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Improvement of rhizobium-soybean symbiosis and nitrogen fixation under drought

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Abstract

The symbiotic interaction between soybean plants and rhizobacteria can be severely affected by drought, which results in a reduction in symbiotic nitrogen fixation and ultimately decreased yields. The aim of our research was to determine whether symbiotically efficient rhizobia that can better tolerate soil water deficits can improve nodule performance in plants subjected to drought. Firstly, rhizobial strains were selected that exhibited differences in tolerance to salt (NaCl) or water deficit (PEG 6000). *Sinorhizobium fredii* strain SMH12 showed the highest tolerance to these treatments while *Bradyrhizobium diazoefficiens* strain WB74-1 showed the lowest tolerance. Greenhouse-grown Prima 2000 soybean plants were then inoculated with either SMH12 or WB74-1 and subjected to two water deficit regimes. Different nodule and plant growth traits were determined, including nodule number, dry weight, water potential, and the accumulation of malondialdehyde and ureide. Plants inoculated with SMH12 had significantly more nodules under water deficit conditions than those inoculated WB74-1, despite having lower root and shoot biomass. SMH12-inoculated plants had higher nodule water potentials and lower malondialdehyde contents than the WB74-1-inoculated plants. These results demonstrate that inoculation of soybean plants with the more water deficit-tolerant *S. fredii* strain improved nodule characteristics when plants were grown under water deficit conditions. However, these improved nodule characteristics do not always directly translate into better plant growth.

KEYWORDS

drought, nitrogen fixation, osmotolerance, rhizobium, soybean

1 | INTRODUCTION

Predicted climatic changes with less water availability for plant growth due to drought conditions will severely affect sustainability of yield of crops such as soybean with a worldwide production of 320.15 million metric tons in 2015/2016 (Foyer et al., 2016). Selection of more drought-tolerant soybean cultivars better tolerating soil water deficit is therefore important to avoid an imminent threat to both food and protein security (Foyer et al., 2016; Ku et al., 2013).

Besides investigating, particularly drought effects on aboveground parts of soybean plants, there is recently an

TABLE 1 Bacterial strains used in this work

Bacterial strain	Place of origin	Source of reference
Sinorhizobium fredii HH103	Hubei province China	Dowdle and Bohlool (1985)
Sinorhizobium fredii HH17	Henan province China	Thomas-Oates et al. (2003)
Sinorhizobium fredii HWG35	Shang Don province China	Thomas-Oates et al. (2003)
Sinorhizobium fredii SMH12	Vietnam	Rodriguez-Navarro et al. (1996)
Bradyrhizobium diazoefficiens WB74-1	Canberra, Australia	Botha, Jaftha, Bloem, Habig, and Law (2004)

increasing interest in studying soybean roots and root nodules of plants exposed to drought (Ferguson et al., 2010; Kunert et al., 2016). However, there is still relatively little interest in investigating how drought affects the symbiotic relationship between nitrogen-fixing soil rhizobacteria and the host plant for biological nitrogen fixation as a low-cost source of nitrogen. In symbiotic nitrogen fixation (SNF), rhizobacteria belonging to the genera *Bradyrhizobium* and *Sinorhizobium* interact under nitrogen-limiting conditions with legume roots to develop symbiotic nodules in which atmospheric nitrogen is reduced to ammonium available for a legume plant like soybean as nitrogen supply. Difference between the two genera is that *Bradyrhizobium* strains, such as *Bradyrhizobium diazoefficiens* (*B. diazoefficiens*) reclassified from (*B. japonicum*; Delamuta et al., 2013), are slow growing on a yeast mannitol medium, whereas *Sinorhizobium* strains, such as *S. fredii and S. xinjiangense*, are fast growing on a yeast mannitol medium (Rodríguez-Navarro, Margaret Oliver, Albareda Contreras, & Ruiz-Sainz, 2011). In America and South Africa, slow-growing rhizobial strains, such as *B. diazoefficiens* and *Bradyrhizobium elkanii*, are predominantly used as commercial soybean inoculants due to their higher nitrogen-fixing efficiency (Hungria, Boddey, Santos, & Vargas, 1998), whereas in China fast-growing rhizobial strains, such as *S. fredii,* are applied. However, *Sinorhizobium* strains can also be applied as a general inoculant, not only in China, but also in other soybean growing regions in case a suitable specific soybean host partner has been identified (Muñoz et al., 2016; Tian et al., 2012).

The symbiotic interaction of soybean plants with rhizobacteria is severely affected by soil water deficit, due to drought conditions, resulting in a reduction of SNF and ultimately soybean yield. Soil water deficit not only reduces the quantity of rhizobacteria but also their development and infection ability (Hungria & Vargas, 2000; Venkateswarlu, Saharan, & Maheswari, 1990). In cells, water stress also causes free radical formation resulting in protein denaturation and lipid peroxidation (Mattos & Moretti, 2015). Rhizobacterium includes many species which survive not only severe salt but also drought stress conditions allowing them to persist in dry soils with improved colonization and infection (Fernandez-Aunión et al., 2010; Mhadhbi et al., 2011; Vriezen, Bruijn, & Nusslein, 2006). For example, a highly salt-tolerant strain (4H41) belonging to the species *Sinorhizobium meliloti* has already been isolated from common bean root nodules grown in soil samples originating from an oasis in Tunisia (Mnasri, Mrabet, Laguerre, Aouani, & Mhamdi, 2007). This strain was more competitive and more effective in nitrogen fixation within common bean nodules under soil water deficiency than the commonly used inoculant *Rhizobium tropici* CIAT899. This inoculant nodulates a variety of legumes and produces nodulation factors under abiotic stress conditions such as acidity or a high salt concentration (del Cerro et al., 2017). In general, more stress-tolerant rhizobial strains induce the formation of nodules which have a better structure and a more efficient metabolism for fixing nitrogen (Mhadhbi et al., 2011). Among the stress adaptive mechanisms in these rhizobial strains are the biosynthesis of compatible solutes balancing the internal and external water potential (Fernandez-Aunión et al., 2010; Mabrouk & Belhadj, 2012; Paul, 2012).

For severely drought-stressed environments in Africa, with an increasing salinity of agricultural soils and frequent incidents of drought periods, and where subsistence farmers also cannot afford the high cost of chemical N-fertilization (Chibeba, Kyei-Boahen, Guimarães, Nogueira, & Hungria, 2017), it is essential to improve legume yield by selecting more salt- and drought-tolerant rhizobial strains to enhance soybean productivity. Ideally, application should involve a combination of both stress-tolerant legume cultivars and stress-tolerant rhizobia used as inoculants. Utilizing a more stress-tolerant rhizobial strain has already been found to better maintain SNF and also achieving higher yield under salt stress (Bertrand et al., 2015; Elsheikh & Wood, 1995; Hungria & Vargas, 2000; Pimratch et al., 2007). Unfortunately, both the impact of drought conditions on growth of rhizobial strains and whether a more drought-tolerant rhizobial strain provides a benefit for plant growth under drought conditions has so far rarely been investigated (Romdhane, Trabelsi, Aouani, Lajudie, & Mhamdi, 2009; Elboutahiri, Thami-Alami, & Udupa, 2010).

In our study, we therefore asked the question if inoculation of soybean plants with a selected more drought-tolerant rhizobial strain more tolerant to water deficit improves plant nodulation and growth under soil water deficit caused by drought. We were further interested to investigate the symbiotic compatibility of *S. fredii* strains with the South African soybean cultivar Prima 2000. For selection of a drought-tolerant strain, we first tested growth of different *S. fredii* strains and the strain *B. diazoefficiens* (WB74-1), used as a control, on media containing different amounts of polyethylene glycol 6000 (PEG 6000) to identify the most PEG 6000-tolerant

strain. PEG 6000 treatment changes the osmotic potential in cells and is applied to simulate water deficit conditions (Michel & Kaufmann, 1973). The strain WB74-1 is commercially applied as a soybean inoculant in South Africa. The South African soybean cultivar Prima 2000 was then inoculated with the most PEG 6000-tolerant strain to test if soybean inoculation with a more drought-tolerant selected rhizobial strain is a useful strategy to enhance nodulation and nitrogen fixation as well as plant growth under drought conditions. In comparison to *Bradyrhizobium* strains, *Sinorhizobium* strains (Table 1), as previously described in the literature, are fast growing and are also highly salt-tolerant under free-living conditions with salinity often associated with drought conditions (Roumiantseva & Muntyan, 2015). The use of fast-growing rhizobial strains for inoculation has additional advantages with bacterial culture cultivation and risk of contamination reduced due to a shorter generation time (Albareda, Rodríguez-Navarro, & Temprano, 2009). However, fast-growing strains, such as *S. fredii* USDA 123, exhibit a high level of host specificity due to the presence of the *Rfg1* gene and might, therefore, nodulate only a limited number of genotypes (Fan et al., 2017).

2 | MATERIALS AND METHODS

2.1 | Bacterial strains and culture conditions

Sinorhizobium fredii strains were provided by Prof Ruiz Sainz (Universidad de Sevilla, Spain), and the *B. diazoefficiens* strain was obtained from the South African Rhizobium Culture Collection (SARCC). All bacterial strains used in this study are listed in Table 1. The purity of the cultures was confirmed by repeatedly streaking the bacteria on a yeast extract mannitol agar (YMA) medium (Somasegaran & Hoben, 1994) and verifying a single colony morphology and absorption by Congo red (25 mg/ml) staining. For storage, bacteria, grown in yeast extract mannitol broth (YEM), were mixed with glycerol (1:1, v:v) and stored at −80°C. Working cultures were maintained on YMA slants at 4°C.

2.2 | Cell viability test

The effect of salt on the growth of the rhizobial strains was evaluated by determining the growth in yeast extract mannitol (YEM) broth supplemented with NaCl concentrations ranging from 0% to 3% (wt/vol) corresponding to an osmotic potential of 0 to −2.3 MPa. After autoclaving, YEM cultures were inoculated with the bacterial strains cultures at a concentration of 10^8 cells/ml. Cultures were grown at 28°C at 150 rpm on an orbital shaker, and the final optical density (OD) was measured after 5 days at 600 nm. In order to compare differences in the strains toward the salinity tolerance, optical density values were converted into percentage values, considering growth at control conditions as 100%. Three replicas per treatment were done.

All rhizobial strains were tested for drought tolerance on the basis of their growth on polyethylene glycol (PEG) 6000 added to yeast extract mannitol (YEM) broth (Somasegaran & Hoben, 1985). Fresh inoculum of each strain was prepared in a 100-ml conical flask containing 50 ml sterilized YEM media and incubated for 3 days on an orbital shaker at 28°C at 150 rpm. Growth media were prepared by adding 0, 100, 150, and 200 gl^{-1} PEG 6000 to YEM medium. Osmotic potential of these media were −0.04, −0.89, −1.23, and −1.57 MPa, respectively, determined with a WP4 dew-point potentiometer (Decagon). Osmotic potential of the media containing PEG was measured before and after autoclaving to check any change in developed potential. Freshly prepared inoculum (0.5 OD) of each strain was then inoculated (0.5 ml) with different PEG amounts in a triplicated set of sterilized test tubes containing 5 ml of YEM medium and then incubated on an orbital shaking incubator at 28°C and 150 rpm. An un-inoculated control set of test tubes at each PEG amount was also maintained with three repeats. After 5 days of incubation, the OD of cell suspensions was measured with a spectrophotometer at 600 nm. The cell viability of rhizobia to salt and PEG was also measured and confirmed by their colony growth on YEMA medium plates supplemented with (0–500 mM NaCl) and (0%–20% PEG 6000) in triplicates. The osmotic potential that reduces 50% of bacterial cell growth (IC50) was also determined.

2.3 | Plant material and growth

Soybean seeds (*Glycine max* L. Merr.; cultivar Prima 2000) were obtained from Pannar Seed. Seeds were surface-sterilized in a solution of 2.5% sodium hypochlorite for 15 min and then rinsed five times with sterilized distilled water and left to imbibe for 3–5 hr. Seeds were then pregerminated for 2 days on Petri dishes containing sterile water-agar. Seedlings were grown in large pots (17.5 cm × 20 cm diameter) in fine-grade vermiculite (Mandoval PC), where each seed was treated with 1 ml of the bacterial inoculum containing 10^8 cells/ml of strain WB74-1 or SMH12. Each strain, *S. fredii* SMH12 and the *B. diazoefficiens* WB74-1, was grown before inoculation in YEM for 5 days at 28°C, and cultures were adjusted to a concentration of 10^8 cells/ml. The bacterial suspension (1 ml) was placed onto each seed in a pot. Plants were then grown under controlled greenhouse conditions with a 13/9 hr light/dark cycle extended with artificial lights, at 27°C/25°C day/night temperature, 600 μmol m^{-2} s^{-1} photosynthetically active radiations (PAR) and 60% relative humidity. Plants were watered twice a week with de-ionized water and once a week with a nitrogen-free Hoagland solution to obtain nodule formation.

2.4 | Drought treatment

To evaluate the effect of water deficit caused by drought on plants derived from inoculated seeds, inoculated plants were grown until they reached the same vegetative growth stage (plastochron index of 3.6) as described by Erickson and Michelini (1957) using 25 mm as the reference lamina length. The plastochron index was calculated as follows: Plastochron index = n + (logLn − logR)/(logLn − logLn + 1), where n is the youngest trifoliate leaf which is longer than the reference value of R = 25 mm counting acropetally from the cotyledonary node. Ln and Ln + 1 are the lengths of the trifoliate leaves in mm of n and n + 1. To reduce error, only the central pinna was measured from the base to the tip (Hanada & Son, 1974). Half of all grown plants were then exposed to drought by completely withholding watering of plants until the vermiculite water content (VWC) reached 60% (9 days) and 30% VWC (21 days). The respective VWC was calculated as follows: VWC = (fresh mass − dry mass)/fresh mass) × 100. The initial dry mass used in each pot was 300 g of dry vermiculite. Control plants were further watered every second day using a nitrogen-free Hoagland nutrient solution.

2.5 | Biomass determination

For biomass determination, all vegetative aboveground plant parts (shoot biomass) and all below-ground (root biomass) were harvested. Nodule biomass was determined separately after removing the nodules from the plant roots. Dry biomass of shoots, roots, and nodules was determined after drying plants in a drying oven (Type U 40, Mommert) at 60°C for 48 hr. Three individual plants (replicates) were harvested and used for distractive biomass measurements.

2.6 | Nodule water potential

Water potential of crown nodules was determined with the WP4 Dew Point Potential meter (Decagon). Nodules were collected and counted. Nodule water potential (ΨNod) was determined immediately after harvesting 100 mg of nodules from three plants with the WP4 Dew Point Potential meter (Decagon) as described by Guerin, Trinchant, and Rigaud (1990).

2.7 | MDA determination

Lipid peroxidation in nodules was assayed by determining the malondialdehyde (MDA) content with the thiobarbituric acid (TBARS) method modified according to Singh, Verma, and Dubey (2012). Ground frozen nodules (100 mg) were homogenized in five volumes of a 6% (w/v) trichloroacetic acid (TCA) solution. The homogenate was centrifuged at 10,000 g for 10 min, and 0.2 ml of the supernatant was added to 0.3 ml of 0.5% TBA. The reduction mixture was incubated at 90°C for 20 min and subsequently incubated on ice for 10 min. The absorbance of the supernatant was determined at 532 nm. The value for nonspecific absorption measured at 600 nm was subtracted. The amount of MDA formed was calculated applying the extinction coefficient of 155 mM^{-1}/cm.

2.8 | Ureide determination

For determining biological nitrogen fixation, the ureide content measured as allantoin production of nodules was assayed. After determining the weight of nodule and leaf tissues (100 mg), ureides were extracted with 100 µl of 0.2 M NaOH. Samples were then boiled for 20 min to convert allantoin to allantoic acid. Samples were cooled and centrifuged at 10,000 g for 10 min where after 5 µl of the supernatant together with 35 µl of H_2O were used for further analysis according to Young and Conway (1942). The diluted plant extract (40 µl) was boiled together with 8 µl of 0.5 M NaOH for another 10 min whereafter 16 µl of a mixture of a 1:1 ratio of 0.33% phenylhydrazine (Sigma) and 0.65 M HCl was added and boiled for another 2 min. A 40 µl solution of 1.67% potassium ferricyanide (Sigma) and HCl (36.5%–38.0%, used for molecular biology) were incubated together with the plant mixture for 10 min before the absorbance was measured at 525 nm. A standard curve was set up with 1, 2, 4, 6, and 8 µg of allantoin (Sigma) to calculate the ureide content.

2.9 | Statistical analysis

A general linear model was performed to assess the effect of strain (WB74-1 and SMH12) and drought treatment (well-watered and 60%, or well-watered and 30% drought), and their interactions on all growth parameters (Table S1). Separate analyses were run for measurements at 9 days (60% drought treatment) and at 21 days (30% drought treatment) to reflect the different age of the plants. If the interaction was significant, Tukey's multiple comparison test was used to assess differences between treatments. If the interaction was nonsignificant, analyses were re-run without the interaction term. The response data were transformed prior to analysis where required to meet model assumptions (Table S1).

3 | RESULTS

3.1 | Salt and PEG treatment of rhizobial strains

We first determined if the different *Sinorhizobium* strains were indeed salt (NaCl)-tolerant. Tested strains generally varied in their response to salt treatment. Strain SMH12 tolerated best the salt treatment, and a 50% growth

inhibition was only obtained when the medium had an osmotic potential as low as −1.21 MPa (Figure 1A). In comparison to all other tested strains, cells of strain SMH12 were still able to grow on a medium containing 500 mM NaCl corresponding to an osmotic potential in the medium of −2.3 MPa (Table 2). Cells of strain *B. diazoefficiens* WB74-1, used as a reference, were the most salt-sensitive and showed growth inhibition of 50% at an osmotic potential of -0.61MPa (Figure 1A).

We then tested the survival of cells of these different strains on a PEG 6000-containing medium to test if survival on a salt medium is directly related to survival on a PEG-containing medium. PEG treatment (15%) corresponding to an osmotic potential of −1.23 MPa in the medium decreased bacterial growth by 50% in all tested strains including the control strain *B. diazoefficiens* (Figure 1B; Table 2). Only

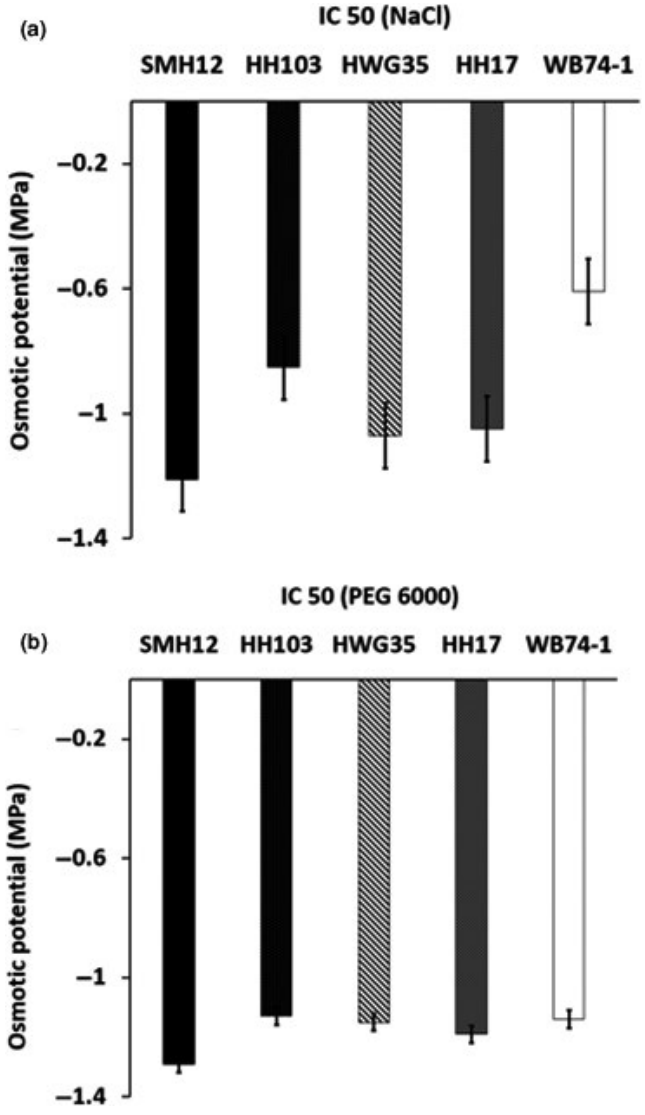

FIGURE 1 Osmotic potential (MPa) required in medium to obtain a 50% inhibition (IC 50) of cell growth of different rhizobial strains after treatment with NaCl (A) or PEG 6000 (B)

TABLE 2 Cell viability of rhizobial strains on solid YEMA medium supplemented with PEG 6000 or NaCl. Reduction in bacterial growth from control in % is indicated in brackets

Bacterial strain	Number of colonies						
	PEG 6000 (%)				NaCl (mM)		
	Control	10 (−0.9 MPa)	15 (−1.23 MPa)	20 (−1.57 MPa)	100 (−0.45 MPa)	300 (−1.35 MPa)	500 (−2.3 MPa)
Sinorhizobium fredii							
SMH12	198 ± 5.8	138 ± 3.5 (30)	96 ± 6.5 (52)	33 ± 5.8 (83)	145 ± 6.9 (27)	68 ± 5.7 (65)	21 ± 2.9 (89)
HH103	147 ± 8.2	105 ± 5.7 (29)	68 ± 2.9 (54)	ND (100)	83 ± 7.2 (44)	8 ± 0.8 (96)	ND (100)
HWG35	184 ± 15.5	118 ± 4.2 (36)	82 ± 4.5 (55)	6 ± 1.6 (97)	130 ± 10.6 (29)	20 ± 4.8 (89)	ND (100)
HH17	104 ± 8.0	92 ± 3.2 (12)	56 ± 5.3 (46)	ND (100)	78 ± 4.0 (25)	11 ± 0.8 (89)	ND (100)
Bradyrhizobium diazoefficiens							
WB74-1	87 ± 9.0	63 ± 7.4 (28)	41 ± 6.1 (53)	ND (100)	ND (100)	ND (100)	ND (100)

Note: ND, not detectable.

FIGURE 2 Effect of drought on nodule abundance on soybean roots inoculated with *Sinorhizobium fredii* strain SMH12 at 21 days after drought was initiated

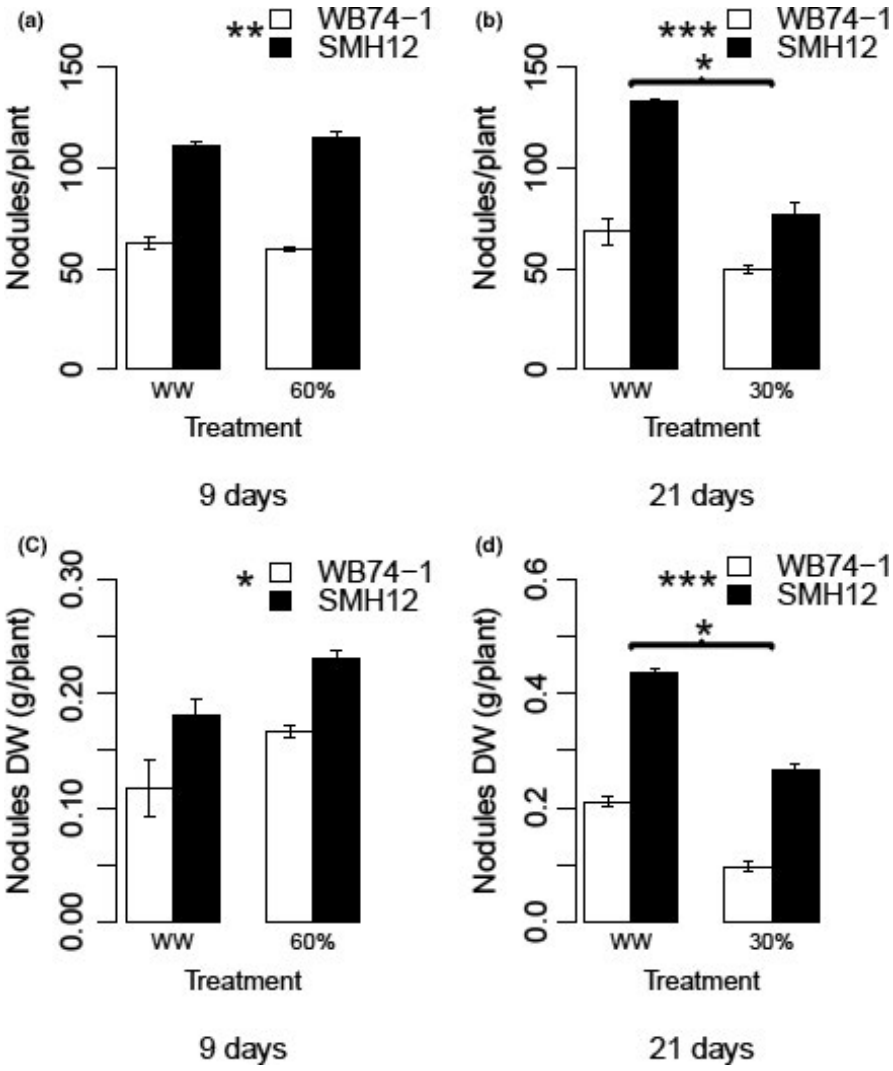

FIGURE 3 Number of nodules (A, B) and nodule dry weight (C, D) in soybean plants inoculated with *Sinorhizobium fredii* SMH12 and *Bradyrhizobium diazoefficiens* WB74-1. Plants were grown either under well-watered (WW) or exposed to drought conditions until 60% VWC (9 days drought exposure) (A, C) or until 30% VWC (21 days drought exposure) (B, D). Data represent the mean ± SE of nodules from three different plants. A significant difference between the strains is indicated by (*) on the strain legend. A significant difference between drought treatments is indicated by a bracket above bars with * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$)

when the medium contained a higher PEG amount (20%) corresponding to an osmotic potential of −1.57 MPa in the medium, *S. fredii* strain SMH12 survived better PEG treatment, with a 17% survival, when compared to all other tested strains (Table 2).

3.2 | Soybean nodulation

Nodulation of soybean plants was generally greatly reduced due to exposure of plants to water deficit for 21 days and when the vermiculite water content (VWC) was only 30% (Figure

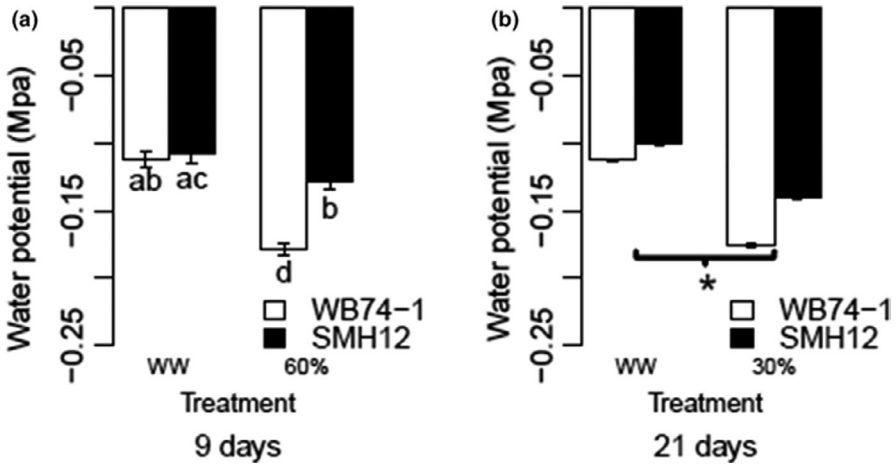

FIGURE 4 Effect of water deficit on nodule water potential (ΨNod), (A, B) of soybean plants inoculated with *Sinorhizobium fredii* SMH12 strain and *Bradyrhizobium diazoefficiens* WB47-1 strain. Plants were grown either under well-watered (WW) or exposed to drought conditions until 60% VWC (9 days drought exposure) or until 30% VWC (21 days drought exposure). Data represent the mean ± SE of three different plants. A significant difference between drought treatments is indicated by a bracket above bars with * ($p \leq 0.05$). Where the interaction between drought and strain was significant, differences are represented by letters above the bar, with different letters denoting statistically significant difference

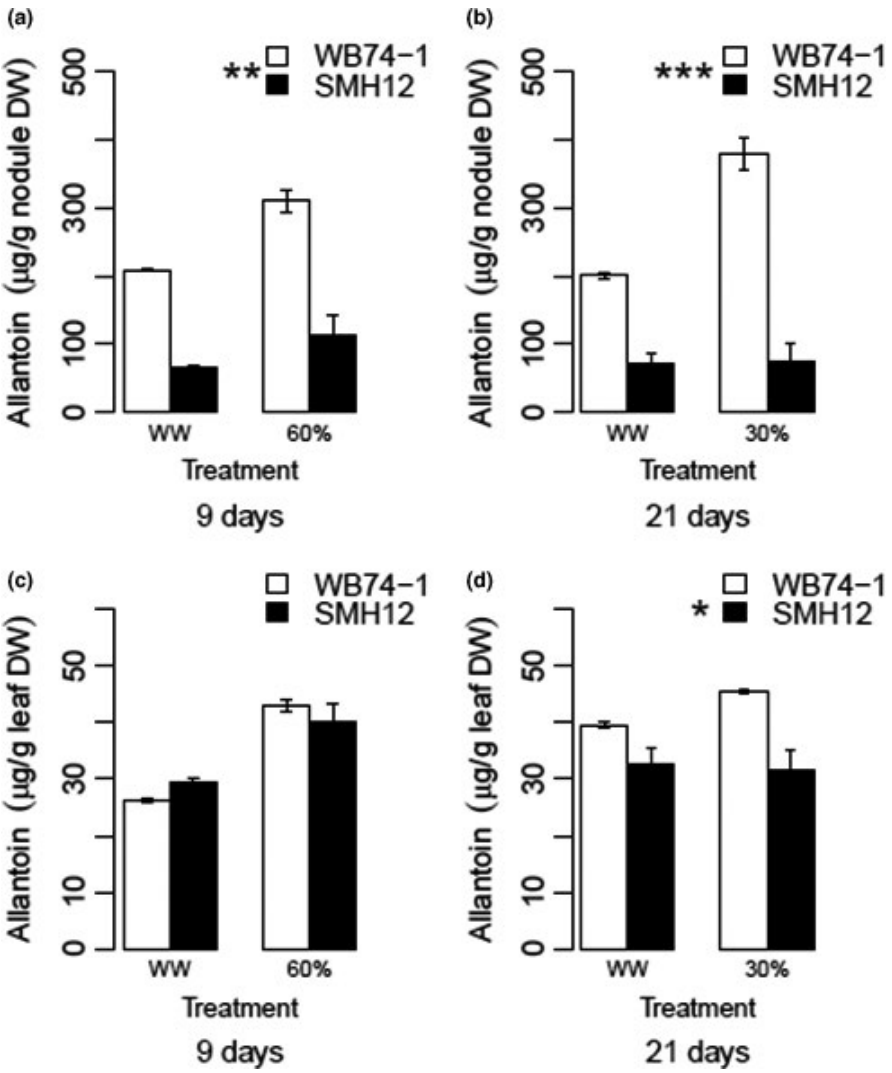

FIGURE 5 Nodule ureide (A, B) and leaf ureide content (C, D) of soybean plants inoculated with *Sinorhizobium fredii* strain SMH12 and *Bradyrhizobium diazoefficiens* strain WB74-1. Plants were grown either under well-watered (WW) or exposed to drought conditions until 60% VWC (9 days drought exposure) (A, C) or until 30% VWC (21 days drought exposure) (B, D). Data represent the mean ± SE from three different plants. A significant difference between the strains is indicated by (*) on the strain legend with * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$)

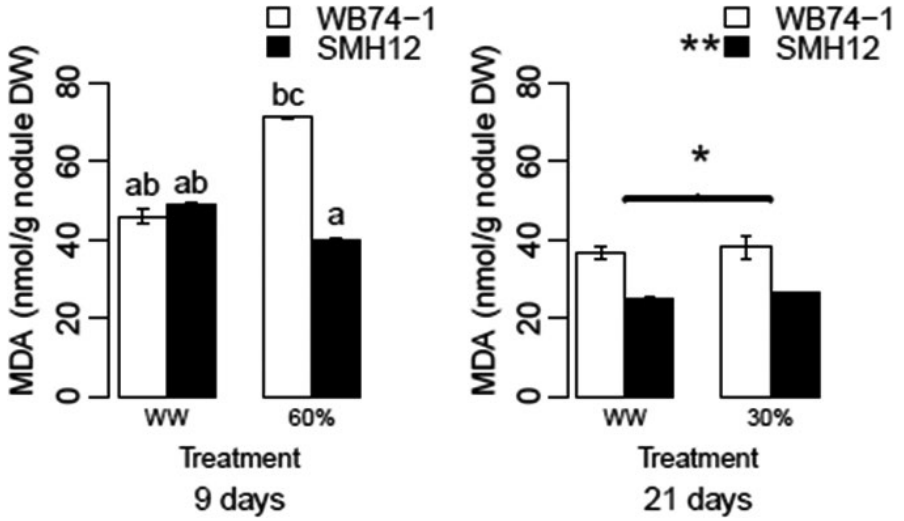

FIGURE 6 Malondialdehyde formation in soybean nodules (A, B) inoculated with *Sinorhizobium fredii strain* SMH12 strain and *Bradyrhizobium diazoefficiens* strain WB47-1. Plants were grown either under well-watered (WW) or exposed to drought conditions until 60% VWC (9 days drought exposure) or until 30% VWC (21 days drought exposure). Lipid peroxidation was measured as MDA-TBA abducts. Data represent the mean ± SE from three different plants measured in duplicate. A significant difference between the strains is indicated by (*) on the strain legend with * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$). A significant difference between drought treatments is indicated by a bracket above bars. Where the interaction between drought and strain was significant, differences are represented by letters above the bar, with different letters denoting statistically significant difference

2). SMH12 plants had, however, significantly ($p \leq 0.01$) more nodules and a higher nodule dry weight ($p \leq 0.05$) than WB74-1 plants at 60% VWC (Figure 3A,3; 9 days). When plants were either becoming older or were exposed to more severe drought conditions (30% VWC; Figure 3B,3; 21 days), SMH12-inoculated plants had again significantly more nodules ($p \leq 0.001$) with significantly higher nodule dry weight ($p \leq 0.001$) than WB74-1-inoculated plants. However, nodules from SMH12 plants were generally smaller in size when compared to nodules from WB74-1-inoculated plants (data not shown).

3.3 | Water potential, ureide content, and lipid peroxidation

Under well-watered conditions, the water potential of nodules was not significantly different between nodules from SMH12- and WB74-1-inoculated plants ($p \geq 0.05$). Exposure to drought conditions (60% and 30% VWC) generally decreased the water potential (Figure 4A,B). However, SMH12 nodules maintained a significantly higher water potential ($p \leq 0.05$) compared to WB74-1 nodules under 60% VWC water deficit conditions. Further, we also found a SMH12 strain and water deficit treatment interaction at 60% VWC (Figure 4A), with a much lower decrease in water potential in SMH12 nodules than in WB74-1 nodules.

We also measured the ureide content, as allantoin formation, in both nodules and leaves. Ureide content was determined as a measure for fixed nitrogen of SMH12- and WB74-1-inoculated plants. Nodules from WB74-1-inoculated plants had under all conditions a significantly ($p \leq 0.01$) higher ureide content than nodules from SMH12-inoculated plants (Figure 5A,B), with an even highly significant difference ($p \leq 0.001$) at 30% VWC. When we measured the ureide content in soybean leaves, leaves from WB74-1-inoculated plants had again generally a higher leaf ureide content than leaves from SMH12-inoculated plants (Figure 5C,D). This difference was also significantly different ($p \leq 0.05$) when leaves derived from plants that were either older or have been exposed to 30% VWC (Figure 5D).

We further determined if nodules from SMH12- and WB74-1-inoculated plants differ in their MDA content, as a measure for peroxidative processes (Figure 6A,B). Nodules from SMH12-inoculated plants had, except for well-watered younger nodules, a significant ($p \leq 0.05$) lower MDA content than nodules from WB74-1-inoculated plants. This result indicates that inoculation of plants with strain SMH12 very likely protected nodules against peroxidative processes. However, exposure to water deficit increased MDA formation particularly in nodules of WB74-1-inoculated plants at 60% VWC. Under these conditions, MDA formation decreased in nodules of SMH12-inoculated plants indicating again a SMH12 strain and water deficit interaction at 60% VWC (Figure 6A).

3.4 | Shoot and root biomass

We finally also determined shoot and root biomass measured as dry weight production of SMH12- and WB74-1-inoculated

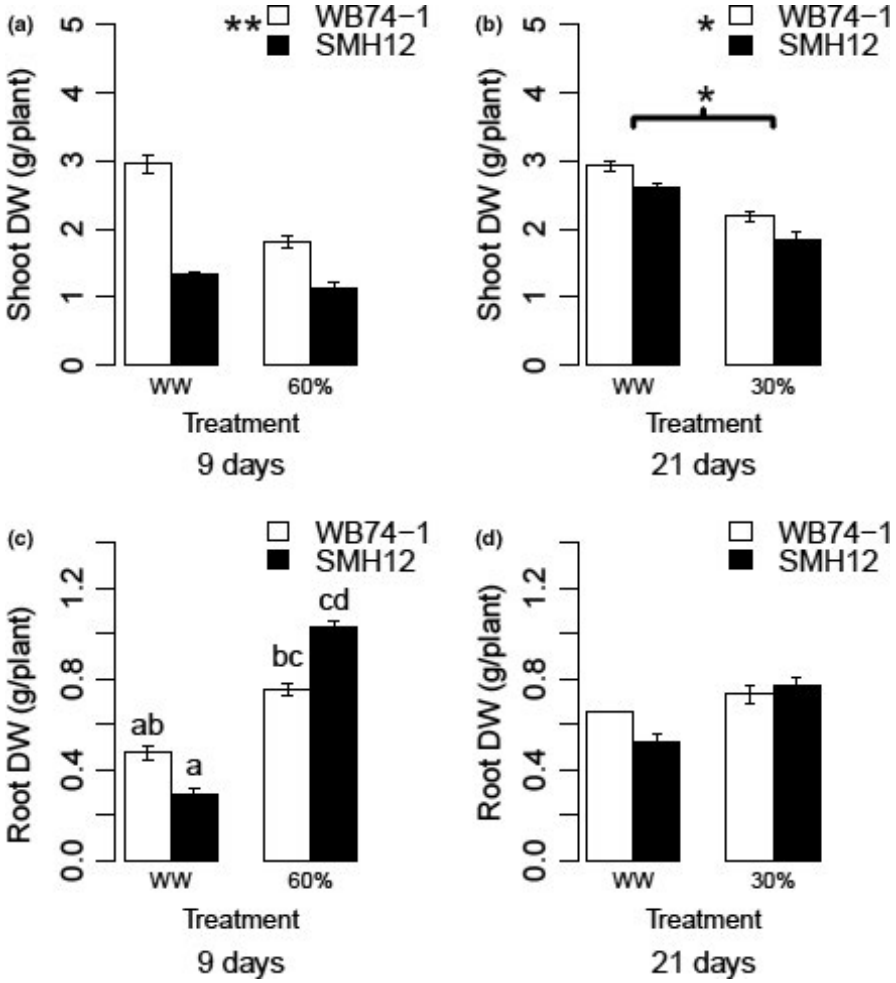

FIGURE 7 Shoot dry weight (A, B) and root dry weight (C, D) in soybean plants inoculated with *S. fredii* strain SMH12 and *Bradyrhizobium diazoefficiens* strain WB74-1. Plants were grown either under well-watered (WW) or exposed to drought conditions until 60% VWC (9 days water deficit exposure) (A, C) or until 30% VWC (21 days water deficit exposure) (B, D). Data represent the mean ± SE from three different plants. A significant difference between the strains is indicated by (*) on the strain legend with * ($p \leq 0.05$), ** ($p \leq 0.01$), *** ($p \leq 0.001$). A significant difference between drought treatments is indicated by a bracket above bars. Where the interaction between drought and strain was significant, differences are represented by letters above the bar, with different letters denoting statistically significant difference

plants. WB74-1-inoculated plants generally had a higher significant shoot biomass compared to SMH12 plants (Figure 7A,B). Exposure to 30% VWC significantly ($p \leq 0.05$) decreased shoot biomass for both strains compared to well-watered control plants. Also, exposure to drought increased the root biomass in both roots of SMH12- and WB74-1-inoculated plants when compared to same age roots from well-watered plants. The highest significant ($p \leq 0.001$) biomass increase in SMH12 roots was at 60% VWC (Figure 7C), and this increase was much higher than the increase for WB74-1-inoculated plants (Figure 7C). This finding also indicates a likely interaction between the strain SMH12 and water deficit at 60% VWC. More severe water deficit (30% VWC) had no significant effect on root biomass, but this was found for both SMH12- and WB74-1-inoculated plants (Figure 7D).

4 | DISCUSSION

Studies investigating the contribution of rhizobia to nitrogen fixation, nodule development, and related plant growth during water deficit caused by drought conditions have been rarely done. We, therefore, investigated if inoculation of soybean with a rhizobial strain more tolerant to water deficit will provide a benefit for soybean plants due to changes in nodulation and plant growth. In our study, cell growth for all rhizobial strains tested was reduced due to salt treatment and treatment with PEG applied to simulate water deficit conditions. All *S. fredii* strains, in particular strains SHM12 and HWG35, were more tolerant to these treatments in comparison to the *B. diazoefficiens* WB74-1 reference strain. *Sinorhizobium fredii* strains HH103 and SHM12 are known to be fast-growing rhizobial strains able to nodulate soybean and also form, depending on the plant species, determinate or indeterminate nodules (Margaret et al., 2011; Rodriguez-Navarro et al., 2014). Our finding that SHM12 survives better salt treatment, with growth even under high salt conditions (500 mM in a medium), further confirms the previously reported high salt tolerance of *S. fredii* strains SMX11 and SMH12 (Rodriguez-Navarro et al., 1996). We also found that strain SMH12 was, in comparison to *B. diazoefficiens* strain WB74-1, more tolerant to PEG 6000 treatment when a high amount of PEG 6000 (20%) was used in the medium which considerably lowered the osmotic potential of the medium.

Application of nonpermeating PEG, such as PEG 6000, lowers the water potential in cells and reduces the water availability by binding water molecules without penetrating the cell wall thereby reducing cell growth (Abdel-Salam, Ibrahim, Abd-El-Halim, Badawy, & Abu-Aba, 2010; Cytryn et al., 2007; Belal, Hassan, & El Ramady, 2013; Mhamdi, Nouairi, Hammouda, Mhamdi, & Mhadhbi, 2015). Bacterial cells generally prevent dehydration by accumulating osmolytes that are low-molecular weight organic solutes. Glycine, betaine, proline, and trehalose are among the major osmolytes in rhizobial osmo-adaptation allowing to balance the internal and external water potential with particularly trehalose playing a major role (Fernandez-Aunión et al., 2010; Mabrouk & Belhadj, 2012; Madkour, Smith, & Smith, 1990; McIntyre et al., 2007; Paul, 2012). More osmo-tolerant rhizobial strains maintaining a positive turgor multiply better in the rhizosphere of a host plant and by withstanding large modifications in osmolality prevent a decrease in the number of viable cells (Abdelmoumen, Filali-Maltouf, Neyra, Belabed, & Missbah El Idrissi, 1999; Bouhmouch, Souad-Mouhsine, Brhada, & Aurag, 2005; Singleton, Swaify, & Bohlool, 1982).

More robust rhizobial strains persisting for longer in dry soils are further important contributors to the rhizobium–cultivar interaction. Recently, a more salt-tolerant *S. meliloti* strain (4H41) was found to be more competitive and more effective in nitrogen fixation of common bean nodules under water deficiency than the commonly used inoculant *Rhizobium tropici* CIAT899 (Mnasri et al., 2007). In particular, such more salt-tolerant strains improving the soybean-rhizobia symbiosis contribute to better drought tolerance of a legume plant (Mhadhbi et al., 2011). The selected rhizobial strain *S. fredii* SHM12, which was the most PEG- and salt-tolerant strain, was also in our study more effective in nodulating soybean under soil water deficit conditions. Formation of more nodules, due to inoculation of soybean with a rhizobial *S. fredii* strain (HH103) similar to our result with strain SMH12, has also been reported by Videira, Pastorino, and Balatti (2001). Hyper-nodulation does not always translate into higher grain yield and some hyper-nodulating soybean genotypes showed high nitrogen-fixing ability only in the early growth stages (Herridge & Rose, 2000; Song, Carroll, Gresshoff, & Herridge, 1995; Wu & Harper, 1991). Hyper-nodulation incites a re-routing of carbohydrates to maintain the metabolic activities of a larger nodule biomass resulting in reduced shoot biomass production (Videira et al., 2001). However, improved vegetative growth in shoots and roots, due to enhanced nitrogen-fixing ability per plant, was found in the hyper-nodulating soybean cultivar Sakukei 4, especially after flowering when compared to conventional hyper-nodulating cultivars (Takahashi, Shimada, Nakayama, & Arihara, 2005). Despite the yield constraints, hyper-nodulating genotypes have beneficial effects as green manure or in intercropping systems.

Nodules of SMH12-inoculated plants also maintained a higher nodule water potential and had a much less decrease in the water potential than WB74-1 nodules at 60% VWC. However, the exact reason for obtaining such higher water potential is still unclear and requires further investigation. Higher nodule water potential might, for example, be related to better survival of SMH12 cells under water deficit, similar to our PEG 6000 findings. Also, the flow of water in the phloem might play a role influencing the water status and the flow of nitrogen from the nodule (Serraj, Sinclair, & Purcell, 1999). It would therefore be useful to also measure in the future the total plant nitrogen content, as a measurement of integrated N fixation, but also the leaf and soil water status to determine how plants are supplied with water when treated with different inoculants.

However, better SMH12 nodulation was not translated into either higher root or shoot biomass, except for a higher root biomass at 60% VWC of SMH12-inoculated plants when compared to the root biomass of WB74-1-inoculated plants. Better survival of SMH12 cells under water deficit and better root colonizing under these conditions might contribute to the formation of more root biomass under water deficit conditions. Any better tolerance to water deficit and also better root colonizing is likely due to a better quorum sensing of SMH12 where bacteria produce and release chemical signal molecules (auto-inducers). Their increase in concentration, as a function of cell density, leads to an alteration in gene expression (Miller & Bassler, 2001). Production of exopolysaccharide (EPS), which are hydrated compounds with 97% of water in a polymer matrix, is important in the formation of biofilms for adaptation to water deficit during quorum sensing. This type of adaptation is essential for survival in bacteria of the genera *Mesorhizobium* and *Sinorhizobium* as well as *Bradyrhizobium* (Pérez-Montaño et al., 2014; Zubair et al., 2014). Water-limiting conditions generally trigger EPS production, and EPS enhance bacterial surface attachment allowing bacteria to better colonize roots more efficiently under soil water deficit conditions (Pérez-Montaño et al., 2014; Saleem, Arshad, Hussain, & Bhatti, 2007). Studies with a *nodD1* mutant, and also with a quorum sensing-defective strain, recently demonstrated that biofilm formation is indeed crucial for optimal root colonization and symbiosis between *S. fredii* and soybean plants (Pérez-Montaño et al., 2014). NodD1 is a member of the NodD family of LysR-type transcriptional regulators (LTTRs) and mediates nodulation (*nod*) gene expression (Peck, Fisher, Bliss, & Long, 2013). The higher efficiency of strain SMH12 to colonize roots very likely contributes to better execute beneficial plant growth promoter actions. These actions include influencing cellulase, protease, lipase, and β-1,3 glucanase productions allowing better plant protection (Gopalakrishnan et al., 2015). In our study, this could explain the lower MDA production found due to less peroxidation of lipids. Lower MDA production

indicates a more healthy state of cell membranes conserving better the oxygen balance in nodules (Mhadhbi et al., 2011).

In our study, the ureide amount also increased in soybean roots and shoots due to drought conditions. Ureide increase coinciding with a decline in N_2 fixation has previously been reported by King and Purcell (2005). Since the ureide amount reflects the availability of nitrogen for growth and development (Todd et al., 2006), we used the ureide content of nodules and leaves as an indicator for the nitrogen fixation status of our plants. However, any improved nodule characteristics, due to soybean inoculation with strain *S. fredii* SMH12, did not translate in our study into a higher soybean ureide amount or higher root or shoot biomass when compared to WB74-1-inoculated plants. The exact reason for such lower SMH12 efficiency, despite that SMH12-inoculated plants had considerably more nodules with higher nodule biomass even under drought conditions, is still unclear. One possibility is that soybean cultivar Prima 2000 applied in South Africa, with an initial germplasm introduction from the US, is better adapted to slow-growing *Bradyrhizobium* but not to *Sinorhizobium* strains that are generally applied with Chinese cultivars. Prima 2000 therefore lacks the ability to efficiently translate hyper-nodulation provided by a *Sinorhizobium* strain into better nitrogen fixation and biomass production. Previous studies have already found that nodulation and nitrogen fixation efficacy is not only directly related to the rhizobial species alone but also to an optimal rhizobium–cultivar interaction (Clua, Roda, Zanetti, & Blanco, 2018).

In conclusion, selection of new rhizobial strains, particularly those tolerating dryer soil conditions, is important in Africa as it is often exposed to severe drought conditions. In this regard, it might be essential when improving soybean yield in a stressed environment to involve a combination of stress-tolerant cultivars and stress-tolerant rhizobia to obtain better drought tolerance in soybean (Romdhane et al., 2009). In the past, fast-growing rhizobia, like SMH12, for effective symbiosis have been regarded as poor N_2-fixers in South Africa with limited value for a commercial inoculant (Keyser, Bohlool, Hu, & Weber, 1982). Our study is among the first to test a highly PEG and salt-tolerant fast-growing *S. fredii* strain for effective symbiosis with a commercially used South African soybean cultivar. Specifically, the *S. fredii* strain SMH12 more effectively induced nodulation under drought when compared to *B. diazoefficiens* strain WB74-1. However, better nodulation did not translate into higher nitrogen fixation, or biomass production likely due to a nonoptimal rhizobium–Prima 2000 cultivar interaction. Any future work will therefore focus on finding more appropriate soybean cultivar partners for strain SMH12. This will particularly include more inoculation trials with different soybean cultivars. Such trials should include amore drought-tolerant soybean cultivar as well as different water-deficit levels to elucidate the efficacy and potential of inoculating soybean with *Sinorhizobium* strains.

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CONFLICT OF INTEREST

None declared.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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