Changes in environmental CO_2 concentration can modify *Rhizobium*-soybean specificity and condition plant fitness and productivity.

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Keywords

Bradyrhizobium japonicum, elevated [CO_2], rhizobium-soybean fitness, photosynthesis, N_2-fixation, nodule, soybean, specificity

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Highlights

- Strains isolated at Ele[CO_2] produce a negative fitness response in soybean grown at Amb[CO_2].
- The negative fitness response is associated with a deficient/slow nodulation.
- Deficient nodulation reduced soybean's N_2-fixation and growth.
- Deficient nodulation may be associated with a change in root exudates caused by the change in [CO_2].
- The strain USDA 110 shows higher N_2-fixation than native strains isolated at SoyFACE.

Abstract

Over the past 10 years, it has been demonstrated in the literature that legume responses to elevated [CO_2], whether positive, negative, or null, are in part dependent on the *Rhizobium* species and genotypes that establish symbiosis with the plant. However, all the strains used in these past experiments were isolated in field conditions at ambient [CO_2]. We studied for first time the fitness response of soybean inoculated with a *Rhizobium* strain that has been previously isolated from nodules of plants grown at elevated [CO_2] in field conditions at a FACE site. In experiments developed in controlled growth, soybean's response at elevated [CO_2] following inoculation with a strain isolated at elevated [CO_2] was very similar to the response of other strains isolated at ambient [CO_2], indicating a stimulation of photosynthesis and growth. However, when the plants were grown at ambient [CO_2], those inoculated with the strain isolated at elevated [CO_2] showed slower and stunted growth, associated with deficient N_2-fixation and slower nodule formation. In fact, when plants were grown at ambient [CO_2] in a field setting with no history of soybean cultivation, those inoculated with the strain isolated at elevated [CO_2] showed the same response as plants without inoculation. We hypothesize that elevated [CO_2] changed the composition of exudates produced by the root, attracting different rhizobia than at ambient [CO_2]. This study showed that the strains isolated in nodules at elevated [CO_2] are not capable of properly nodulating soybean plants grown at ambient [CO_2]. However, more research is needed in order to understand how changes in environmental conditions can affect the symbiotic relationship and ultimately how we can improve plant fitness in a changeable world.

1. INTRODUCTION

The atmospheric carbon dioxide concentration [CO_2] has increased from 320 μmol mol^{-1} in 1960 to 404.2 μmol mol^{-1} in 2016 (www.co2.earth), and it is projected to increase to 550 μmol mol^{-1} by 2050 and to 730-1000 μmol mol^{-1} by 2100, depending on the climate scenario considered, and the trends in atmospheric CO_2 emissions (Ciais et al., 2013). It is widely known that in the last 30 years the increase in atmospheric [CO_2] has been accompanied by a significant increase in yield gains in C3 crops (Specht et al., 1999; McGrath & Lobell, 2013; Sakurai, et al., 2014; Specht et al., 2014); as a consequence of photosynthesis stimulation and photorespiration reduction (Drake, et al., 1997; Long, et al., 2004; Aranjuelo et al., 2013).

In soybean, genotypic yield variations under elevated [CO_2] have been demonstrated in field conditions using free air CO_2 enriched technology (FACE), detecting cultivars that show a positive yield response to elevated [CO_2], while others are irresponsive (Bishop et al., 2015). In a subsequent experiment investigating the physiological traits behind this differential yield response to elevated [CO_2], Sanz-Saez et al. (2017) found that a combination of higher light interception efficiency (LIE), and radiation use efficiency (RUE) were the main traits responsible for the higher yield under elevated [CO_2]. It has been theorized that LIE is very close to its theoretical maximum, and improvements in RUE will most likely come from the use of transformation technologies in order to ameliorate the photosynthetic apparatus (Zhu et al., 2010; McGrath et al., 2015; Glowacka et al., 2017). Aside from improving above-ground traits, Ainsworth et al. (2012) suggested that ameliorating the response of the unknown below-ground organs of soybeans, roots and nodules, to elevated [CO_2], could help to increase yields *via* an increase in sink capacity and N_2-fixation.

Soybean forms symbiotic associations with the soil N_2-fixing bacteria, *Bradyrhizobium japonicum*, with the plant forming a nodule in the root and providing carbohydrates to the bacteria in exchange for N compounds derived from atmospheric N_2-fixation. In this process, *Bradyrhizobium* bacteria can consume 4-11% of the carbohydrates fixed through photosynthesis, increasing the plant's energy needs for N_2-fixation (Kaschuck et al., 2009). This extra C consumption in the nodules can increase plant sink capacity and stimulate legume photosynthesis and growth under elevated [CO_2] (Ainsworth et al., 2004; Kaschuck et al., 2009), avoiding the sink limitation and photosynthesis down-regulation sometimes observed in plants grown at elevated [CO_2] (Ainsworth et al., 2004). As in plants, there is genotypic variation response depending on the strain of *Bradyrhizobium* that establishes the symbiosis. The carbohydrate consumption in the nodule depends in part on the strain of *Bradyrhizobium*, and this can increase or decrease the efficiency of N_2-fixation (Kaschuck et al., 2009). Other factors that modulate the efficiency of N_2-fixation include plant genotype, plant genotype x bacterial genotype interactions (Heat et al., 2010), and plant performance, which at the same time can be influenced by environmental conditions (Aranjuelo et al., 2014).

Since the discovery of symbiotic N_2-fixation and the isolation of the first rhizobium bacteria, scientists have been selecting for more efficient rhizobia (Schubert et al., 1978; Postgate, 1982). In soybean, selected *B. japonicum* strains such as USDA110, have greater N_2-fixation potential, resulting in higher production compared to unselected or native strains (Schubert et al., 1978). It has been theorized that inoculation of selected bacteria in soybeans grown under elevated [CO_2] in open field conditions could increase photosynthesis and yield due to their higher N_2-fixation potential in comparison to plants nodulated with native strains (Sanz-Saez et al., 2015). However, this theory was only true when plants were grown in sterile soils where competition with native rhizobia did not

displace USDA110 from the nodules, and reduce N_2-fixation and biomass response to elevated [CO_2] (Sanz-Saez et al., 2015). Reduction in N_2-fixation by competition with native rhizobium strains has been documented before (Hagen & Hamrick 1996; Silva et al., 1999). This phenomenon is explained by the higher metabolic investment of native strains in multiplication as a survival trait, compared with the higher investment in N_2-fixation of selected strains (Kiers et al., 2007; 2010; Friesen et al., 2012). In addition, because soil microorganisms increase their numbers and diversity at elevated [CO_2] (Wang et al., 2017), it is expected that competitiveness for nodule occupation will be increased (Sugawara & Sadowsky, 2013).

In fact, Sugawara & Sadowsky (2013) identified different native *B. japonicum* strains collected from nodules isolated from soybeans grown at ambient (390 μmol mol^{-1} CO_2, strain SFJ4-24) and elevated (600 μmol mol^{-1} CO_2, strain SFJ14-36) [CO_2] at the SoyFACE facility at the University of Illinois. These authors found that the strain isolated at elevated [CO_2] (SFJ14-36) significantly over expressed genes encoding for nodulation and N_2-fixation at elevated [CO_2] in comparison to the strain isolated at ambient [CO_2] (SFJ4-24) and the strain used as a control (USDA110). On the other hand, in several experiments performed in the field and growth chambers, it has been shown that *Bradyrhizobium* strains that showed higher N_2-fixation at ambient and elevated [CO_2] tended to be accompanied by higher levels of photosynthesis and biomass accumulation in their soybean host (Bertrand et al., 2011; Sanz-Saez et al., 2015).

Due to the lack of physiological measurements in Sugawara's study, it is paramount that more research is performed to understand the plant-rhizobia fitness response to elevated [CO_2] with these strains. In addition, because the strain SFJ14-36 showed a higher expression of nodulation and N_2-fixation genes than USDA110 in the experiment performed by Sugawara & Sadowsky (2013), we hypothesize that this strain

could show potential as inoculum at ambient and elevated [CO_2] for its possible greater competitiveness and N_2-fixation compared to USDA110. For these reasons, a growth chamber experiment was performed in order to test the physiological response of soybean (photosynthesis, N_2-fixation and biomass) inoculated with these 3 strains (SFJ4-24, SFJ14-36, and USDA110) in sterile soil conditions and grown at ambient (400 μmol mol^{-1} CO_2) and elevated [CO_2] (700 μmol mol^{-1} CO_2). Additionally, a field experiment was performed at ambient [CO_2] and in a field setting with no history of soybean planting (no rhizobia competition) to test whether these strains (SFJ4-24, SFJ14-36, and USDA110) produced similar results to those obtained in the growth cabinet experiment at ambient [CO_2] in sterile soil with no other microorganisms present.

2. MATERIALS AND METHODS

2.1. Growth Chamber Experiment

2.1.1. Plant and bacterial material

To avoid problems of compatibility between the soybean cultivar and the *Bradyrhizobium japonicum* strains isolated at the SoyFACE facility at the University of Illinois Urbana-Champaign (https://soyface.illinois.edu) by Sugawara & Sadowsky (2013), we used the same soybean cultivar (*Glycine max* cv. 93B15; Pioneer Hi-Bred) and the same *B. japonicum* strains isolated by Sugawara & Sadowsky, (2013). The *B. japonicum* strains selected were SFJ4-24 (serogroup 123) isolated from nodules of soybean grown at ambient [CO_2] (390 ppm of CO_2), while SFJ14-36 (serogroup 38) was a strain isolated from soybean nodules grown at elevated [CO_2] (550 ppm of CO_2). In addition, we used USDA110 as a control strain because it has demonstrated high soybean performance at ambient and elevated [CO_2] at SoyFACE (Sanz-Saez et al., 2015). These strains were provided by Prof. Michael Sadowsky (University of Minnesota; SFJ4-24, and SFJ14-36) and by the USDA-ARS Rhizobium Germplasm Resource Collection in Belstville, MD (USDA110). The different *B. japonicum* strains were plated on modified arabinosegluconate media (MAG, modified arabinose gluconate media, USDAversion; van Berkum, 1990) in Petri dishes and grown at 30 °C for 7 days. Isolated colonies were transferred to 5 mL MAG liquid media and grown at 30 °C and 4 g in an orbital shaker (Sanyo Orbital Incubator), for 7 d. When the culture was in an exponential growth phase (approximately 10^8 cells mL^{-1}), it was stored at 4 °C for later use as a stock culture.

2.1.2. Seed and plant inoculation

Soybean seeds were surface sterilized with sodium hypochlorite (1%) for 10 min and rinsed with sterile water until the smell to bleach disappeared. For the seed inoculation, 200 mL of MAG medium liquid culture containing ≈ 5 x 10^9 cell mL^{-1} of

each individual *B. japonicum* strain was centrifuged for 15 min at 5100 *g* to separate the bacteria from the media. The bacterial pellet was re-suspended in 2 mL of sterile deionized water containing 2% PVPP (polivinylpolypyrrolidone), reaching a concentration of ≈ 10^{11} cell mL^{-1}. Then 200 seeds were placed in a 500 mL sterile beaker containing the 2 mL of concentrated inoculum on a rotatory shaker overnight, and immediately planted (adapted from Yokoyama et al., 2006). For the liquid inoculation, 1L of MAG liquid culture containing ≈ 5 x 10^9 cell mL^{-1} was centrifuged and re-suspended as above to a final concentration of 10^8 cell mL^{-1}.

2.1.3. Plant growth conditions and treatments

Five inoculated soybean seeds per pot were planted in 10 L pots containing a mixture of peat moss, perlite, and vermiculite in a 1:1:1 v/v/v ratio, which had been previously sterilized as described in Sanz-Saez et al. (2015). One week after emergence plants were thinned to one plant. After plants emerged from the pot they were inoculated 3 times at 2, 9 and 16 days after emergence (DAE) with the liquid inoculum from the appropriate *B. japonicum* strain (USDA110, SFJ4-24 and SFJ14-36). In addition, in order to have a non-inoculated control, one set of plants was not inoculated but was supplemented with 15mM of NH_4NO_3, and this treatment was named Non-Inoc. +N. All plants were watered alternatively with Evans Ns-free solution and distilled water to avoid salt accumulation (Evans, 1974), with the exception of Non Inoc. +N plants ,which were watered with Evans + 15 mM of NH_4NO_3 instead of the Evans N free solution. From the beginning of the experiment plants were grown in two growth chambers, one maintained at ambient [CO_2] (≈ 400 μmol mol^{-1} CO_2), and the other maintained at elevated [CO_2] (700 μmol mol^{-1} CO_2). Both chambers were maintained at 60/70% relative humidity and 25/22°C day/night temperature and a photosynthetic photon flux density (PPFD) of 1200 μmol m^{-2} s^{-1} from 7:00 to 22:00 h, until developmental stage V5 (Fehr et al., 1971), when

the day length was decreased by 2 h to induce flowering. Every two weeks, plants and treatments were rotated among and within chambers in order to reduce potential chamber effects. Within each chamber we grew 16 pots of each inoculation treatment to ensure that there were enough plants to collect sequential harvests and perform physiological measurements at R2 (6 pot/reps), R4 (6 pots/reps), and R7 (4 pots/reps).

2.1.4. Photosynthetic parameters

Soybeans had open flowers on upper nodes in the R2 developmental stage and had seeds developing at the R4 developmental stage (Fehr et al., 1971) at the time of photosynthetic measurements. These developmental stages were chosen for gas exchange based on previous evidence that these stages represent a range in N fixation capacity and leaf C and N responses to elevated [CO_2] (Rogers & Ainsworth, 2006). Midday photosynthetic measurements (photosynthetic rate and stomatal conductance) were performed in a fully expanded young leaf, using a portable infrared gas exchange system (LI-6400), maintaining the same environmental conditions in the gas analyser as in the growth chamber with a light intensity of 1200 μmol m^{-2} s^{-1}, block temperature 25ºC, RH ≈ 60%. The [CO_2] in the gas analyser was changed from ambient (400 µmol mol^{-1} CO_2) to elevated [CO_2] (700 µmol mol^{-1} CO_2) depending on the atmospheric [CO_2] in each chamber. Night respiration measurements were performed during the dark period in the chamber in the same leaf as above while maintaining light intensity at 0 μmol m^{-2} s^{-1}, block temperature at 22ºC, RH ≈ 70% and changing the [CO_2] to ambient (400 µmol mol^{-1} CO_2) or elevated [CO_2] (700 µmol mol^{-1} CO_2) depending on the atmospheric [CO_2] in each chamber. In addition to these measurements, the leaf CO_2 assimilation rate or photosynthesis (*A*) in response to changes in the intercellular [CO_2], (Ci) curves were performed on fully expanded leaves. To do so, photosynthesis was initially induced at the growth [CO_2], either 400 or 700 µmol mol^{-1} CO_2, depending on the [CO_2] of growth.

Next, the [CO_2] was reduced stepwise to the lowest concentration of 50 μmol mol^{-1}, and then increased stepwise to the highest CO_2 concentration of 1500 μmol $mol^{-1,}$ using a total of 12 [CO_2] points. During the measurements, leaf temperature and photosynthetic photon flux density (PPFD) were maintained at 25 °C and 1750 μmol m^{-2} s^{-1} respectively. The maximum rate of carboxylation of Rubisco ($V_{c,max}$) and the RuBP regeneration rate (J_{max}), were estimated from the Ci curves using equations developed by Farquhar *et al*. (1980) with the temperature functions of Bernacchi *et al*. (2001, 2003).

2.1.5. Biomass sampling and final harvest

When plants reached the R2 and R4 developmental stages, 6 plants per inoculation and [CO_2] treatment were harvested and separated into organ samples; leaves, stems, roots, nodules, and pods. In addition, nodule numbers were recorded for each plant. Each organ sample was oven dried at 65 °C for at least 72 h and then weighed. The data were organized as total dry weight (Total DW, the sum of all the organs harvested), aboveground biomass (the sum of leaf, stem and pod weights), and nodule dry weight (Nodule DW). At the R7 developmental stage, 4 plants per inoculation and [CO_2] treatment were harvested and seed weight was recorded (seed DW, g of seed $plant^{-1}$).

^{15}N labelling experiment and N concentration analysis

To test if the inoculation and [CO_2] treatment affected N_2 fixation, we performed a ^{15}N labelling experiment in plants that were at the R2 developmental stage. Three plants (there was one plant per pot) per inoculation and [CO_2] treatment were labelled, while 3 plants were used as unlabelled controls, and harvested at the same time as the labelled plants. The $^{15}N_2$ labelling was accomplished by injecting labelled gas into the root zone using handmade labelling pots following the procedure of Sanz-Saez et al. (2015). Plants were grown in these pots for the duration of the experiment. On the night preceding the

labelling experiment, the pots were sealed with plastic lids in order to avoid the escape of the labelled gas. To perform the $^{15}N_2$ labelling, 10% $^{15}N_2$ enriched gas was prepared in Supelco-Inert Foil Gas Sampling Bags (Sigma-Aldrich, St Louis, MO, USA) by mixing the $^{15}N_2$-labelled gas enriched at 99% with ambient air ($\delta^{15}N_2$ at 0‰). Two hundred mL of $^{15}N_2$ (10%) mixed gas was injected into the labelling pots using a gas syringe (SGE; Sigma-Aldrich) 2 and 4 h after the lights were turned on, coinciding with the period of greatest N_2-fixing activity (Molero et al. 2014). After 24 h of labelling, the plants were harvested and separated into leaves, stems, roots, and nodules, then dried at 65 °C for at least 72 h. The dried organs were weighed and ground to a 1mm of particle size. The samples were analyzed in a Costech 4010 elemental analyzer coupled in continuous flow with a Thermo Fisher Delta V Advantage Isotope Ratio Mass Spectrometer (IRMS Thermo Scientific, Waltham, MA, USA). All deltas were measured relative to laboratory standards that were calibrated with international standards VPBD for air N_2. The $^{15}N/^{14}N$ ratio (R) in plant material was expressed in δ notation ($\delta^{15}N$, ‰) with respect to atmospheric N_2, with an analytical precision of 0.1‰:

$$\delta^{15}N = \left(\frac{R_{sample}}{R_{standard}} \right) - 1$$

The nitrogen isotope composition was then converted to a percentage: $\%^{15}N = R_{sample}/(1+R_{sample})$ where R_{sample} was obtained with $R_{standard} \times (\delta^{15}N+1)$, with $R_{standard} = 0.003667$. The amount of ^{15}N fixed during the day of the labelling experiment was calculated as ^{15}N fixed in each organ:

$$^{15}N\ fixed\ by\ organ\ (mg\ ^{15}Norgan^{-1}day^{-1}) = total\ N\ (mg\ organ^{-1}) \times \%^{15}N\ excess$$

where $\%^{15}N$ excess was calculated as $\%^{15}N$ of the labelled sample -$\%^{15}N$ of the non-labelled sample, as has been previously described by Bei et al. (2013). After the

calculation of ^{15}N fixed in each organ, the total amount of ^{15}N fixed by the plant was calculated as the sum of each organ's value. In addition to this analysis, N% was also measured in each plant organs harvested at R2 and R4 stages. Biomass was ground to a fine powder, weighed and analysed using an elemental analyser (Costech 4010) as described above.

2.2. Field Experiment

Soybean seeds (*Glycine max* L.) of cultivar 93B15 (Pioneer Hi-Bred) were sown on May 1st 2015 at the Neiker-Tecnalia research centre in Arkaute (Alava, Spain), in two fields, one managed with conventional practices, and the other managed with organic practices in order to obtain more variability between field conditions. In each field, soybeans seeds inoculated with the three studied *B. japonicum* strains (USDA110, SFJ14-36, and SFJ24-4) as described above, were sown. A non-inoculated treatment was included as control (Non-Inoc.). Seeds were sown in 4 row plots 6 m long, and with a row spacing of 0.38 m. The experiment was performed as a randomized block design with inoculation as the main effect, and 4 plots as blocks. At the R2 and R5 developmental stages, chlorophyll content was estimated by using a SPAD meter (SPAD -502Plus, Konica Minolta Inc.), measuring three plants per plot, and averaging five measurements per fully expanded young leaf. When plants reached maturity, 5 plants per plot were randomly selected and measured for plant height. Then, the two central rows of each plot were selected, and eliminated a 1 m band of plants on each side of the plot. A two-row combine harvester (Plot Master Combine – PMC20, Almaco, USA) was used to harvest the four metre-long central two rows of the plot.

2.3. Statistical Analysis

In the growth chamber experiment, all parameters were tested using a mixed model analysis of variance (PROC MIXED, SAS 9.4, Cary, NC, USA). The [CO_2] and

inoculation treatments were considered fixed effects, while the replicates were the random effect. When the main effect of the [CO_2], inoculation treatment, or their interaction was significant, least square means post-hoc tests were performed to compare the means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

In the field experiment, all parameters were tested using a mixed model analysis of variance (PROC MIXED, SAS 9.4, Cary, NC, USA). The management (field) and the inoculation treatments were considered fixed effects, while block was the random effect. When the main effect of inoculation or management, or their interaction was significant, least square means post-hoc tests were performed to compare the means (LSMEANS, SAS 9.4, SAS Institute, Cary, NC, USA).

3. RESULTS

Elevated [CO_2] significantly increased ^{15}N-labelled biomass, a parameter equivalent to N_2 fixation (Sanz-Saez et al., 2015), in plants inoculated with USDA110 and SFJ14-36 but not in SFJ4-24, which showed similar values to plants grown at ambient [CO_2] (Fig. 1). Under elevated [CO_2], USDA110 inoculated plants showed approximately 40% higher ^{15}N content than SFJ14-36 and SFJ4-24. At ambient [CO_2], plants inoculated with SFJ14-36, which is the *B. japonicum* strain that was isolated under elevated [CO_2] at SoyFACE, showed the lowest N_2-fixation (Fig. 1).

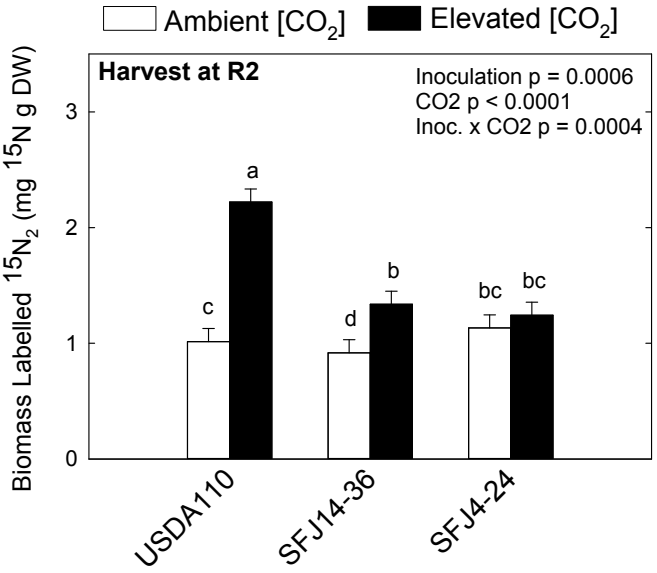

Figure 1: Biomass labelled $^{15}N_2$ values (‰), an estimation of N_2-fixation, measured in soybean plants grown at ambient (400 µmol CO_2 mol^{-1}), and elevated (700 µmol CO_2 mol^{-1}) [CO_2] and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24). The bars represent means ± standard error (n = 3). Empty bars represent plants grown at ambient [CO_2], while filled bars represent plants grown at elevated [CO_2]. Means showing the same lower case letter are not significantly different (P =0.05) in the LSD test.

Elevated [CO_2] tended to increase soybean´s aboveground and total dry weight at all harvest stages, with the Non-Inoc. +N plants accumulating more biomass at both levels of [CO_2] (Fig. 2, Table 1). Among the inoculated plants, the ones inoculated with SFJ14-36 showed the greatest increase in biomass accumulation at elevated [CO_2] when compared to ambient [CO_2], reaching levels similar to plants inoculated with USDA110

at R4 and R7 (Fig. 2, Table 1). This impressive difference between ambient and elevated [CO_2] biomass accumulation was due to SFJ14-36 being associated with the lowest biomass values at ambient [CO_2] in all harvests with the exception of R7. Plants inoculated with the SFJ4-24 strain did not show significant differences in biomass between ambient and elevated [CO_2], either in the aboveground biomass (Fig. 2) or in the total biomass (Table 1), reaching similar values to the other strains only at R7 (Fig. 2). The seed weight at R7 followed a similar trend to aboveground biomass in which Non Inoc. +N plants showed the highest production at both [CO_2] levels followed by plants inoculated with USDA110. Plants inoculated with the SFJ14-36 and SFJ4-24 strains produced slightly lower seed weight than USDA110 inoculations at ambient [CO_2], but the difference was not significant (Table 1). It is worth noting that although plants inoculated with strain SFJ14-36 showed the greatest response in total biomass to elevated [CO_2], there were no significant differences in seed production between ambient and elevated [CO_2].

Elevated [CO_2] increased nodule dry weight (DW) by 43.7%, 825%, 269%, and nodule number by 39.7%, 255.5%, 148.3% at R2 stage in plants inoculated with USDA110, SFJ14-36, and SFJ4-24, respectively (Table 1). This strong boost of nodule characteristics at elevated [CO_2] in plants inoculated with SFJ14-36 could be explained by their low performance at ambient [CO_2] where they had the lowest nodule DW, which was ten times lower than in plants inoculated with USDA110 (Table 1). In fact, nodule number and dry weight got similar values at elevated [CO_2] whatever the bacterial strains were used. At the R4 developmental stage, nodule DW and nodule number were affected by the interaction of [CO_2] and strain inoculation. Elevated [CO_2] significantly increased nodule DW in USDA110 and SFJ14-16 inoculated plants, with USDA110 resulting in higher nodule DW under both elevated and ambient [CO_2] levels (Table 1). In contrast,

plants inoculated with USDA110 showed the largest nodule number under ambient [CO_2], but not under elevated [CO_2] which did not stimulate the nodule number (Table 1). However, plants inoculated with SFJ4-24 and SFJ14-36 showed the lowest nodule number at ambient [CO_2] and the highest nodule number at elevated [CO_2] at R4, with plants inoculated with SFJ14-36 having a slightly lower nodule number and weight than plants inoculated with SFJ14-36 at ambient [CO_2].

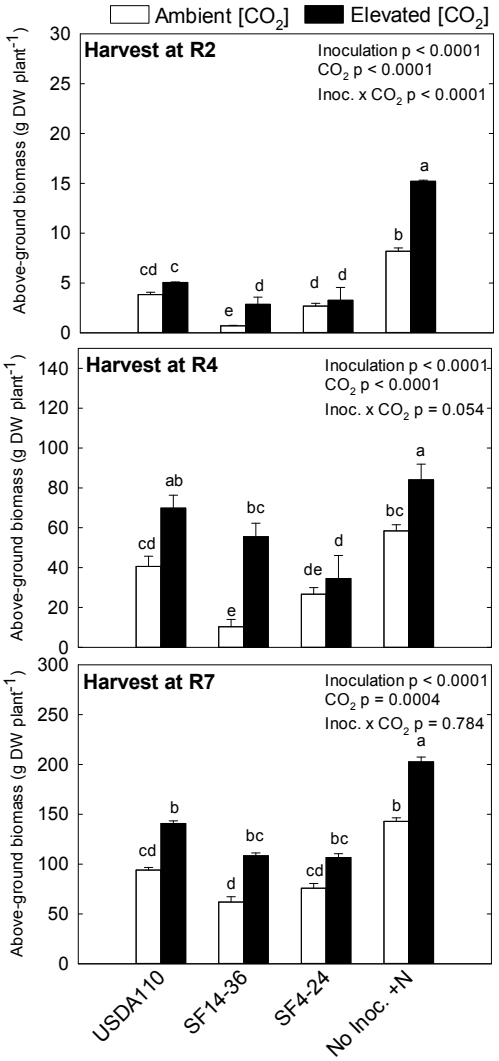

Figure 2: Aboveground biomass measured in soybean plants grown at ambient (400 µmol CO_2 mol^{-1}), and elevated (700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were harvested at R2, R4, and R7 developmental stages. The bars represent means ± standard error (n = 6). Empty bars represent plants grown at ambient [CO_2], while filled bars represent plants grown at elevated [CO_2]. Means showing the same lower case letter are not significantly different (P =0.05) in the LSD test.

Following a similar trend as biomass, midday photosynthesis at R2 and R4 was increased significantly by elevated [CO_2] in all *B. japonicum* treatments (Fig. 3). At R2, the differences between strains were significant, as well as the interaction between [CO_2] and the inoculated strain. At elevated [CO_2] all the strains resulted in the same

Harvest at R2						
	Total DW		Nodule DW		Nodule Number	
B. japonicum	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2
USDA110	5.43 cd	7.04 c	0.320 abc	0.436 a	174.3 b	243.6 a
SFJ14-36	0.92 e	3.91 d	0.032 d	0.296 abc	64.0 b	227.3 a
SFJ4-24	3.76 d	4.46 d	0.104 cd	0.384 ab	86.3 b	214.6 a
Non-Inoc. +N	11.27 b	20.01 a	-	-	-	-
Effect	F	*p-value*	F	*p-value*	F	*p-value*
Inoculation	113.50	≤0.0001	3.26	0.081	2.25	0.156
CO_2	40.10	≤0.0001	10.18	0.009	19.64	0.001
Inoc x CO_2	10.63	0.0004	0.57	0.581	1.02	0.395
Harvest at R4						
	Total DW		Nodule DW		Nodule Number	
B. japonicum	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2
USDA110	49.20 cd	76.79 b	1.49 b	2.08a	1237.2 b	1324.8 b
SFJ14-36	17.88 e	67.32 bc	0.71 c	1.79b	575.5 c	1860.6 a
SFJ4-24	35.14 de	30.05 e	1.27 bc	1.77b	535.5 c	2293.3 a
Non-Inoc. +N	65.92 bc	99.24 a	-	-	-	-
Effect	F	*p-value*	F	*p-value*	F	*p-value*
Inoculation	22.19	0.0006	1.57	0.005	0.54	0.592
CO_2	31.15	0.0002	0.91	0.356	44.01	≤0.0001
Inoc x CO_2	5.89	0.0018	1.51	0.006	9.99	0.0017
Harvest at R7						
	Seed DW					
B. japonicum	Amb CO_2	Ele CO_2				
USDA110	45.10 cd	71.55 ab				
SFJ14-36	35.64 cd	52.54 bcd				
SFJ4-24	29.23 d	54.46 bc				
Non-Inoc. +N	70.05 ab	93.87 a				
Effect	F	*p-value*				
Inoculation	10.22	0.0008				
CO_2	16.03	0.0013				
Inoc x CO_2	0.14	0.936				

Table 1: Mean values and ANOVA results (F, *p-value*) of total, nodule, and seed DW, and nodule number in soybean plants grown at ambient (Amb, 400 µmol CO_2 mol^{-1}), and elevated (Ele, 700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were harvested at R2, R4 and R7 developmental stages. Means showing the same letter are not significantly different (P =0.05) in the LSD test (n = 6).

photosynthetic rate, however, at ambient [CO_2], SFJ14-36 inoculation led to the lowest rate followed by SFJ4-24, and USDA (Fig. 3). At R4, the effect of the strain on photosynthesis was slightly significant ($p < 0.1$), with SFJ14-36-inoculated plants showing lower photosynthesis rates than USDA110 when measured at ambient [CO_2]. Stomatal conductance (g_s) was not affected by [CO_2] or inoculation at the R2 developmental stage. However, at R4, elevated [CO_2] significantly decreased g_s in all inoculation treatments except in SFJ4-24-inoculated plants (Fig. 3). Respiration was decreased under elevated [CO_2] at the R2 stage, but was not altered at the R4 stage. At R2, plants inoculated with the SFJ4-24 strain showed the highest respiration rates at ambient and elevated [CO_2] (Fig 3). The maximum rate of carboxylation of Rubisco

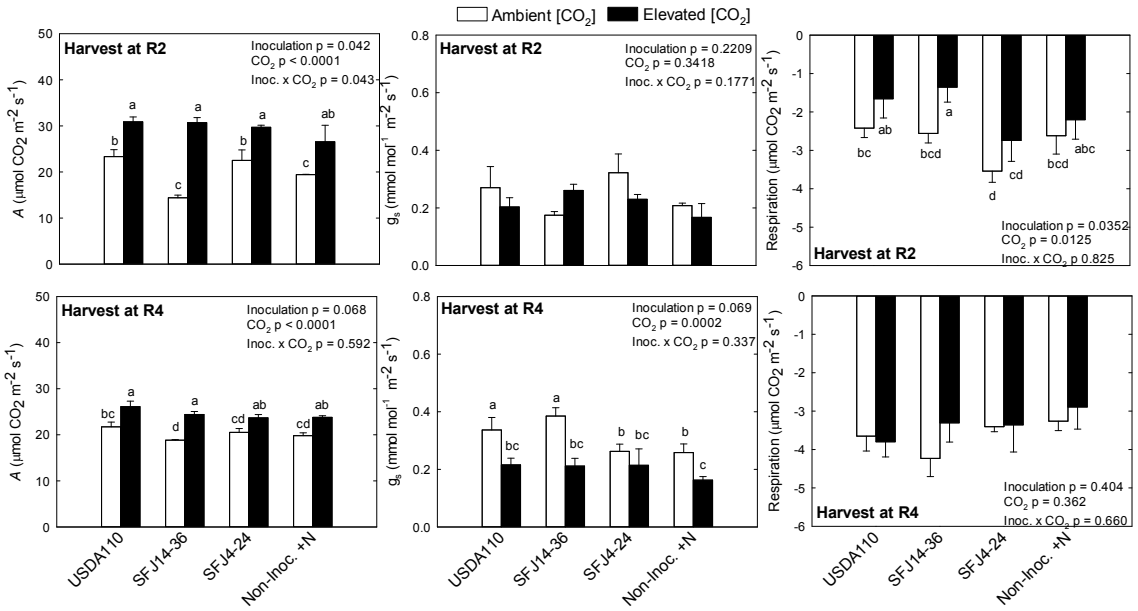

Figure 3: Midday photosynthesis (*A*), stomatal conductance (g_s), and night respiration measured in soybean plants grown at ambient (400 µmol CO_2 mol^{-1}) and elevated (700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were measured at R2 and R4 developmental stages. The bars represent means ± standard error (n = 6). Empty bars represent plants grown at ambient [CO_2], while filled bars represents plants grown at elevated [CO_2]. Means showing the same lower case letter are not significantly different (P =0.05) in the LSD test.

($V_{c,max}$) was affected by the interaction of [CO_2] and inoculation at the R2 stage (Fig. 4). At R2 under elevated [CO_2], no differences were found in the responses of plants inoculated with any of the *B. japonicum* strains. However, the Non-Inoc. +N plants showed a significant decrease in $V_{c,max}$ at elevated [CO_2] (Fig. 4). On the other hand, when the plants were measured at the R4 stage, there were no differences between the inoculation and [CO_2] treatments (Fig. 4). The RuBP regeneration rate (J_{max}) measured at R2 was significantly decreased by elevated [CO_2] in plants inoculated with SFJ14-36 and in Non Inoc. +N plants. However, at the R4 stage, the effect of elevated [CO_2] and the inoculated strain disappeared (Fig. 4).

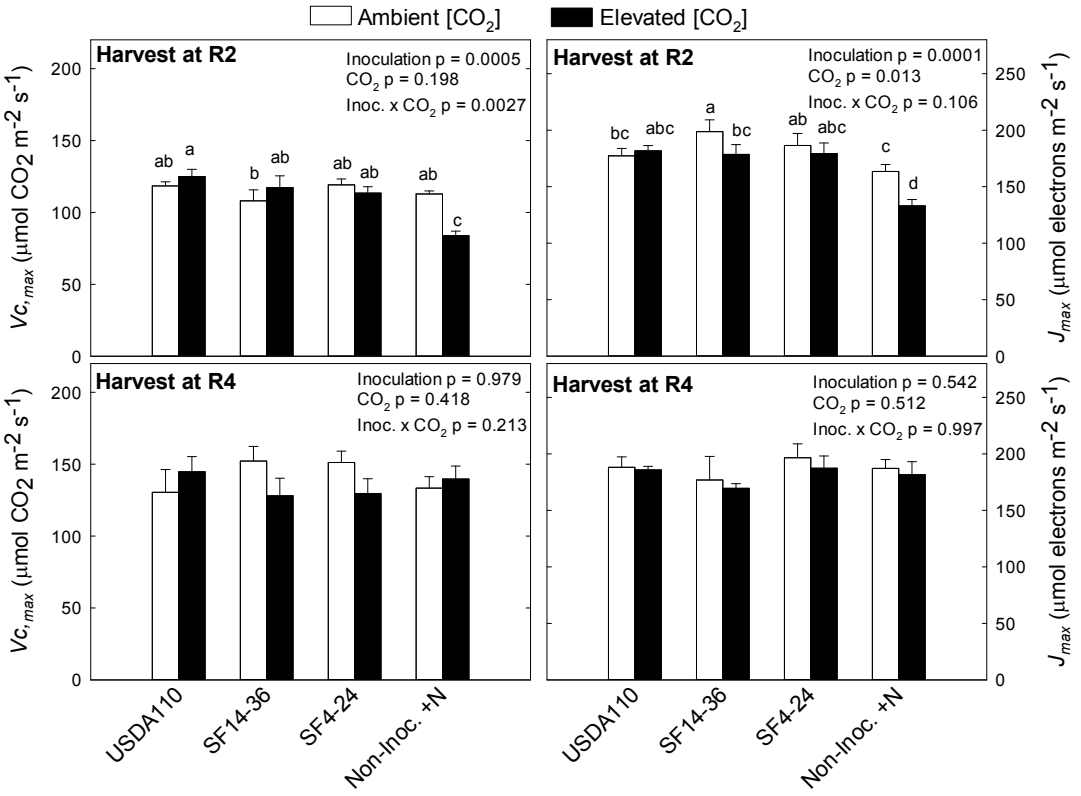

Figure 4: Rubisco maximum rate of carboxylation ($V_{c,max}$) and RuBP regeneration rate (J_{max}).measured in soybean plants grown at ambient (400 µmol CO_2 mol^{-1}) and elevated (700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were measured at R2 and R4 developmental stages. The bars represent means ± standard error (n = 6). Empty bars represent plants grown at ambient [CO_2], while filled bars represents plants grown at elevated [CO_2]. Means showing the same lower case letter are not significantly different (P =0.05) in the LSD test.

Elevated [CO_2] did not modify the leaf N% at the R2 developmental stage, and Non Inoc. +N plants showed lower N% than the inoculated plants. At the R4 developmental stage there were no differences between inoculation treatments, but elevated [CO_2] decreased leaf N% (Table 2). Stem and root N% showed interactive effects with [CO_2] and inoculation at the R2 stage. Plants inoculated with USDA110 or SFJ4-24 showed no decrease in their stem, root or nodule N% at elevated [CO_2]. However, plants inoculated with SFJ14-36 showed the highest N% at ambient [CO_2], decreasing their stem and root N% at elevated [CO_2] to similar percentages as USDA110 and SFJ4-24 inoculated plants (Table 2). In the nodules, the opposite trend was observed with SFJ14-36 plants showing the lowest N% at ambient [CO_2], but having a similar value to the other strains at elevated [CO_2]. At the R4 stage, the leaf %N decreased under elevated [CO_2] in all treatments, whereas stem %N only decreased under elevated [CO_2] in USDA inoculated plants, however, these plants showed the highest N% value at ambient [CO_2], and a similar N% to the other treatments at elevated [CO_2]. Elevated [CO_2] did not decrease root N% in any of the inoculated treatments (Table 2). In the R4 stage nodule N% was not modified by [CO_2] or inoculation treatments (Table 2).

Overall, elevated [CO_2] boosted more DW accumulation and nodule formation in plants inoculated with SFJ14-36, although they ended up producing a similar amount of biomass than other strains at the end of plant growth. However, at ambient [CO_2], plants inoculated with SFJ14-36 showed the lowest aboveground and nodule biomass in comparison to the other strains. In addition, nodules of SFJ14-36 grown under ambient [CO_2] were smaller and seemed less functional than the nodules inoculated with USDA110 or SFJ4-24 (data not shown). Plants inoculated with SFJ14-36 and grown at ambient [CO_2] also showed lower ^{15}N accumulation and therefore lower N_2-fixation than SFJ4-24. It also seemed that at ambient [CO_2], SFJ14-36 needed more time to nodulate

Harvest at R2								
	Leaf N %		Stem N %		Root N %		Nodule N %	
B. japonicum	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2
USDA110	3.34 a	3.50 a	1.88 b	2.01 b	1.30 cd	1.56 abc	6.25 ab	6.74 a
SFJ14-36	3.57 a	3.66 a	3.05 a	1.95 b	1.82 a	1.31 cd	4.65 c	6.18 ab
SFJ4-24	3.42 a	3.12 a	1.91 b	2.14 b	1.53 bc	1.33 cd	5.73 b	5.99 ab
Non-Inoc. +N	3.20 b	2.18 b	1.73 bc	1.33 c	1.74 ab	1.22 d	-	-
Effect	F	*p-value*	F	*p-value*	F	*p-value*	F	*p-value*
Inoculation	5.35	0.0115	12.05	0.0004	0.93	0.4506	5.96	0.0197
CO_2	2.45	0.1402	6.24	0.0255	14.39	0.002	8.75	0.0143
Inoc x CO_2	2.43	0.1080	7.16	0.0038	7.95	0.0024	2.29	0.1516
Harvest at R4								
	Leaf N %		Stem N %		Root N %		Nodule N %	
B. japonicum	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2
USDA110	4.89 a	3.56 b	2.62 a	2.12 b	1.54 a	1.60 a	5.67	6.11
SFJ14-36	4.72 a	3.69 b	1.96 b	2.01 b	1.49 a	1.46 ab	4.53	5.22
SFJ4-24	4.38 a	3.47 b	2.19 b	1.98 b	1.45 ab	1.20 bc	5.42	5.21
Non-Inoc. +N	4.49 a	3.27 b	2.19 b	1.95 b	1.39 ab	1.11 c	-	-
Effect	F	*p-value*	F	*p-value*	F	*p-value*	F	*p-value*
Inoculation	1.99	0.1610	3.54	0.0426	5.48	0.0106	1.73	0.2271
CO_2	75.69	≤0.0001	6.15	0.0264	4.08	0.0628	0.47	0.5071
Inoc x CO_2	0.52	0.6760	1.49	0.2613	1.76	0.1999	0.36	0.7056

Table 2: Mean values and ANOVA results (F, *p-value*) of leaf, stem, root, and nodule N content (%) of soybean plants grown at ambient (Amb, 400 µmol CO_2 mol^{-1}) and elevated (Ele, 700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were harvested at R2 and R4 developmental stages. Means showing the same letter are not significantly different (P =0.05) in the LSD test (n = 6).

because at R4, these plants had increased their nodule number. These observations made us suspect that SFJ14-36 had some problems to nodulate at ambient [CO_2], or that the bacteria were not functional at fixing atmospheric N_2. For that reason a field experiment at ambient [CO_2] and in soil where there was no history of soybean plantation (and therefore an absence of competitive rhizobia) was performed to determine whether SFJ14-36 could nodulate and produce yields similar to SFJ4-24 and USDA110.

Soybean plants were grown at the Neiker Research Centre under two management techniques, conventional and organic, in two fields in which no history of soybean had ever been recorded and after a corn-cereal rotation. The results show that yield was not affected by the field management technique (Table 3). In both fields, USDA110 and

SFJ4-24 were the most productive strains, with the Non Inoc. treatment and SFJ14-36 showing the lowest yields. Similar results were obtained when the plants height was measured at the R7 stage (Table 3). In a general way, plants grown in the conventional field were taller than the ones grown in the organic field. At the R2 developmental stage, plants were pulled from the field to check for root nodules. Plants inoculated with USDA110 and SFJ4-24 showed abundant nodules, whereas plants inoculated with SFJ14-36 and the Non Inoc. treatment had no nodules in both fields (data not shown). SPAD measurements, which are an estimation of leaf chlorophyll content, showed lower values in the Non Inoc. and SFJ14-36, in comparison with USDA110 and SFJ4-24 (Table 3), suggesting a N deficiency as a consequence of the lack of nodulation and thus N2-fixation.

	Yield		**Height at R7**		**SPAD at R2**		**SPAD at R5**	
B. japonicum	Conventional	Organic	Conventional	Organic	Conventional	Organic	Conventional	Organic
USDA110	4036 A	3414 a	86.5 A	77.6 a	43.08 A	42.23 a	46.05 A	45.74 a
SFJ14-36	2552 C	2128 b	77.4 AB	66.5 b	43.12 A	38.85 a	40.93 B	36.80 c
SFJ4-24	3205 B	3210 a	82.1 A	76.6 a	42.72 A	39.73 a	44.34 AB	41.76 b
Non-Inoc. +N	1830 D	2261 b	69.3 B	65.0 b	41.96 A	37.73 a	36.65 C	39.18 bc
Effect	F	*p-value*	F	*p-value*	F	*p-value*	F	*p-value*
Inoculation	29.75	≤0.0001	8.83	0.0006	1.87	0.165	17.72	≤0.0001
Management	1.16	0.2932	10.43	0.004	13.4	0.0015	1.61	0.218
Inoc. x Manag.	2.73	0.694	0.44	0.7281	0.91	0.4542	2.68	0.0732

Table 3: Mean values and ANOVA results (F, *p-value*) of yield, plant height (measured at R7), and SPAD (measured at R2 and R5) of soybean plants grown under two management techniques, conventional and organic, at ambient [CO_2] (~400 µmol CO_2 mol^{-1}), and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated (Non-Inoc.). Means showing the same letter are not significantly different (P =0.05) in the LSD test (n = 4). Conventional and organic practices were not compared between each other at the level of the inoculation treatment, therefore the LSD values comparing inoculation treatments are in capital (Conventional) and lower case (Organic) letters to differentiate between the two practices.

4. DISCUSSION

According to the expression data obtained by Sugawara & Sadowsky (2013), we hypothesized that at elevated [CO_2] the plants inoculated with SFJ14-36, which is the strain isolated at elevated [CO_2] at SoyFACE, would show higher nodule number and N_2-fixation rates than SFJ4-24 (strain isolated at ambient [CO_2]) and USDA110, due to its higher expression of nodulation genes detected under these conditions. Our data confirmed that plants inoculated with SFJ14-36 developed similar or greater numbers of nodules than USDA110 at elevated [CO_2] but only at R4 (Table 1). However, plants inoculated with SFJ14-36 and SFJ4-24 fixed less N_2 than USDA110 at elevated [CO_2] at R2, therefore contradicting the expression data published by Sugawara & Sadowsky (2013) and our hypothesis (Fig. 1), although at R4 %N was similar in SFJ14-36 and in USDA110 . The higher N_2-fixation of USDA110 could be explained by the fact that the plants inoculated with this strain showed higher nodule biomass and lower nodule numbers, suggesting that these plants possess larger nodules than SFJ14-36 and SFJ4-24 plants. It has been reported that soybean genotypes with larger nodules usually have higher N_2-fixation rates because of their bigger infection zone, greater bacteroid numbers and thus increased nitrogenase content (Purcell et al., 1997; King & Purcell, 2001). Thus, the higher N_2-fixation rate of the USDA110 plants observed in this study might be associated with them having fewer but larger nodules than SFJ14-36 and SFJ4-24 plants. The high efficiency in N_2-fixation of USDA110 was demonstrated previously because USDA110 fixed more N_2 than unknown native bacteria isolated from SoyFACE soils Sanz-Saez et al. (2015).

Nevertheless, these results do not explain the incongruence between the expression data published by Sugawara & Sadowsky (2013) and the nodule numbers and N_2-fixation reported in this study. One of the possible causes of this discrepancy is that

the expression data are usually reported as fold changes in gene expression at elevated [CO_2] relative to expression at ambient [CO_2]. If we do the same exercise and compare the percentage change in nodule dry weight from ambient to elevated [CO_2] in plants inoculated with USDA110 (43.7%) and SFJ14-36 (825%) (Table 1), it seems that SFJ14-36 is more effective at nodulation than USDA110 due to its higher percentage. However, this difference in the percentage value was caused by the low nodule dry weight at ambient [CO_2] because the weight values are not significantly different at elevated [CO_2]. In fact, the plant performance (considered as above-ground biomass accumulation) of plants inoculated with SFJ14-36 and grown at ambient [CO_2] was significantly lower than the USDA110 and SFJ4-24 plants (Fig. 2). This phenomenon could be the consequence of deficient nodulation and thus lower N_2 fixation, as observed in our growth chamber experiment under ambient [CO_2] where SFJ14-36 plants showed the lowest N_2-fixation rate (Fig. 1). In addition to this data, another sign of deficient N_2-fixation at ambient [CO_2] can be the stem N accumulation observed in SFJ14-36 inoculated plants (Table 2) that can be showed as ureids accumulation in legumes in which N_2-fixation is being affected by other abiotic stresses such as drought (Purcell et al., 2000; Coleto et al., 2014). *The* deficient nodulation, or the incapability of SFJ14-36 to nodulate plants at ambient [CO_2] in the growth chamber experiment, was confirmed in the field trials in which plants inoculated with SFJ14-36 did not have any nodules, and the yield was lower than USDA110 and SFJ4-24, and even the same as non-nodulated plants in organic management (Table 3). These results suggest that SFJ14-36 does not nodulate properly at ambient [CO_2], and that its proper nodulation only happens at elevated [CO_2]. However, the growth chamber experiment confirms that the nodulation is not completely defective, because even though the nodule dry weight is the lowest at R2, by R4 it had recuperated and was very similar to SFJ4-24, therefore indicating more of a delay in

nodulation than a complete inefficiency in SFJ14-36. However, in the field experiment, the lack of nodulation in the plants inoculated with SFJ14-36 might be due to the lack of efficiency of SFJ14-36 under ambient [CO_2]; and to the fact that in the field experiments the plants were only inoculated in the seed before planting, in comparison with the growth chamber experiment, in which the plants were inoculated by liquid inoculum during three different dates.

Nodule formation depends on a complex and elaborate signal exchange between the plant and the bacteria, that starts with the secretion of several substances by the plant's roots such as sugars, amino acids, proteins, phenols, and flavonoids among others (Broughton et al., 2003; Cooper, 2007; De-la-Peña et al., 2008, 2010; Badri et al., 2009; Badri & Vivanco, 2009). The secretion of different compounds attracts different species of bacteria, with different plant cultivars determining strain specificity (Hungria et al., 1991, 1992; Dakora et al., 1993). However, it has been demonstrated that different environmental factors such as drought, temperature, salinity, etc., can produce a change in the amount and composition of root exudates, modifying the cultivar by strain specificities (Pan & Smith, 1998; Hatimi, 1999; Hungria & Vargas, 2000; Lira et al., 2005; Andres et al., 2012; Lira et al., 2015). It has been reported in soybean, white clover, and common bean, that elevated [CO_2] in field conditions changes the population structure of *Rhizobium* strains living in the rhizosphere and in the nodules (Montealegre et al., 2000; Hasse et al., 2007; Sugawara & Sadowski, 2013; Wang et al., 2017). This strain specificity has been related to an increase and/or a signal change in root exudates (Hasse et al., 2007; Prevost et al., 2010; Bertrand et al., 2011, Sugawara & Sadowski, 2013; Wang et al., 2017). Therefore, the reason for the nodulation failure or delay of SFJ14-36 at ambient [CO_2] might be a change in the root exudates that hampers the attraction or infection of that bacterium. However, at elevated [CO_2], the conditions at

which this strain was isolated at SoyFACE (Sugawara & Sadowski, 2013), the nodule dry weight and numbers were very similar to the ones observed in our USDA110 control strain, suggesting that at these conditions there is no problem with the chemotaxis or infection of the bacteria. To our knowledge, this is the first study describing an efficiency/inefficiency nodulation response that is indirectly dependent on the atmospheric [CO_2], and might have implications for how we understand plant-rhizobium interactions in a changing environment.

One of the hypotheses of this study was that because SFJ14-36 showed higher expression of Nod genes than USDA110, it might show a higher N_2-fixation that would translate to higher levels of photosynthesis and growth at elevated [CO_2]. However, in the current experiment, as shown previously in soybean inoculated with USDA110 grown in sterile conditions and compared with SoyFACE's native bacteria, nitrogen fixation, photosynthesis, and growth were greater in the presence of USDA110 (Fig. 1; Fig. 3) than native soil bacteria from SoyFACE soil, which probably included a mixture of SFJ14-36, SFJ4-24, and other *Rhizobium* bacteria (Sugawara & Sadowsky, 2013; Sanz-Saez et al., 2015). Therefore, SFJ14-36 did not out-perform USDA110, making our hypothesis invalid. It is worth noting that the lower photosynthesis and biomass accumulation of SFJ14-36 observed at R2 in plants grown under ambient [CO_2] conditions could be related directly and/or indirectly to its lower N_2-fixation rate. To be directly related to the lower N_2-fixation rate, the N concentration or content should be lower in SFJ14-36 plants, affecting the protein concentration etc., and then reducing photosynthesis and growth. In our study, the N% in the leaves for SFJ14-36 plants at ambient [CO_2] was the same as USDA110, however, if we calculate the total N content (N% multiplied by dry weight biomass); USDA110 plants had higher N content than SFJ14-36 plants (Supplemental Table 1). In that case, it could be considered that the lower N_2-fixation of SFJ14-36 was

affecting directly the photosynthetic functions and growth. The low N_2-fixation of SFJ14-36 could also affect photosynthesis indirectly. During flowering (R2), soybean's photosynthesis can be sink-limited as the pods have not been developed yet and carbohydrates tend to accumulate in the leaves and down-regulate photosynthesis via a substrate feedback mechanism (Kaschuk et al., 2012). It has been demonstrated that high rates of N_2-fixation that need to be fuelled by substantial amounts of carbohydrates would eliminate the sink limitation and therefore boost photosynthesis and growth (Kaschuk et al., 2012; Sanz-Saez et al., 2015). In this study, it seems that the lower N_2 fixation may not be enough to compensate for sink limitation, and this could be one of the causes of the low photosynthesis associated with SFJ14-36 at ambient [CO_2]. The fact that SFJ14-36 is not associated with lower photosynthesis than USDA110 at elevated [CO_2] supports this theory. Under these conditions, SFJ14-36 was able to fix more N_2 than under ambient [CO_2], and this might have eliminated the sink limitation, increasing plant growth but not enabling the same biomass accumulation as USDA110. During the reproductive stages (R4), the continuous development of pods may have enhanced C demand and the importance of nodules as sinks may have been reduced (Rogers & Ainsworth, 2006, Rogers et al., 2006), eliminating the differences that existed in photosynthesis between plants inoculated with SFJ4-24 and SFJ14-36 in comparison to USDA110.

In our present study, plants inoculated with USDA110 showed a slightly increased photosynthesis and growth relative to SFJ14-36 at ambient and elevated [CO_2] at the R2 developmental stage (Fig. 2, Fig. 3). In any case, the plants inoculated with native strains isolated by SoyFACE (SFJ4-24, and SFJ14-36) did not out-perform the seed weight production of USDA110, although they showed good performance under these conditions. Contrastingly, plants inoculated with SFJ4-24 and SFJ14-16, produced 26.5% and 23.8% lower seed weight than USDA110 at elevated [CO_2] (Table1). These results

follow a similar trend to that reported by Sanz-Saez et al. (2015), in which plants grown in sterile soil and inoculated with USDA110 showed greater dry weight accumulation than a unknown mixture of native soil bacteria of the same origin as those studied in this experiment. On the other hand, the slightly better plant fitness response of the plants inoculated with SFJ14-36 under elevated [CO_2] in comparison to SFJ4-24 (and vice versa for ambient [CO_2]), could explain why Sugawara & Sadowsky (2013) found this strain in the nodules of plants grown at elevated [CO_2] and not SFJ4-24. This could indicate that plants can discriminate among rhizobium strains depending the plant fitness benefit, as demonstrated by several authors (Kiers et al., 2003; Friesen, 2012; Kiers et al., 2013). However, the change in the rhizobium strain depending on the atmospheric [CO_2] seems to involve more factors than plant fitness, such as root signalling (Haase et al., 2007; Prevost et al., 2010; Bertrand et al., 2011; Sugawara & Sadowski, 2013; Wang et al., 2017) and microorganism competition (Denison & Kiers, 2004; Kiers et al., 2013).

4.1. Conclusion

In this study we showed that the strain isolated at elevated [CO_2] (SFJ14-36) in the SoyFACE at the University of Illinois, produced high levels of nodulation and a strong good plant fitness response similar to inoculation with a reference strain (USDA110), only when grown at elevated [CO_2] conditions. When the plants were grown at ambient [CO_2], those inoculated with SFJ14-36 showed a poor plant fitness response (plant N_2-fixation, photosynthesis, and growth), suggesting that under ambient [CO_2], nodulation and thus plant growth is suppressed or at least delayed. We hypothesized that this poor fitness response at ambient [CO_2] is caused by a change in the secretion of root exudates between elevated and ambient [CO_2] that delays or inhibits nodule formation and thus N_2-fixation and growth. However, more research is needed in order to understand how

changes in environmental conditions can affect this symbiotic relationship and ultimately how we can improve plant fitness in a changeable world.

Authors contributions

ASS, and ML conceived the experiment. ASS, ML, UPL, AMP, AMR performed the experiments. All the authors interpreted data and contributed to the drafting of the manuscript. ASS also supervised the whole project and wrote the manuscript. ML, IA gave experimental advice.

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Supplemental Table 1: Mean values and ANOVA results (F, *p-value*) of total N content (g of N in the total biomass) of soybean plants grown at ambient (Amb, 400 µmol CO_2 mol^{-1}) and elevated (Ele, 700 µmol CO_2 mol^{-1}) [CO_2], and inoculated with 3 *Bradyrhizobium japonicum* strains (USDA110, SFJ14-36, and SFJ4-24) or non-inoculated and supplemented with N (Non-Inoc. +N). Plants were harvested at R2 and R4 developmental stages. Means showing the same letter are not significantly different (P =0.05) in the LSD test (n = 6).

Total N content (g N)				
	Harvest at R2		**Harvest at R4**	
B. japonicum	Amb CO_2	Ele CO_2	Amb CO_2	Ele CO_2
USDA110	0.161 b	0.227 a	1.311 b	2.242 a
SFJ14-36	0.028 e	0.113 d	0.375 c	1.633 b
SFJ4-24	0.109 d	0.128 d	0.794 c	0.857 bc
Non-Inoc. +N	0.321 b	0.497 a	2.231 a	2.445 a
Effect	F	*p-value*	F	*p-value*
Inoculation	70.41	≤0.0001	35.67	≤0.0001
CO_2	23.53	≤0.0001	27.53	≤0.0001
Inoc x CO_2	3.41	0.0470	5.90	0.0080