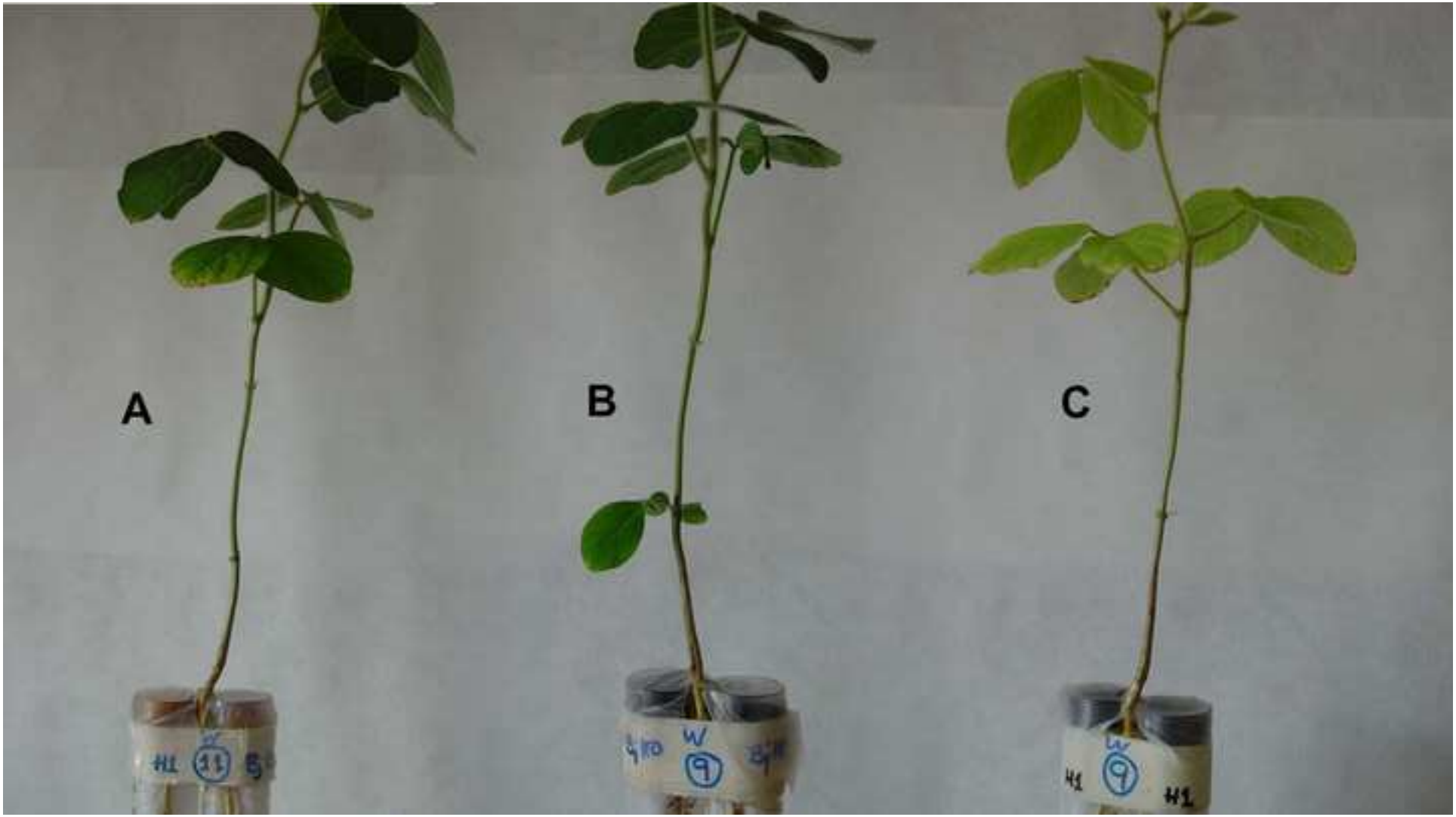
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