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Role of fixing nitrogen in common bean growth under water deficit conditions

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Abstract

In common bean (*Phaseolus vulgaris* L), limited information is available if potential of nodulation and symbiotic nitrogen fixation is associated with the expression of traits that confer better adaptation to drought conditions. Adaptive traits were therefore measured in N-fixing bean lines BAT 477, DOR 364, and PAN 185 and in non-nodulating lines BAT 477-NN and DOR 364-NN. Drought affected growth in all bean lines and decreased chlorophyll content but more in DOR 364-NN and BAT 477-NN, and also reduced nodule size with highest reduction in DOR 364 (21.6%). Loss of nitrogen fixation under drought conditions was further associated with lower water use efficiency, measured as carbon isotope ratio of $^{13}C/^{12}C$, and lower %N in shoots when compared to capability to fix nitrogen. Loss of symbiotic nitrogen fixation in mutant bean lines DOR 364-NN and BAT 477-NN negatively affected root architectural traits under drought. Under drought conditions, line BAT 477-NN had about 50% lower values for all root architectural traits, such as root angle, strongly emphasizing an association of capability to fix nitrogen with root development. An association between capability to fix nitrogen and maintaining a better harvest index and seed yield was also found in the study. Under well-watered conditions, all N-fixing lines had a higher harvest index and seed yield (2.6–2.8 t of seed yield/ha) in comparison with the two nonfixing bean lines (1.4 t/ha). Although the harvest index and seed yield declined due to drought in all tested lines, this decline was only significant for line DOR 364-NN (harvest index) and lines DOR 364-NN and BAT 477-NN (seed yield). Our study has overall demonstrated an important association between the capability of maintaining atmospheric N-fixation and expression of traits conferring better adaptation to drought conditions with any change in nitrogen fixation affecting these traits.

KEYWORDS
common bean, drought, nitrogen fixation, nodulation, root architecture, water deficit

1 | INTRODUCTION

Legumes, like common bean (*Phaseolus vulgaris* L), represent some of the most important crop species worldwide. Common bean is also an important subsistence crop for African smallholding farmers with a significant role in human nutrition (Broughton et al., 2003; Petry, Boy, Wirth, & Hurrell, 2015). The bean is often referred to as the meat of the poor due to its

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high protein content and affordability and the bean is further a vital source of micronutrients, such as iron, in an otherwise starch-based diet of the poor. Common bean is therefore a key crop for food security in Africa (Asare-Marfo et al., 2011; Kalyebara & Buruchara, 2008).

The bean is mostly grown under rain-fed conditions where drought can limit sufficient soil water supply. Drought conditions in these agro-ecologies can, therefore, severely affect bean growth (Fenta, Driscoll, Kunert, & Foyer, 2011; Devi et al., 2013; Beebe, Rao, Blair, & Acosta-Gallegos, 2013; Polania, Poschenrieder, Beebe, & Rao, 2016b). For drought adaptation, beans possess traits for more efficient use of soil water and also for maintaining better photosynthesis (Basu, Ramegowda, Kumar, & Pereira, 2016; Polania, Poschenrieder, Beebe, et al., 2016b). In particular, root architectural traits are important to provide not only more water uptake but also better mobilization of photosynthates as well as better mobilization of fixed nitrogen to the seed to maintain productivity (Beebe et al., 2006; Lynch, 2007; Zhao et al., 2004). Such root traits improving water acquisition include more small fine roots as well as higher root length and density (Comas, Becker, Cruz, Byrne, & Dierig, 2013). In common bean, the ability to produce a high root mass is further associated with higher harvestable yield (White & Castillo, 1991) and effective use of water correlates with a deep and dense root system, or high root mass, to produce seed under drought conditions (Fenta et al., 2011; Kobata, Okuno, & Yamamoto, 1996; Mohamed, Keutgen, Tawfik, & Noga, 2002; Polania et al., 2017).

Low soil fertility in marginal areas is, however, a major limitation to common bean yield. Smallholder systems in Africa are particularly affected by low soil fertility due to both nitrogen depletion in the soil and limited application of nitrogen fertilizer (Rao et al., 2016). Beans can access part of their required nitrogen from the atmosphere through symbiotic nitrogen fixation. Common bean has, however, relatively low symbiotic nitrogen-fixing capacity with low rates of nitrogen (N) fixation even under well-watered conditions with an estimated mean value of nitrogen derived from the atmosphere (Ndfa) of 39% (Peoples, Murray John, & David, 2009; Devi et al., 2013). This value is much lower than the mean values of 54%–65% of other widely grown legume crops and specifically the values for soybean and faba bean with 68% and 75%, respectively (Peoples et al., 2009). The best N-fixing bean lines under water deficit are further more drought-tolerant (Polania, Poschenrieder, Rao, & Beebe, 2016a and Polania et al. 2016b). However, even more drought-tolerant bean genotypes are severely affected by nitrogen deficiency (Beebe, Rao, Devi, & Polania, 2014; Devi, Sinclair, Beebe, & Rao, 2013).

Soil water deficit has a particular negative effect on the nodulation process and also rhizobium bacteria (Sinclair & Vadez, 2012). In nodules, rhizobium bacteria carry out biological nitrogen fixation and also carbon–nitrogen metabolism and metabolite transport across cell membranes occur (Liu, Contador, Fan, & Lam, 2018a). Root nodules therefore facilitate carbon influx and nitrogen compound efflux, whereas the plant provides sucrose as energy to fix atmospheric nitrogen (Day et al., 2001). Nodule formation is further largely under plant control depending on environmental conditions and the physiological state of the host plant. Phytohormones are important regulators of root nodule symbiosis (Liu, Zhang, Yang, Yu, & Wang, 2018b; Ryu, Cho, Choi, & Hwang, 2012). Still limited information also exists on a possible association of capability to fix atmospheric nitrogen and traits, such root architectural traits that confer better adaptation to soil water deficit. Such association is still poorly understood, and only a few studies have been so far carried out to simultaneously explore such association and also the genetics behind it (Yang et al., 2017).

Unfortunately, symbiotic atmospheric nitrogen fixation, which is more difficult to measure, is often neglected as an important trait by most bean improvement programs when selecting for drought tolerance. As a result, modern bean breeding programs do rarely focus on breeding genotypes efficient in atmospheric nitrogen fixation. Such focus might be, however, particularly useful in Africa where chemical nitrogen fertilization is costly and drought a severe problem in common bean production. The objective of our study was therefore to investigate a possible association between capability to fix atmospheric nitrogen in nodules and traits that confer better adaptation to soil water deficit due to drought. We specifically asked the question whether bean plants fixing atmospheric nitrogen in their nodules will better express these traits and whether these plants will ultimately also have improved yield than plants without nodules and having lost the capability to fix nitrogen. For our study, we had the particular working hypothesis that in common bean, the capability to fix nitrogen is directly associated with better expression of traits that confer adaptation to drought which will ultimately also result in higher yield. We measured therefore in our study several traits which can be affected by drought, such as water use efficiency and root architectural traits as well as traits for maintaining photosynthesis (chlorophyll). We further used the N-fixing bean lines BAT 477 and DOR 364 and another N-fixing bean line PAN 185 as a control and compared these lines with two nonfixing mutant bean lines BAT 477-NN and DOR 364-NN not producing any nodules (Diaz et al., 2017; Polania, Poschenrieder, Rao, et al., 2016a). Previous research has already shown that N-fixing line BAT 477 has deep rooting ability and also fixes nitrogen under soil water deficit conditions (Castellanos, Peña-Cabriales, & Acosta-Gallegos, 1996; Sponchiado, White, Castillo, & Jones, 1989). In contrast, N-fixing bean line DOR 364 is regarded as a “good yielder” under well-watered conditions but sensitive to soil water deficit conditions (Beebe, Ochoa, Skroch, Nienhuis, & Tivang, 1995).

For the determination of atmospheric nitrogen fixation, we applied in our study the ^{15}N natural abundance method often applied for the estimation of atmospheric nitrogen fixation ability of legumes in the field (Holdensen, Hauggaard-Nielsen, & Jensen, 2007; Polania, Poschenrieder, Rao, et al., 2016a). There are two types of nitrogen isotopes, ^{14}N and ^{15}N. SNF is determined by the $^{15}N/^{14}N$ ratio when the plant ^{15}N concentration is different from the concentration in the surrounding environment and $\delta^{15}N$ is thereby a measure of the ratio of the two stable nitrogen isotopes. In general, non-N-fixing plants have greater $\delta^{15}N$ values than N-fixing plants. In addition to atmospheric nitrogen fixation determination, we also employed in our study the carbon isotope ratio of $^{13}C/^{12}C$ as an indirect indicator of water use efficiency (WUE), a trait sensitive to drought stress (Beebe et al., 2013). Since WUE determination is difficult and time-consuming in particular under field conditions, the carbon isotope ratio of $^{13}C/^{12}C$ in plant tissue, as measured also in our study, has been previously found as an indirect indicator of WUE (Rytter, 2005).

2 | MATERIALS AND METHODS

2.1 | Experimental site and planting procedure

An off-season field study was conducted at Ukulima Root Biology Center (URBC), operated by the Pennsylvania State University in Limpopo Province, South Africa (24°032.002’S, 28°007.427’E and 1237m above sea level). The area had a total rainfall during the growing season when the experiment was carried out of 34 mm with an average maximum/minimum temperature of 26-33/14-17°C.

The soil texture of the field was sandy according to the USDA soil classification (USDA, 2011). Since we worked on a shallow-rooted crop, a soil depth profile was, however, not specifically established. Prior to experiments, a soil analysis for both macro- and micronutrients was conducted by the Alpha Agric PLC soil analysis laboratory, Nylstroom, South Africa. Nutrient analysis by extracting soil sample in diethylenetriaminepentaacetic acid (DTPA) revealed values for pH (in KCl) of 5.82, available P 18 mg/kg, K 50 mg/kg, Na 12 mg/kg, Ca 196 mg/kg, Mg 57 mg/kg, Fe 4.62 mg/kg, Mn 2.37 mg/kg, Cu 0.15 mg/kg, Zn 0.85 mg/kg, and a cation exchange capacity (CEC) of 1.63. Based on the recommendation made by the laboratory, 4 kg/ha boron, 1 kg/ha zinc sulfate, and 25 kg/ha potassium sulfate were applied to overcome nutrient limitations in the soil.

2.2 | Plant material

Common bean (*Phaseolus vulgaris* L.) lines used in this field experiment were inbred line BAT 477, with deep rooting ability (Sponchiado et al., 1989) and also good N-fixing ability under moisture stress (Castellanos et al., 1996), and also inbreed line DOR 364 with good yielding under well-watered condition but drought-sensitive (Beebe et al., 1995). Also used in the experiment were two mutant lines that have lost nodulation capacity (DOR 364-NN and BAT 477-NN) acquired from CIAT (Polania, Poschenrieder, Rao, et al., 2016a and Polania et al. 2016b; Diaz et al., 2017) as well as one commercial nitrogen-fixing cultivar widely grown in South Africa (PAN 185).

2.3 | Pest control

Before land preparation, a post-emergence non-selective herbicide Agroquat (Syngenta Crop Protection, Inc.) and Roundup (Monsanto Plc) at 3 L/ha were applied to kill all aboveground green tissue of actively growing plants on the field. The land was prepared by plowing and row-making applying a tractor with mounted farming implements. Before planting, pre-emergence herbicides Unimoc (Meridian Agrochemical Company (Pty) Ltd) EC 800 ml/ha and Imazethapyr (American Cyanamid Co.) 400 ml/ha were applied to control both grass and broadleaf weeds. Frequent hand weeding was also carried out as needed. To prevent nematode infestation, the nematicide Oxamyl (SinoHarvest Agrochemical Manufacturer) (3 L/ha) was applied immediately after planting and also after at one month of planting.

2.4 | Experimental design and data collection

The experimental design was a randomized complete block with two water treatments. Plants were grown in one block under adequate water supply where plants were irrigated at a regular interval to keep the soil moisture status near field capacity. During the first four weeks of growth, plants were watered regularly (8 mm/day) applying pivot sprinkler irrigation to maintain optimum growth. The second treatment block received a limited water supply, and water stress was initiated one month after planting. The trial was, however, exposed to three days of rain at 7th, 19th, and 26th days after commencement of drought with 14, 9, and 11 mm (a total of 34 mm rain), respectively. The rain did not affect the drought experiment, since irrigation had also be planned once per week for drought plots. Drought stress lasted for one month.

Plants for each treatment were established in six rows with spacing of 75 cm × 10 cm between rows and plants, respectively. Row length was 4 m with a single plot size of 15 m^2. Distance between rows was deliberately increased to facilitate root sampling at harvest and allowing use of farm implements. Four rows were used for data collection, and the outside rows were used as a border. Each treatment had also three replicates. Between plots, 75 cm space was left and 1.2 m between replication and 1 m as border. The two water regimes were separated by 4 m space. One seed per position was planted with a jab planter which allowed to plant with a uniform 5 cm depth.

Volumetric water content was measured to evaluate the water status of the soil at the initiation of the drought treatment and every five days during crop development. Soil sampling was conducted by taking a soil core with a steel corer lined with a plastic tube (60 cm length and 42 mm diameter) acquired from Giddings Machine Company Inc. The corer is also used as a standard core sampler by the Pennsylvania State University Root Biology Center and International Center for Tropical Agriculture (CIAT) to allow taking a soil core of 60 cm in length sufficient for working with a shallow-rooted crop like common bean. Four samples per replication (twelve samples) were taken from each irrigation regime. Soil samples were not mixed and were independently oven-dried to allow independent calculations. After determining the mass of wet soil, the soil was oven-dried for 48 hr at 105°C. Finally, the volumetric water content was calculated according to Brady and Weil (2008).

2.5 | Chlorophyll content

Three plants of each variety per plot (nine plants per water regime treatment) were sampled at the beginning and at the end of water deficit treatment using the central leaflet with same age of the 3rd and 4th trifoliate leaf. Chlorophyll content of leaves was measured using the Chlorophyll Meter SPAD-502 (Konica Minolta Sensing, Inc.), and chlorophyll content was determined nondestructively by taking the average of three individual SPAD chlorophyll meter readings (SCMR). Before measurement, the SPAD-502 meter was first calibrated by taking extracted chlorophyll values from individual cultivars applied in the study (Markwell, Osterman, & Mitchell, 1995).

2.6 | Root architecture

Soil coring was carried out for quantifying root distribution across soil depth. Three soil samples were taken for each plot under well-watered and drought conditions. Samples were collected applying the identical procedure already described for the determination of soil moisture content by placing the soil core at a point mid-way between two plants (5 cm from each plant with a total distance between plants of 10 cm).

After coring with a steel corer lined with a plastic tube (60 cm length and 42 mm diameter) to allow taking a soil core 60 cm in length sufficient for working with a shallow-rooted crop like common bean, the soil core of three soil samples from each plot was cut into 10 cm pieces (up to 40 cm soil depth) with a fifth cut of 20 cm (40 to 60 cm soil depth). Each segment was washed using a 2 mm size mesh. Separated roots were kept in plastic vials containing water with 25% ethanol. The washed and preserved roots were scanned with the root scanner Epson Perfection V 700 Photo/V 750 Pro (Seiko Epson Corporation 2005). Scanned images were analyzed by image analysis applying the winRHIZO 2008a software specially designed for root architecture measurements (Regent Instruments Canada Inc.). With the help of this software, root architectural characteristics, such as root length, average diameter, total area, and volume, which are considered as drought adaptive root traits, were determined.

Phenotyping for root architecture (Figure 1) was carried out at flowering stage of plants by taking six representative individual plants per plot for each water regime. For the determination of architecture, roots were carefully harvested by applying a "Shovelomics" technique (Lynch, 2011; Trachsel, Kaeppler, Brown, & Lynch, 2011). This technique involves extracting roots with a shovel and gently washing the roots using water. Tap root width (thickness) was determined by measuring the diameter of the tap root 2 cm away from the root origin using an Electronic Digital Caliper 5HA 1890 Model (Omni-Tech electronic Co. Limited). Branching density was determined by counting the lateral roots on a 2 cm root segment from the tap root. The number of whorls (Figure 1) was measured by counting. The whorl angle was determined by displaying the root on 1,800 protractor sketched board (similar to the root in the soil) where the stem is at 0^0 and the angle is measured from the soil surface. For measurement, the protractor is placed on a cardboard and then the lines for each angle were extended by drawing with the help of a ruler to create a bigger size sketch of the protractor. The angles on both sides of the stem were measured, and the mean of measurements was determined. The total number of basal roots was recorded by counting from the whorls. Basal and adventitious root diameter (thickness) was calculated by selecting representative basal/adventitious roots or by taking the mean of the diameter of two or three basal/adventitious roots 2 cm away from the root origin. Branching density was determined by taking a representative area from the basal/adventitious root and counting the healthy lateral roots emerging within a 2 cm root segment for three randomly selected basal/adventurous roots.

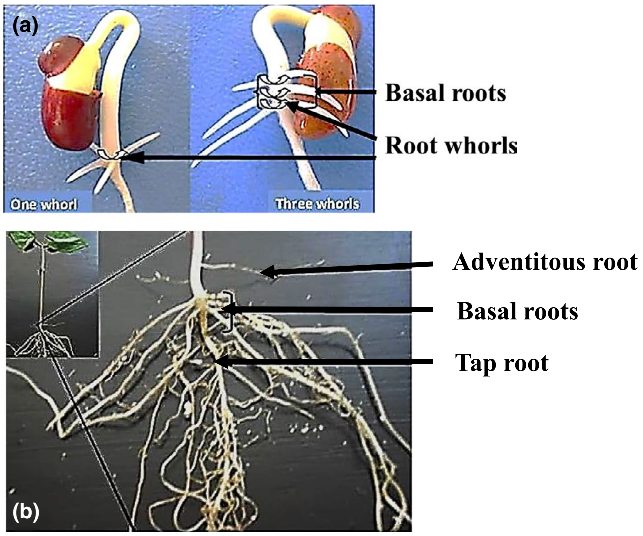

FIGURE 1 Schematic representation of common bean root system architecture with root whorl number (a) and main root types (b)

2.7 | Biomass partitioning and seed yield

Whole aboveground plant samples of six representative individual plants per plot for each water regime were harvested at flowering and at mid-pod filling stage. The vegetative parts were carefully separated into leaves, stems, and pods (at mid-pod filling stage). Dry mass was determined by drying plant material in an oven (TERM-O-MAT LABOTEC, South Africa) at 60°C for 48 hr. For determining seed yield from each plot per treatment, two rows of 3 m length (4.5 m^2 area) were used, disregarding a border (0.5 m) on both extremes of the rows. Harvested plants were counted and applied to calculate the exact area according to the number of the plants harvested to standardize the plot area using the formula:

$$\text{Seed yield per plot} = \frac{\text{Measured seed yield per plot} * \text{plot area}}{\text{Calculated harvested area}}$$

The calculated harvest area was determined by the formula:

$$\text{Calculated harvested area} = \frac{\text{Plot area } (4.5\,\text{m}^2) \times \text{counted number of plants at harvest}}{\text{Total number of plants per } 4.5\,\text{m}^2 \text{ plot } (60)}$$

Seed yield was determined by adjusting the seed moisture content at 10% with the following formula.

$$\text{Adjusted seed yield per plot (g)} = \frac{\text{Seed yield per plot (g)} \times 10\%}{\text{Measured seed moisture content (\%)}}$$

Productivity/ha was calculated with the equation:

$$\text{Seed yield (kg/ha)} = \frac{\text{Adjusted seed yield per plot (kg)} \times 10{,}000 \left(\text{m}^2\right)}{\text{Plot area } \left(\text{m}^2\right)}$$

Harvest index (HI), as a measure of productivity and success in partitioning assimilated photosynthates, was further determined as seed biomass dry weight at harvest/total shoot biomass dry weight at mid-pod filling x 100. The shoot biomass at mid-pod filling stage was calculated by sampling six plants per plot and adjusting to a common area, that is, two rows plot area. For determining the biomass partitioning ability of plants of various lines, all plants from one row (3 m length) were counted and harvested independently and then the pod wall and seed were separated carefully by splitting by hand. Samples were dried in an oven at 60°C for 2 days, and the dry mass was determined. Data were used to calculate the pod harvest index (PHI) with the following formula (Beebe et al., 2013).

$$\text{PHI} = \frac{\text{Seed biomass dry weight at harvest (g)}}{\text{Pod biomass dry wight at harvest (g)}} * 100$$

2.8 | Nitrogen fixation

For the measurement of nodule size, we first made a millimeter scale sketch on a board and then we placed nodules on the board and the average size was determined. For the analysis of CID and ^{15}N natural abundance, the plant samples previously used for dry mass determination of aboveground parts (both leaf and stem) were ground to fine powder with a grinder (A 11 basic Analytical Mill, IKA® Works, Inc). The three plants per plot were bulked and ground to make up one replicate. Three replications per treatment were used for analysis. The samples were analyzed with an isotope-ratio mass spectrometer (Thermo electron, Bremen, Germany) at Cape Town University, Department of Archaeology. Nitrogen was expressed in terms of its value relative to atmospheric nitrogen, while carbon was expressed in terms of its value relative to Pee Dee Belemnite. The following procedures were applied to determine these isotopes. Stable carbon isotope ratio was determined with the following equation:

$$\delta^{13}\text{C}‰ = \frac{\mathbf{R}\text{sample} - \mathbf{R}\text{standard}}{\mathbf{R}\text{standard}} * 1000$$

R_{sample} and R_{standard} are the abundance ratio of ^{13}C /^{12}C (δ^{13}C) of the sample and the standard Pee Dee Belemnite (Mostajeran & Rengel, 2007; Rytter, 2005). The value of CID was determined from δ^{13}C of each plant sample, assuming the atmospheric δ^{13}C (δair) was −8‰ (Farquhar, Ehleringer, & Hubick, 1989) with the following formula (Kondo, Pablico, Aragones, & Agbisit, 2004):

$$\text{CID}(‰) = \frac{(\delta\,\text{air} - \delta\,\text{plant})}{(1 + \delta\,\text{plant})} * 1000$$

Natural abundance of δ^{15}N was calculated with the following formula (Valles-De La Mora, Cadisch, & Aluja-Schunemann, 2003):

$$\delta 15\text{N}(‰) = \frac{\%\,\text{atom } 15\text{N (sample)} - \%\,\text{atom } 15\text{N air } (0.36637)}{\%\,\text{atom } 15\text{N air } (0.36637)} * 1000$$

To calculate the percentage of the nitrogen derived from the atmosphere (Ndfa), the following formula was applied (Shearer & Kohl, 1986):

$$\%\text{Ndfa} = \frac{\delta 15\text{N (reference plant)} - \delta 15\text{N (Nfixing plant)}}{\delta 15\text{N (reference plant)} - \text{B}}$$

For calculation of %Ndfa, a reference plant is required which can be either a nonfixing mutant plant or a plant of another nonfixing crop. In our study, we used as reference plant our nonfixing DOR 364-NN and BAT 477-NN mutant plants. B represents the value obtained from the legume that grows in the medium where atmospheric N_2 is the only source of N to the plant. The B value was obtained with the same four bean lines and rhizobium strain applied in the field trial replicated four times (16 samples) and completely dependent on atmospheric N_2 fixation for growth for plants grown under controlled conditions in N-free medium and with N-free nutrient solution. The plant samples for analysis for $\delta^{15}N$ were collected at flowering time from the field trial. The B value obtained was −4.10882. Plant biomass at flowering was measured with six plants per bean line. Calculations were done for two rows with 3 m length to determine the plant N for harvestable area according to Peoples et al. (2009).

2.9 | Statistical analysis

Data were analyzed with the JMP® 9.0 statistical package (SAS Institute Inc.). Analysis of variance was applied to determine significance, and LSmeans Student's *t* test was applied to compare bean lines for measured traits. Multivariate Pearson's correlation analysis was further applied for determining the relationship (correlation) between measured traits.

3 | RESULTS

3.1 | Volumetric soil water content

Before exposure of different bean lines to water deficit, 14% of total soil was water for both the well-watered and drought blocks (Figure 2). This is comparable to the field capacity for sandy soil (Brady & Weil, 2008). The soil water percentage progressively decreased during drought exposure of plants and was only 7.4% after 4 weeks (Figure 2). This was a 45% reduction in the amount of soil water in water deficit plots when compared to the well-watered plots. The water deficit treatment was, however, only effective for 4 weeks after which rain occurred and the soil water status immediately increased.

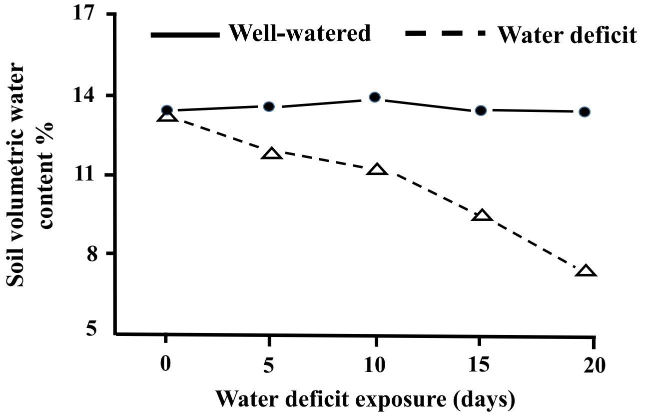

FIGURE 2 Soil volumetric water content (SWC) values for water deficit and well-watered blocks of bean plants. Values represent the mean ± SEM of four soil samples per replication (twelve samples) for each water regime

3.2 | Leaf chlorophyll content and nodule size

We first measured the chlorophyll content of leaves of the different bean lines to determine any effect in plants due to drought exposure. The leaf chlorophyll content was about 10% lower in all the N-fixing lines after 4 weeks water deficit exposure when compared to lines grown under well-watered conditions. This decrease was, however, even higher (19.3%) in the two nonfixing lines, DOR 364-NN and BAT 477-NN (data not shown).

We next determined any water deficit effect on the size of nodules but only in N-fixing lines since the two mutant non-N-fixing lines had no measurable nodules. Previous research suggests that nitrogen-fixing activity of root nodules is closely related to their size and nodule size can be a rapidly measurable effective index of the nitrogen-fixing activity of nodules (King & Purcell, 2001; Tajima, Lee, Abe, Lux, & Morita, 2007). Under well-watered conditions, plants of line BAT 477, and also of the commercial bean line PAN 185, had significantly larger nodules ($p \leq .05$) than plants of line DOR 364 (Table 1). Water deficit reduced the nodules size in all test lines with the highest reduction in plants of line DOR 364 (21.6%). This line is also considered as drought-sensitive (Beebe et al., 1995). The lowest reduction (2%) in nodule size due to water deficit was, however, found in plants of the reference line PAN 185 (Table 1).

TABLE 1 Nodule size of bean lines grown under well-watered and water deficit conditions

	Nodule size (mm)		
Lines	Well-watered	Water deficit	Reduction (%)
DOR 364	2.1 ± 0.3c	1.7 ± 0.2c	21.6
BAT 477	3.0 ± 0.2a	2.4 ± 0.2ab	18.7
PAN 185	2.9 ± 0.3ab	2.9 ± 0.3a	2.0
Significance	*	*	

Note: Data represent mean ± SEM of four plants per plot with twelve individual plants per line. Significance level was determined using ANOVA (**p* < .05). Difference between treatment means was determined using the LSmeans Student's *t* test. Reduction (%) calculated as reduction in nodule size due to water deficit treatment. Means followed by the same letter within the column are not significantly different.

3.3 | Carbon isotope discrimination and nitrogen fixation

In a further step, we determined carbon isotope discrimination (CID), as an indirect measure for water use efficiency (WUE). When a treatment comparison was made for CID of shoot samples under well-watered conditions, all nitrogen-fixing and nonfixing tested lines performed equally with no significant ($p \geq .05$) differences in CID values (Table 2). Water deficit decreased CID values in all tested bean lines indicating a higher WUE in these lines. However, the mutant, nonfixing line BAT 477-NN had the highest CID value under drought when compared to N-fixing lines and also line DOR 364 which had the lowest WUE under drought.

We then measured the nitrogen fixed in plants of the different N-fixing and nonfixing lines. Significant differences were found for tested bean lines for $\delta^{15}N$, %N, and fixed N/plot (4.5 m^2) under both well-watered and drought conditions, with the exception for %N under well-watered conditions (Table 2). In general, a low $\delta^{15}N$ value indicates high symbiotic nitrogen fixation (SNF) and a high $\delta^{15}N$ value low SNF. However, there was no significant difference ($p > .05$) among N-fixing lines for $\delta^{15}N$ under well-watered conditions (Table 2), but nonfixing bean lines had higher $\delta^{15}N$ values than N_2-fixing lines (Table 2). Under water deficit, N-fixing lines significantly differed ($p < .05$) in their $\delta^{15}N$ with N-fixing line BAT 477 having the lowest (more SNF) and the two non-N-fixing lines DOR 364-NN and BAT 477-NN (low SNF) the highest $\delta^{15}N$ values (Table 2). The amount of N-fixed per plot (g/4.5 m^2) further revealed that reference line PAN 185 and the N-fixing line BAT 477 were the best N-fixing lines (3.2–4.9 g/4.5 m^2) under well-watered conditions. Drought severely affected N-fixation. Plants of N-fixing line DOR 264 fixed the lowest amount of nitrogen (0.31 g/plot m^2) and were the most affected plants by drought when compared to all other tested N-fixing lines. Plants of line DOR 264 had also the lowest %N of shoot samples (1.69) of all N-fixing lines under drought (Table 2).

3.4 | Root morphology

In a next step, we wanted to know whether different nitrogen-fixing capacity also affects root characteristics. We therefore measured root length, surface area, volume, and also average diameter in both the N-fixing and nonfixing bean lines as well as in the reference N-fixing line PAN 185 under well-watered conditions and after exposure to drought (Table 3). The two non-N-fixing bean lines (DOR 364-NN and BAT 477-NN) had about 50% lower values for all root morphological traits when compared to the N-fixing bean plants. These much lower values in mutant plants are also a clear indication for the critical importance of capability to fix nitrogen for root growth and development.

TABLE 2 CID ($^{13}C/^{12}C$ carbon isotope ratio), $\delta^{15}N$ ($^{15}N/^{14}N$ nitrogen isotope ratio), fixed nitrogen (N)/ plot (g/m^2), and % nitrogen (N) of shoot samples in nitrogen (N)-fixing and nonfixing bean lines

Lines	**CID (Δ)**		**$\delta^{15}N$**		**Fixed *N* (g/ plot m^2)**		**%*N***	
	WW	**WD**	**WW**	**WD**	**WW**	**WD**	**WW**	**WD**
N-fixing								
DOR 364	22.3 ± 0.26a	20.9 ± 0.53bc	−1.40 ± 0.28c	0.59 ± 0.75bc	2.8 ± 0.27bc	0.30 ± 0.18c	2.2 ± 0.26b	1.6 ± 0.29cde
BAT 477	21.8 ± 0.24abc	20.7 ± 0.13c	−0.40 ± 0.31bc	−0.53 ± 0.48d	3.2 ± 0.12ab	2.2 ± 0.35b	2.6 ± 0.17ab	2.1 ± 0.18b
PAN 185	21.8 ± 0.5abc	20.7 ± 0.27c	0.06 ± 1.14abc	0.58 ± 0.44bc	4.9 ± 1.50a	3.9 ± 0.55a	3.3 ± 0.04a	2.7 ± 0.33a
Nonfixing								
DOR 364-NN	22.2 ± 0.04ab	21.3 ± 0.01ab	0.41 ± 0.16ab	2.8 ± 0.06a	nd	nd	1.4 ± 0.06c	1.5 ± 0.14de
BAT 477-NN	22.2 ± 0.29ab	21.6 ± 0.73a	1.73 ± 0.25a	1.6 ± 0.04b	nd	nd	1.4 ± 0.14c	1.3 ± 0.30e
Significance	*	*	*	*	*	*	*	*

Note: Result mean of three replicates with each replicate from a composite sample of three plant samples for each bean lines for each water regime. Significance level was determined using ANOVA ($^{**}p < .01$, $^{*}p < .05$), and difference between treatment means was determined using the LSmeans Student's *t* test. Means followed by the same letter within the column are not significantly different.

Abbreviations: Nd, not determined. WW, well-watered; WD, water deficit.

TABLE 3 Differences in root morphology traits of nitrogen (N)- and non-N-fixing bean lines grown under water deficit

Lines	Root length (cm)	Root surface area (cm^2)	Root volume (cm^3)	Average root diameter (mm)
N-fixing lines				
DOR 364	57.2 ± 14.2bc	7.0 ± 1.7bc	0.07 ± 0.02cd	0.38 ± 0.02
BAT 477	132.1 ± 23.4a	15.7 ± 2.5a	0.16 ± 0.03a	0.41 ± 0.02
PAN 185	83.8 ± 13.5b	11.1 ± 1.9ab	0.12 ± 0.02ab	0.45 ± 0.02
Nonfixing lines				
DOR 364-NN	55.9 ± 11.2bcd	6.2 ± 1.3c	0.06 ± 0.01d	0.44 ± 0.03
BAT 477-NN	52.2 ± 12.1cd	7.3 ± 1.3bc	0.07 ± 0.01cd	0.41 ± 0.02
Significance	*	*	*	ns

Note: The root image was taken by a root scanner, and analysis was made by using the winRHIZO 2008a software after 4 weeks of drought. Values reported are per plant. Significance level was determined using ANOVA ($^*p < .05$, and ns $p > .05$), and difference between treatment means was determined using the LSmeans Student's *t* test. Means followed by the same letter within the column are not significantly different. The result is the mean ± SEM of four replicates for each treatment acquired from soil cores up to 60 cm soil depth.

Relative to the nonstressed treatment, common bean lines subjected to water deficit responded by increasing their root length, area, and volume by between 15% and 20% when compared to well-watered conditions (data not shown). Among the tested lines, N-fixing line BAT 477 was superior to the other two tested lines under drought conditions with significantly ($p \leq .05$) longer roots, and higher root surface area and root volume (Table 3). In addition, this line had under drought a significantly ($p \leq .05$) higher 1st whorl basal root angle, tap root branching density, basal root number as well as higher basal root branching density (Table 4A, 4). In contrast, we found no significant ($p \geq .05$) differences for all lines when we measured average root diameter, basal root whorl number, tap root diameter, basal root diameter, or adventitious root number. However, BAT 477 superiority was no longer evident in the mutant non-N-fixing bean line BAT 477-NN. Values were no longer significantly different ($p \leq .05$) to values obtained for the line DOR 364-NN (Table 4). This result also indicates that the potential to fix nitrogen is associated with root development.

3.5 | Days to maturity, biomass, and yield

In final experiments, we also wanted to know whether lack of N-fixation affects the days to maturity, biomass, and yield in N-fixing and nonfixing bean lines. No significant ($p \leq .05$) difference between N-fixing and nonfixing bean lines was found for days to maturity under drought conditions which was very similar to days required under well-watered conditions (data not shown). All tested lines matured in 81–96 days with plants of reference line PAN 185 at 96 days the latest. The rather small difference in days between well-watered and drought conditions might have also be determined by rainfall occurring during the field experiment.

We then also wanted to know whether lack of N-fixation measured affects biomass at flowering, mid-pod filling and also at harvest stage. Measuring pod dry mass and total mass (biomass) at pod filling stage is required for the calculation of harvest index. The measured pod harvesting index (PHI) value further reflects the biomass partitioned to seed as a proportion of total pod biomass (Table 5). According to Beebe, Rao, Blair, & Acosta-Gallegos, (2010), measuring the biomass at mid-pod filling stage for common bean is essential to get a reliable biomass for common bean since at this stage, the plant has maximum biomass. After this stage, the plant will start shattering leaves which will not allow to obtain sufficient and reliable data.

In general, all tested lines had lower values for all measured parameters including PHI due to drought. The two non-N-fixing mutant bean lines had further also lower values for all parameters under well-watered conditions clearly indicating the importance of N-fixation for plant growth under well-watered conditions. In addition, the N-fixing reference line PAN 185 had the highest total dry mass at flowering (Table 4). N-fixing line DOR 364 had, however, the most significant decrease due to drought (46%) for total dry mass at flowering and also pod dry mass (30%). Dry mass after drought treatment in the N-fixing line DOR 364 was, however, not significantly ($p \geq .05$) different to the dry mass in the non-N-fixing DOR 364 mutant line. Any decrease in dry mass due to drought in the N-fixing line DOR 364 was therefore related to water deficit-induced lack of nitrogen fixation.

Finally, we also measured the harvest index and seed yield to find out whether nitrogen fixation in plants of lines changes the harvest index and also results in higher seed yield independent of the environmental conditions used. Under well-watered conditions, all N-fixing lines had indeed a higher harvest index and seed yield (2.6–2.8 t of seed yield/ha) in comparison with the two nonfixing bean lines (1.4 t/ha) (Figure 3a,b). In contrast, the harvest index and seed yield declined due to drought but this was only significant ($p \leq .05$) for line DOR 364 (harvest index; Figure 3a) and lines DOR 364 and BAT 477 (seed yield, Figure 3b).

TABLE 4 Differences in root architecture traits of nitrogen (N)-fixing and non-N-fixing bean lines grown under water deficit

Lines	Basal root whorl number	1st whorl basal root angle (^{0})	2nd whorl basal root angle (^{0})	Tap root diameter (mm)	Tap root branching density
(A)					
N-fixing					
DOR 364	2.0 ± 0.0	36.7 ± 3.0bcd	47.3 ± 2.6ab	1.7 ± 0.2	8.0 ± 0.8bc
BAT 477	2.0 ± 0.1	49.7 ± 2.6a	43.3 ± 3.5abc	1.7 ± 0.2	8.7 ± 0.6ab
PAN 185	1.9 ± 0.2	42.3 ± 2.6abc	40.0 ± 3.3abc	1.7 ± 0.2	6.6 ± 0.5bcd
Nonfixing					
DOR 364-NN	1.7 ± 0.1	35.7 ± 2.4cd	43.0 ± 3.2abc	1.5 ± 0.2	7.4 ± 1.2bcd
BAT 477-NN	1.8 ± 0.1	35.0 ± 4.0cd	37.3 ± 2.2bc	1.0 ± 0.1	5.2 ± 0.5d
Significance	ns	*	*	ns	*

Lines	Basal root number	Basal root diameter (mm)	Basal root branching density	Adv. root number	Adv. root diameter (mm)	Adv. root branching density
(B)						
N-fixing						
DOR 364	6.3 ± 0.4bc	0.9 ± 0.1	6.6 ± 0.7b	7.3 ± 1.0	0.30 ± 0.1d	2.7 ± 0.7e
BAT 477	7.6 ± 0.4a	0.7 ± 0.1	9.5 ± 1.2a	9.6 ± 0.8	0.65 ± 0.04ab	5.7 ± 0.9bcd
PAN 185	6.8 ± 0.6abc	1.1 ± 0.1	8.0 ± 0.4ab	8.4 ± 1.2	0.80 ± 0.06a	6.0 ± 0.9bc
Nonfixing						
DOR 364-NN	5.7 ± 0.4c	1.2 ± 0.2	6.8 ± 0.9b	5.5 ± 1.5	0.38 ± 0.05cd	3.4 ± 0.6de
BAT 477-NN	6.3 ± 0.4bc	0.9 ± 0.1	5.4 ± 0.6b	6.6 ± 1.6	0.55 ± 0.11bc	3.9 ± 0.9cde
Significance	*	ns	*	ns	*	*

Note: (A) Significance level was determined using ANOVA ($^{*}p < .05$, and ns $p > .05$), and difference between treatment means was determined using the LSmeans Student's *t* test. Means followed by the same letter within the column are not significantly different. The result is the mean ± SEM of six representative plants per plot exposed to 4 weeks of drought. (B) Adv = adventitious roots. Significance level was determined using ANOVA ($^{*}p < .05$ and ns $p > .05$), and difference between treatment means was determined using the LSmeans Student's *t* test. Means followed by the same letter within the column are not significantly different. The result is the mean ± SEM of six representative plants per plot under drought stress condition after the exposure for one month of moisture stress.

4 | DISCUSSION

We originally hypothesized that in common bean, the capability to fix nitrogen is directly associated with better expression of traits that confer adaptation to drought which will ultimately also result in higher yield. With our study, we found support for this hypothesis. Lack of nitrogen fixation in our mutant bean plants was directly associated with a change in traits adaptive to drought. In our view, it is important to first establish such possible association to find out whether indeed lack of nitrogen fixation has any influence on drought adaptive traits before any more in-depth mechanistic studies will be done to explore the exact link between capability of fixing nitrogen and change of traits adaptive to drought. In general, drought exposure reduces nodule size as found in our study for all N-fixing bean lines with line DOR 364 the most severely affected. However, this line had not only nodules with the smallest size but also greatly diminished atmospheric nitrogen fixation. In general, nodules provide reduced nitrogen, in exchange for photosynthates, to the plant in form of ureides (allantoin and allantoic acid) for biomass production and finally seed protein production with the help of UPS1 transporter proteins (Collier & Tegeder, 2012). Drought can specifically impair nodule nitrogenase activity and also the supply of photosynthates to the nodules and causes breakdown of the oxygen diffusion barrier as well as loss of leghemoglobin (King & Purcell, 2006; Gil-Quintana et al., 2013). However, relatively little information is still available how drought particularly affects this symbiotic relationship between nitrogen-fixing soil rhizobia and the host plant (Ferguson et al., 2010; Kunert et al., 2016).

Our results further emphasized an important association between capability to fix nitrogen and maintaining chlorophyll to carry out photosynthesis and also with root traits not only under well-watered but also under drought conditions. Growth of a plant depends very much on efficient root plasticity to adapt to a changed growth condition (Lynch & Ho, 2005; Osmont, Sibout, & Hardtke, 2007). Deeper rooting providing improved drought tolerance and higher productivity has been previously reported for common bean as an adaptation to soil

TABLE 5 Biomass at flowering, mid-pod filling, and at harvest stage under well-watered and water deficit conditions in different bean lines

Lines	Total dry mass (g) (flowering)		Pod dry mass (g) (mid-pod filling)		Total mass (g) (mid-pod filling)		Pod harvest index	
	WW	WD	WW	WD	WW	WD	WW	WD
N-fixing								
DOR 364	11.4 ± 0.3ab	6.8 ± 0.4cd	10.6 ± 0.6ab	7.4 ± 0.6b	63.7 ± 2.7ab	51.9 ± 1.3de	69.4 ± 0.5ab	54.0 ± 1.9cd
BAT 477	11.2 ± 0.3b	10.1 ± 0.4ab	11.8 ± 0.5a	9.9 ± 0.4a	63.3 ± 1.5ab	60.7 ± 1.6a	67.9 ± 1.9abc	63.8 ± 1.abcd
PAN 185	12.3 ± 0.3a	10.9 ± 0.3a	11.5 ± 0.6a	7.8 ± 0.3b	65.1 ± 1.5a	60.5 ± 1.2a	68.3 ± 0.7abc	67.1 ± 1.5ab
Nonfixing								
DOR 364-NN	9.9 ± 0.5c	7.8 ± 0.4c	6.9 ± 0.7cd	6.8 ± 0.4b	55.2 ± 1.3d	53.4 ± 0.8cde	60.6 ± 0.6d	51.8 ± 4.05d
BAT 477-NN	8.8 ± 0.2d	5.9 ± 0.5d	8.3 ± 0.5d	7.4 ± 0.59b	55.0 ± 0.7d	51.2 ± 0.7e	57.9 ± 1.6d	51.9 ± 1.3d
Significance	*	*	*	*	*	*	*	*

Note: Value for biomasses is for mean of six individual plants (g/plant). Different letters within a column denote significant difference ($p < .05$).
Abbreviations: WW, well-watered; WD, water deficit.

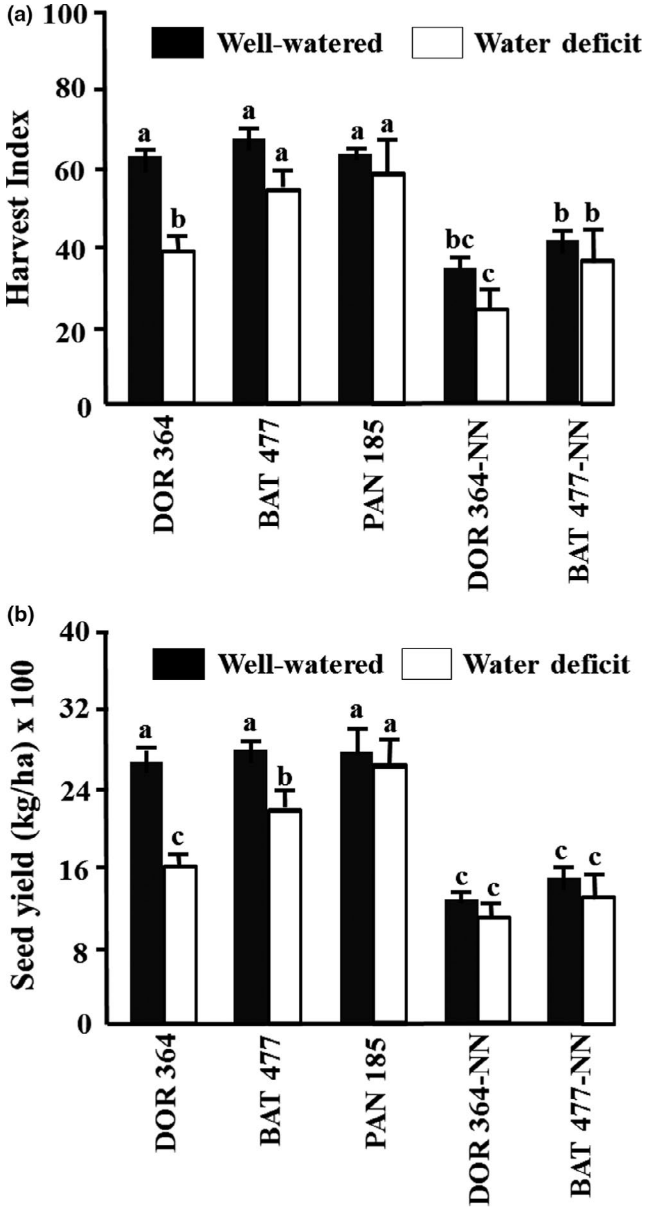

FIGURE 3 Harvest index (a) and seed yield (b) of bean lines grown either under well-watered (closed bars) or water-limited growth condition (open bars). Values represent the mean ± SEM of four plants for each line and water regime. Different letters on bars denote a significant difference ($p < .05$)

water deficit (Sponchiado et al., 1989). In particular, the more drought-tolerant N-fixing line BAT 477 had in our study better root characteristics. The line was also more efficient in fixing nitrogen than the other lines. Our N-fixing lines had, in comparison with the two non-N-fixing lines, further the advantage of a more favorable root angle. Zhao et al. (2004) has previously classified the soybean root angle into three categories: shallow (<40^0), intermediate ($40–60^0$), and deep (>60^0). PAN 185 and BAT 477 exhibited $40^0–60^0$ whorl angles and both have therefore an "intermediate" root architecture. Plants with an intermediate root architecture have already the advantage of better acquiring nutrients and water (Zhao et al., 2004). In contrast, plants with a deeper root and a higher root angle can absorb water from deeper soil layers under water-limited condition (Singh et al., 2010; Zhao et al., 2004). However, we still have to find out whether better drought tolerance of BAT 477 solely depends on a more favorable root angle or whether this line has simply more roots and therefore accesses more water, which in turn would permit more N-fixation.

We were also interested to find a possible association between capability to fix nitrogen and efficiency to use water (WUE), which increases under drought (Hatfield & Dold, 2019). WUE is defined as the amount of carbon assimilated as biomass or grain produced per unit of water used by a crop. Higher WUE is therefore associated with higher photosynthetic CO_2 assimilation per unit of water which ultimately contributes to higher seed yield (Ehleringer, 1990). Passioura (1996) also hypothesized many years ago that productivity particularly under drought is the function of the effective use of water. The ability to convert the photosynthetic assimilate into a harvestable product and WUE only increases if a plant with satisfactory water management traits better exploits water reserves of the soil even under drought conditions (Varga, Vida, Varga-Laszlo, Bencze, & Veisz, 2015). In our study, we measured WUE by carbon isotope discrimination (CID), which can be applied as an indirect indicator for WUE (Martin & Thorstenson, 1988; Rytter, 2005). However, we only determined CID in shoots and not in seeds, since we found a too high variation in values for seeds particularly under well-watered conditions (18.33 to 21.69‰; drought 18.80 to 20.49‰). The difference of CID between shoot and seed is further partly due to the difference in the carbon source. The shoot carbon isotope measurement includes carbon obtained from photosynthetic sugar, whereas in the seed, it includes carbon from starch reserves that have been assimilated from the plant (Deleens, Cliquet & Prioul, 1994). A positive relationship between CID, root length density, and grain yield has been previously found in bush bean under moderate drought or in nonarid environments (Sponchiado et al., 1989; Hall, 2004). In addition, CID measurement also serves as a measure of stomatal behavior. Polania, Poschenrieder, Beebe, et al. (2016b) further classified beans due to their reaction to water deficit as "water spenders" with open stomata, and implicitly not necessarily efficient, and "water savers" with stomata that close more readily, and implicitly more efficient. Stomata that are open permit access to ^{12}C, while stomata that are closed force the photosynthetic apparatus to fix whatever carbon is in the intercellular spaces including ^{13}C. In sugar beet, a stable high carbon isotope ratio (CID) of $^{13}C/^{12}C$ in plant tissue has been found to be negatively correlated to WUE (Rytter, 2005). Selection for low ^{13}C discrimination has been further proposed in C_3 crop breeding as a screen for improved WUE (Easlon et al., 2014). In our study, drought treatment generally decreased the CID value indicating increased WUE in all tested lines. We found, however, under drought the highest CID values in our nonfixing mutant lines. This finding provides strong evidence that loss of capability to fix nitrogen is associated with lower WUE. Maintaining the capability of atmospheric N-fixation in common bean under drought conditions is therefore important for using available soil water more efficiently. However, a future study has still to confirm that our mutant non-N-fixing lines are indeed better "water spenders" with lower WUE and possibly also with more open stomata under water deficit. Plants also produce and accumulate increased amounts of the phytohormone abscisic acid (ABA) in the guard cells under water deficit conditions inducing stomatal closure to conserve water (Lim, Baek, Jung, Kim, & Lee, 2015). An interesting aspect to also explore in the future would be to find out whether lack of capability to fix atmospheric nitrogen in our mutant lines has a direct effect on ABA synthesis causing less efficient closure of stomata (Lu et al., 2015).

When we further investigated any existence of an association between CID and $\delta^{15}N$, we found that our N-fixing bean lines with higher WUE had also higher %N in their shoot than nonfixing lines with lower WUE. This result is consistent with earlier findings of Raven and Farquhar (1990) that a change of CID is due to a change in nitrogen availability to C_3 plants. In plants, the CID value is generally affected by the amount of nitrogen and its metabolism due to the requirement of carbon atoms from CO_2 assimilation in organic N-compounds (Raven & Farquhar, 1990). A complementation of carbon fixation with nitrogen fixation as well as nitrogen metabolism has been also reported for lentil (Knight, Verhees, Kessel, & Slinkard, 1993). Silsbury (1977) further suggested that considerable amount of CO_2 assimilation is indispensable for effective nodule activity.

Finally, we also examined whether capability to fix nitrogen is associated with higher biomass and seed yield. Such an association was indeed found in our study but not only under well-watered conditions but also under drought conditions, despite that atmospheric nitrogen fixation only partially contributes to the nitrogen content of common bean plants (Peoples et al., 2009; Devi et al., 2013). The harvest index and seed yield declined due to drought in all tested lines. But this decline was only significant for mutant line DOR 364-NN (harvest index) and for mutant lines DOR 364-NN and BAT 477-NN (seed yield). This result indicates the importance of capability to fix nitrogen for maintaining a better harvest index and also seed yield under water deficit. Lower reduction of seed yield found in both N-fixing bean lines under drought conditions might further indicate that improved adaptation to drought conditions is due to capability of fixing nitrogen causing change of root traits in these lines. More effective production of auxin might thereby also play an important role. Several studies have already highlighted the involvement of auxin and cytokinin in the regulation of the nitrogen-fixing symbiotic interaction and the positive role of auxins and cytokinins in plant root susceptibility to symbiotic bacteria

(Boivin, Fonouni-Farde, & Frugier, 2016). A more detailed study with our mutant lines might actually also allow to investigate such possible link between auxin production and the capability to fix nitrogen.

In conclusion, our study has contributed with new findings to better understand in common bean a possible association between capability to fix atmospheric nitrogen and expression of traits, particularly root architectural traits, conferring better adaptation to soil water deficit caused by drought. In this regard, the availability and characterization of non-N-fixing mutant bean lines provided an excellent tool to study such association. However, in the future, the exact mechanisms for the link between nitrogen fixation and such change of traits have to be investigated in more detail. Results in other species have also indicated that nonnodulating bean mutants have lost the ability to associate with mycorrhizae (Sagan, Morandi, Tarenghi, & Duc, 1995). It will be therefore worthwhile to also investigate in the future any association between nodulation potential and plant–arbuscular mycorrhizal fungi interaction. Finally, since measurement of atmospheric nitrogen fixation is often neglected as an important trait by most bean improvement programs when selecting beans for more tolerance against drought, we have overall provided with our study strong evidence for the usefulness of inclusion of this trait in breeding programs due to its association with the expression of traits providing better adaptation to drought conditions. Such inclusion would be further particularly useful in Africa where chemical nitrogen fertilization is costly and drought a severe problem in bean production.

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