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DROUGHT STRESS

Effect of *Bacillus paralicheniformis* on soybean (*Glycine max*) roots colonization, nutrient uptake and water use efficiency under drought stress

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Abstract

Spore-forming, plant growth-promoting bacteria (PGPR) offer extraordinary opportunities for increasing plant productivity in climate change scenarios. Plant–water relationships, root development and photosynthetic performances are all key aspects of plant physiology determining yield, and the ability of PGPR to influence these in a coordinated manner is crucial for their success. In this study, we dissected the mode of action of a commercial *Bacillus paralicheniformis* FMCH001 in promoting soybean (*Glycine max*, *seed variety: Sculptor*) establishment in well-watered and drought conditions. We found that FMCH001 colonizes the roots, improved root growth and allowed plants to absorb more nutrients from the soil. FMCH001 inoculation had no effect on abscisic acid in leaf or xylem sap, while significantly improved photosynthesis rate, stomatal conductance and transpiration rate at 28 days after planting when drought stress exposed for 7 days, with depressed leaf water potential and osmotic potential. Moreover, the bacterium increased water use efficiency and the inoculated soybean plants exposed to drought used 22.94% less water as compared to control, despite producing comparable biomass. We propose that the ability of the bacterium to promote root growth and also modulate plant water relations are key mechanisms that allow FMCH001 to promote growth and survival in dicotyledon plants.

KEYWORDS
drought, nutrient uptake, plant growth-promoting rhizobacteria, soybean, water use efficiency

Key points

- *Bacillus paralicheniformis* FMCH001 promoting soybean establishment in well-watered and drought conditions were dissected.
- FMCH001 colonized the roots, improved root growth and allowed plants to absorb more nutrients from the soil.

Jie Liu and Lorenzo Fimognari contributed equally to the work, co-first author.

- FMCH001 inoculation significantly improved leaf gas exchange rates when drought stress exposed for 7 days.
- FMCH001-inoculated plants used less water with comparable biomass, hereby improving plant water use efficiency.

1 | INTRODUCTION

Feeding a growing population under the conditions of climate change is one of the most significant challenges for mankind in the coming decades. Among the most likely outcomes of climate change, water scarcity is of particular concern in many areas of the world since drought negatively impacts plant yield. In soybean, for example, water limitation severely increases pod abortion resulting in lower yield per hectare. The effects of water limitation are especially severe when drought occurs at flowering and early pod development (Liu et al., 2003; Mayla et al., 2021). This is because drought decreased cell wall instability (Mayla et al., 2021), compromises the switch between cell division to cell differentiation/expansion during flowering which is a necessary steps to reach high pod yield (Westgate & Peterson, 1993). Pod development under water-limiting conditions is suggested to be influenced by tissue water status and hormone levels which in turn control cell division and expansion (Saini & Westgate, 1999). If water is limited during the flower/pod setting stages, the combination between decreased water potential and increased levels of the hormone abscisic acid (ABA) in flowers leads to pod abortion (Liu et al., 2003). It is, therefore, of paramount importance to implement strategies to lower drought stress in field crops and help ensure high yield also in dry conditions.

The application of beneficial plant-associated microorganisms, such as *Bacillus* and *Pseudomonas* spp. represents an extremely promising tool in integrated crop management to help ensure high and sustainable crop yield, even under drought stress (Ullah et al., 2021). In climate change scenarios, beneficial bacteria are of particular interest because they can help the plant in a multitude of ways due to their multiple modes of action. Beneficial bacteria have been shown to increase the yield of chickpea (Elkoca et al., 2008; Shahzad et al., 2014), corn (Egamberdiyeva, 2007; Ferreira et al., 2013), peanuts (Dey et al., 2004), rice (Bao et al., 2013; Isawa et al., 2010), soybean (Hungria et al., 2013; Sandip et al., 2011), sugarcane (Beneduzi et al., 2013; Govindarajan et al., 2006) and wheat (Díaz-Zorita & Fernández-Canigia, 2009; Upadhyay et al., 2012). The Gram-positive spore-forming *Bacillus* spp. are of particular interest due to their high stability and long shelf-life (Ulrich et al., 2018). Moreover, *Bacillus* spp. spores can be co-applied with traditional fertilizers and pesticides allowing the formulation of dual products that do not influence conventional farmer practices (Huang & Luo, 2021; Li et al., 2020). *Bacillus* spp. spores have been shown to sustain high/low pH, radiation, heat and desiccation (Brown, 2000; Nicholson et al., 2000; Setlow, 2006; Ulrich et al., 2018). These features make them robust and ideal for agricultural use. When roots emerge and the *Bacillus* spp. spores are provided with both water and root exudates, the spores are then able to germinate and enter a vegetative state of growth. Root colonization by *Bacillus* spp. can take place through a biofilm formation on the root surface and/or by endophytic colonization (Beauregard et al., 2013; Mendis et al., 2018). The ability of a bacterium to colonize roots and express a specific metabolite profile will determine the type of microbe–plant interaction that occurs. Plant growth-promoting rhizobacteria (PGPR) have been shown to increase nutrient acquisition by solubilizing phosphate, fix nitrogen, produce phytohormones, stimulate the plant immune system and directly suppress soil pathogens (Glick, 1995; Hayat et al., 2010; Jogaiah et al., 2010; Kloepper et al., 2004).

In previous work conducted on maize (Akhtar et al., 2020), *Bacillus paralicheniformis* FMCH001 can stimulate plant growth during well-watered and drought conditions, and increase plant water use efficiency up to by 46%. The ability of this PGPR to alter plant water relations is a powerful feature that could have unique applications in the field, especially in drier climates. However, the potential of FMCH001 to influence soybean seedling establishment and water relations during well-watered and drought stress conditions at early developmental stages, especially at the formation of side shoots stage still remains unknown. Therefore, the objective of this study was to investigate the potential of *Bacillus paralicheniformis* FMCH001 to colonize soybean roots, and further *to explore the plant growth-promoting proprieties of the FMCH001 and its effect on plant–water relationships* and water use efficiency. This was accomplished by a combination of in-depth water relation studies, plant physiological measurements, colonization studies and enzymatic analysis in the target plant.

2 | MATERIALS AND METHODS

2.1 | Experimental design

The manuscript contains two independent colonization experiments. *Bacillus paralicheniformis* FMCH001 (provided by Chr-Hansen A/S, Hørsholm, Denmark) was used to colonize soybean roots (*Glycine max, seed variety: Sculptor from Saaten-Union, Eisenstrasse, Isernhagen, Germany*) in São Paulo State University, located in Botucatu-SP, Brazil from January to May of 2019, *and to explore the plant growth-promoting proprieties of the FMCH001 and its effect on plant–water relationships and water use efficiency in greenhouse in* University of Copenhagen's field site at Højbakkegård, Taastrup, Denmark between *June and July 2018*.

2.1.1 | Colonization experiment 1: Bacterial dynamics during soybean cycle under water stress periods

The purpose of this experiment was to evaluate the bacterial dynamic of FMCH001 on the soybean root, with and without drought periods. The experiment was conducted using rhizotron pots under greenhouse conditions. The dimensions of the used rhizotrons were 20 cm of diameter and 1 m of depth. A mixture of soil and a soil conditioner in the proportion 1:1 was used. For the experiments, half of the soybean seeds were treated with FMCH001, whereas control seeds were not inoculated.

Three watering systems were performed in this study: continuous irrigation; drought period during 20 days during the vegetative growth stage (started at 15 days after planting (Dap)); drought period for 20 days from vegetative to reproductive stage (started at 39 Dap). After these drought periods the irrigation was re-established. The water used in all treatments outside of the drought period was 20 mL per day. Three plants were harvested for qPCR quantification at each harvest point representing three biological replicates. All the treatments were harvested at 7, 15, 39, 50 and 90 Dap.

To investigate the dynamics of FMCH001 colonization throughout the soybean roots system, the root sampling was carried out at three different depths: 0–30, 30–60 and 60–90 cm. For the harvest at 7 and 15 Dap, only the collection at 0–30 cm was carried out because the root system was not located in the 30–60 and 60–90 cm layers.

2.1.2 | Colonization experiment 2: Microscopy of bacterial colonization

This experiment aimed to characterize the strain colonization of soybean roots using microscopic observation. For microscopy analysis, soybean seeds treated with FMCH001 were sown in pots under greenhouse conditions with 5 seeds per pots containing 1:1:1 sand/perlite/potting soil and watered once a week. Plants were harvested 23 days post sowing. Roots were fixed 5 h at 4°C in a paraformaldehyde solution (4% w/v in phosphate buffer saline (PBS), pH 7.2) in Eppendorf tubes, then samples were rinsed thrice with PBS. Treatment with lysozyme solution (1 mg mL^{-1} in PBS) was applied for 10 min at 37°C followed by an ethanol dehydration series (25%, 50%, 75% and 99.9%; 15 min each step). The double-labelling-of-oligonucleotide-probes-Fluorescence in situ hybridization (DOPE-FISH) was performed with probes from Eurofins (Austria) labelled at both the 5′ and 3′ ends. A mixture of probes EUB338I, EUB338II and EUB338III (mixEUB) targeting all bacteria (with equivalent mixture of EUB338, EUB338II, EUB338III) coupled with the fluorochrome Cyanine 3 (Cy3) (Amann et al., 1990; Daims et al., 1999) as well as a bacillus 2 (BAC2) probe 5′CCCGAAGGGGAAGCCCTATCT-3′ targeting *Bacillus paralicheniformis* as well as other *Bacillus* spp. coupled with Cyanine 5 (Cy5) were used. The probe was created using stellaris software, in silico analysed using mathfish software, and check was carried out using rdp genes, oligocalc, ncbi and silva databases. The other *Bacillus* spp. were *B. licheniformis*, other members of the *B. subtilis* species complex and some members of the *B. cereus* group. The probe was previously tested on in vitro plantlets inoculated with FMCH001 (unpublished). A nonbacterial probe 338 used as negative control in hybridization experiments (NONEUB probe) (Wallner et al., 1993) coupled with Cy3 and Cy5 was also used independently as a negative control. Hybridization was carried out at 46°C for 2 h with 10–20 µL solution (containing 20 mM Tris–HCl pH 8.0, 0.01% w/v sodium dodecyl sulphate (SDS), 0.9 M NaCl, 20% formamide and 5 ng μL^{-1} of each probe) applied to each plant sample in a Eppendorf tube. Posthybridization was conducted at 48°C for 30 min with a prewarmed post-FISH solution containing 20 mM Tris–HCl pH 8.0, 0.01% SDS, 5 mM EDTA pH 8.0 and NaCl at a concentration corresponding to the formamide concentration. Samples were then rinsed with distilled water before air drying for at least one day in the dark. The samples were then observed under a confocal microscope (Olympus Fluoview FV1000 with multiline laser FV5-LAMAR-2 HeNe(G) and laser FV10-LAHEG230-2). Cy3 signal (all bacteria) was assigned as green and Cy5 as red (*Bacillus paralicheniformis* and some *Bacillus* spp.). Combination of both signals resulted in an orange colour. X, Y, Z pictures were taken at a 60X objective and visualized using IMARIS software. Pictures were cropped, whole pictures were sharpened, and the light/contrast balance improved to better observe the image details. All experiments were repeated on all root samples. Images shown in this paper represent the average of colonization.

2.1.3 | Big pots greenhouse experimental design

With the aim of investigating how the bacteria influence plant growth and physiological parameters, we designed a big pots soybean greenhouse experiment where plants were exposed to either a well-watered or drought-recovery treatment from 4 June to 10 July 2018. The soil used in the experiment was from 0 to 20 cm layer at Højbakkegård, Taastrup, Denmark (University of Copenhagen), classified as sandy loam, soil properties were detailed described in Akhtar et al. (2020). The soybean seeds were coated before the experiment with either FMCH001 or mock (control) and three harvests were performed during the experiment on 27 June, that is, 23 days after planting (*Dap*) when four nodes on the main stem beginning with the unifoliate node (V4), 2 July, that is, 28 *Dap* when five nodes on the main stem beginning with the unifoliate node (V5) and 10th July, that is, 36 *Dap* when seven nodes on the main stem beginning with the unifoliate node (V7) (Figure 1), all these growth periods belong to vegetative stage, that is, N nodes on the main stem beginning with the unifoliate node (VN) according to Fehr et al. (1971). Four plants for each treatment were harvested at each harvest time. In the drought-recovery treatment, water was withheld during the period from V4 to V5 causing plants to progressively be exposed to drought stress. Day 28 was the last

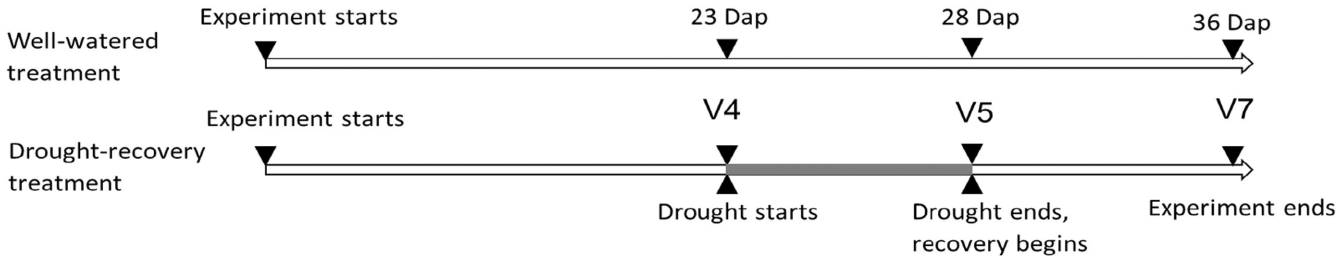


FIGURE 1 Schematic representation of the experimental design. Greenhouse-grown soybean plants were exposed to either a well-watered condition or received a drought-recovery treatment. In both cases, seeds were either inoculated with the beneficial bacterium FMCH001, or were mock inoculated. Grey colour represents the length of the drought treatment. Plants were sampled at three different time point, at the start of drought (4 nodes on the main stem beginning with the unifoliate node (V4)), end of drought (5 nodes on the main stem beginning with the unifoliate node (V5)) and end of recovery (7 nodes on the main stem beginning with the unifoliate node (V7)).

day of drought, after which the drought-recovery treatment was re-watered allowing drought recovery.

The three harvests in the well-watered treatment were designed to shed light on the effect of the bacteria at different developmental stages throughout the vegetative growth of soybean. The harvest at V5 in the drought treatment allowed us to investigate whether the bacterial treatment influenced soybean responses to drought, whereas the V7 harvest in the drought-recovery treatment allowed us to test the ability of the bacteria to change soybean recovery from drought.

2.2 | Measurements

2.2.1 | Root system visualization in rhizoboxes

Soybean plants were grown in rhizoboxes in order to visualize the effect of FMCH001 on the root morphology in soil. The rhizobox setup was designed and custom-built. In short, each rhizobox consists of two sheets of 27×45 cm clear 3 mm acrylic plastic divided with 7 mm foam strips along the edges, forming a 7 mm soil layer, and held together using 20-mm-wide plastic clamps. For the bottom of the rhizobox the foam strips were split into smaller pieces and divided with small distances in between to allow watering from below by soaking. The rhizoboxes were positioned vertically in stands with an angle of 45° which allowed the root system to grow along the bottom plastic sheet. Each rhizobox was covered with a 25 cm×44 cm×1 mm dark polyvinylchloride sheet cover to protect the roots from light during the experiment. A sheet of cellophane was put between the acrylic sheet and the soil to allow the box to open without breaking the roots.

Field soil originating from University of Copenhagen's field site at Højbakkegård, Taastrup, Denmark, was used with a composition of approximate 22.7% clay, 20.5% silt, 14.8% fine sand, 38.0% coarse sand and 3.2% humus. The soil was heat treated at 70°C for 16 h prior to the experiment in order to limit the number of insects and pathogens. Soybean seeds were pregerminated on wet filter paper and transferred and inoculated after 4 days. The soybean seedlings were transferred to a small pocket of pith soil placed on the top of the rhizobox. Bacterial inoculation was performed by pipetting 0.5 mL on the root tip of either FMCH001 spores diluted at OD_{600} = 0.001 in 10 mM magnesium sulphate or without spores (mock). The plants were fertilized by watering and grown in 24°C day/18°C night with 16-hour light cycle at 130 µmol m^{-2} s^{-1}. 25 days after inoculation the bottom side of the rhizoboxes were removed, and pictures of the roots were taken using fixed light and camera settings for all pictures.

The root surface area was calculated using image analysis in the software Fiji (https://imagej.net/software/fiji/). The pictures were renamed with randomized numbers to ensure nonbiased analysis. A fixed threshold value was used for all pictures to isolate white pixels representative of the root. False-positive pixels (stones, reflection, etc.) were manually removed using an editing tool.

2.2.2 | Plant physiological and growth indexes in big pots greenhouse experiment

In the Big pots greenhouse experiment, leaf gas exchange, $\delta^{13}C$ and $\delta^{18}O$ composition, C and N concentration, plant water relations, water use, dry mass and water use efficiency, leaf ABA and xylem ABA concentrations, cation and anion concentration in xylem sap were measured.

Leaf gas exchange

From the first day before drought treatment to the end of the experiment, leaf gas exchange rates including net photosynthetic rate (A_n, µmol m^{-2} s^{-1}), stomatal conductance (g_s, mol m^{-2} s^{-1}) and transpiration rate (T_r, mmol m^{-2} s^{-1}) were measured on upper canopy fully expanded leaves between 9:00 and 11:00 am with a portable photosynthetic system (LiCor-6400XT; LI-Cor). Measurements were performed on one leaf per plant at 25°C chamber temperature and 1200 µmol m^{-2} s^{-1} photon flux density. Intrinsic water use efficiency (WUE_i, µmol mol^{-1}) was calculated as the ratio of A_n to g_s, and instantaneous water use efficiency (WUE_T, µmol $mmol^{-1}$) was calculated as the ratio of A_n to T_r.

$\delta^{13}C$ and $\delta^{18}O$ composition, C and N concentration

Stem, leaf and root samples were thoroughly ground into powder and analysed for carbon and oxygen isotope composition ($\delta^{13}C$ and $\delta^{18}O$) using an elemental PyroCube (Elementar Analysensysteme GmbH). C and N concentrations were determined by a CHNS/O Elemental Analyser (Flash 2000; Thermo Fisher Scientific).

Plant water relations

Plant water relation characteristics were measured at each harvest, that is, 23 Dap, 28 Dap and 36 Dap. Relative water content (RWC) and leaf water potential (Ψ_l) were determined on the same leaf for measuring gas exchange. RWC was determined as:

$$RWC = \frac{W_f - W_d}{W_t - W_d},$$

where W_f was the leaf fresh weight, W_t was the leaf turgid weight determined after immerging in distilled water for 2 h and W_d was the leaf dry weight determined after 48 h drying in an oven at 75°C.

Ψ_l was determined with a pressure chamber (Soil Moisture Equipment). After measuring Ψ_l, the leaf was divided into two parts, immediately packed in aluminium foils, frozen in liquid nitrogen and stored at −80°C for measurement of osmotic potential (Ψ_π) and leaf abscisic acid concentration ($[ABA]_{leaf}$). Ψ_π was measured after thawing the leaf sample at 20°C by using a psychrometer (C-52 sample chambers; Wescor Inc.) connected to a microvoltmeter (HR-33T; Wescor). Turgor press (Ψ_p) was the difference between Ψ_l and Ψ_π. Root water potential (Ψ_r) was determined with a Scholander-type pressure chamber (Liu et al., 2006).

Water use, dry mass and water use efficiency

Water use (WU, cm^3) per plant was estimated as the sum of irrigation water and the change of soil moisture in pot between the beginning of treatment (the first harvest) and the end of the harvest. Dry mass (DM, g) was the total dry mass of leaf, stem and root. Water use efficiency (WUE, $kg\,m^{-3}$) of plant was determined as DM to WU ratio.

Leaf ABA and xylem ABA concentrations

Leaf sample was ground into powder with liquid nitrogen, and 27–33 mg was weighted and added into a 1.5-mL Eppendorf tube. The ABA was extracted with 1.0 mL milli-Q water on a shaker at 4°C overnight. The extracts were centrifuged at 14,000×*g* and 0.7 mL supernatants were collected for $[ABA]_{leaf}$ analysis. At each harvest, xylem sap was collected by pressurizing the potted plant in a Scholander-type pressure chamber following the procedure described by (Liu et al., 2006), then immediately frozen in the liquid nitrogen and stored at −80°C for ABA analysis. $[ABA]_{leaf}$ and xylem sap ABA concentration ($[ABA]_{xylem}$) were assayed by ELISA (enzyme-linked immunosorbent assay) following the protocol described by Asch (2000).

Cation and anion analysis

Concentrations of K^+, Ca^{2+}, Mg^{2+}, NO_3^-, PO_4^{3-} and SO_4^{2-} in xylem sap were determined by ion chromatography (Metrohm AG, Herisau, Switzerland), with a Metrosep C4-100 analytical column (4 mm × 125 mm, 1.7 mM nitric acid/0.7 mM dipicolinic acid eluent) and a Metrosep A supp 4 analytical column (4 mm × 125 mm, 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$ eluent) for cations and anions respectively.

2.3 | Quantitative PCR

For the strain dynamics evaluation, 180 mg of each root sample was weighed and inserted in G2 DNA/RNA Enhancer (Ampliqon A/S) and then DNA extraction was performed using the FastDNA Spin Kit for soil (MP Biomedicals), following the manufacturer's instructions.

To perform the strain quantification, the qPCR method was performed using the CFX96 Touch real-Time PCR Detection System (Bio-Rad Laboratories, Inc.), using the primers FMCH001F (CCCCT TGTTGCGAATCTCAA) and FMCH001R (CCGAAGGAAGCACT AGAAAAGATTA). The qPCR analysis was performed in a 12 µL final volume, which contained 6.25 µL of SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 0.25 µL of each primer at 10 µM. Seven-microlitre aliquots of the master mix were dispensed in the wells and 5 µL of DNA was added as a PCR template. The qPCR cycle conditions were: first denaturation step at 98°C for 3 min, 40 cycles at 95°C for 15 s, 60°C for 30 s and a final step at 72°C for 25 s. Three biological replicates were used and further divided into technical triplicates in the microplate.

The standard curves used for the strain quantification was established by spiking a known amount of bacteria in the soil matrix, as described by (Stets et al., 2015). A volume of 180 µL (dilution range 10^3–10^8 CFU) was added to 180 mg of soil and then well mixed. After 20 min at room temperature the DNA extraction was performed following the method described above. The standard curves were generated by plotting the CT value versus the number of CFU added to each tube. No bacteria were added to the negative control. The detection limit of the primer pair used was 10^3 CFU/g of root in the soil matrix.

2.4 | Enzymatic analysis

Protein extraction was done as previously described (Fimognari et al., 2020). In summary, plant material was grinded in liquid nitrogen. A tip (1–3 mg) of polyvinylpolypyrrolidone and 1 mL extraction buffer (40 mM Tris HCl pH 7.6, 1 mM benzamidine, 24 µM NADP, 14 mM β-mercaptoethanol) were added to 0.5 g grinded material following extraction for 30 min at 4°C in rotary shaker. The samples were centrifuged and the supernatant dialyzed overnight in dialysis membrane tubes in 20 mM phosphate buffer pH 7.6. Extracts were stored at −80°C until use. Protein content in each sample was quantified using Bradford assay with bovine serum albumin (BSA) for standard curve (Bradford, 1976). UDP-glucose pyrophosphorylase (UGPase) and fructokinase (FK) kinetic assays were performed according to (Jammer et al., 2015).

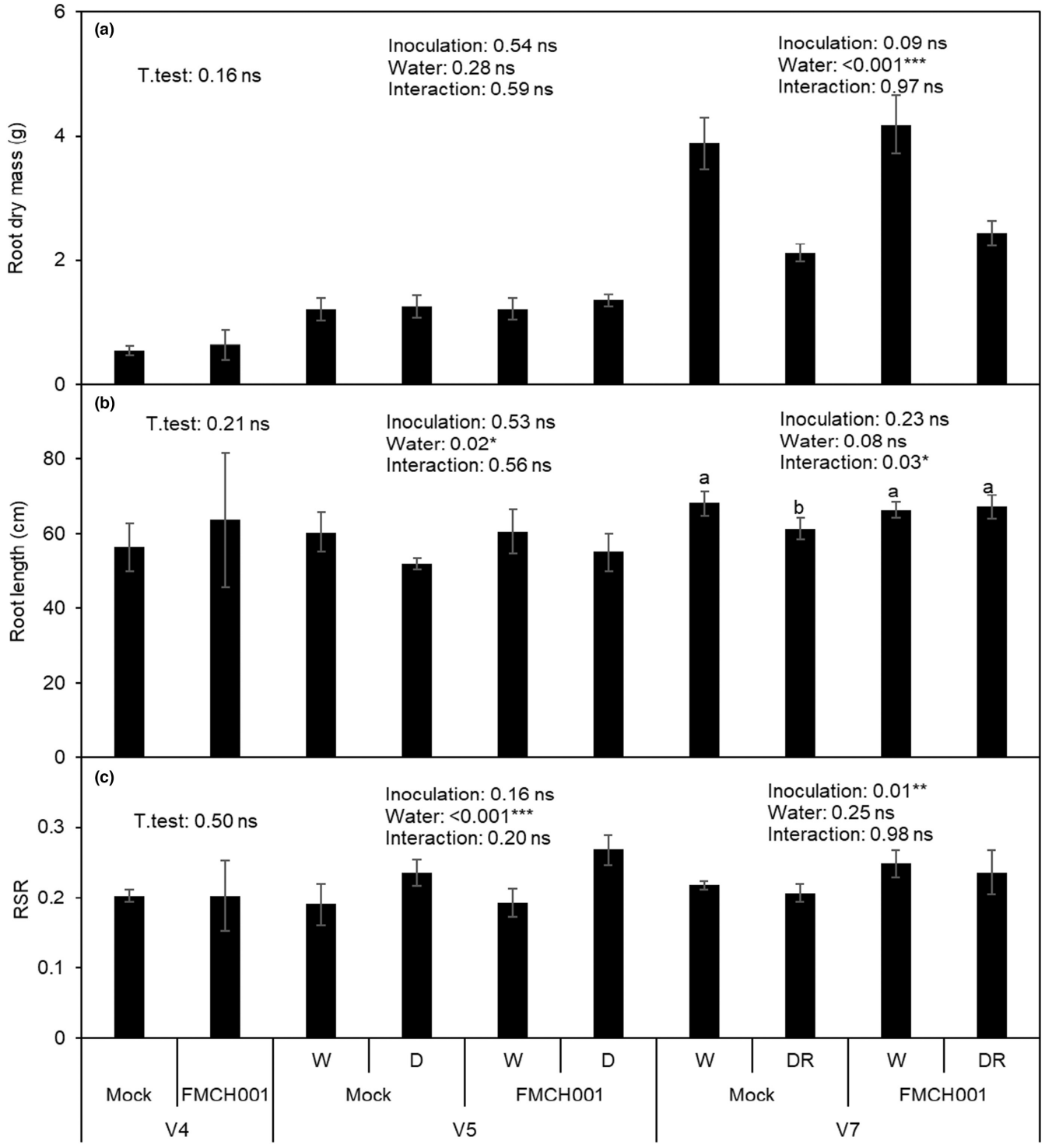


FIGURE 2 The change in soybean root dry mass (a), root length (b) and root to shoot ratio (RSR, C) at stage when 4 nodes on the main stem beginning with the unifoliate node (V4, before drought), 5 nodes on the main stem beginning with the unifoliate node (V5, drought stress exposed) and 7 nodes on the main stem beginning with the unifoliate node (V7, recovery from drought). Plants were either exposed to a negative control treatment or to the bacterium FMCH001. *, ** and *** indicate statistically significant difference based on the Student's *t*-test or the two-way ANOVA on four biological replicates at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. D, drought plants; DR, drought-recovery plants; W, well-watered plants. Bars refer to standard deviation, $n = 4$.

The UGPase assay was done by adding the extract into assay solution (0.44 mM EDTA, 5 mM $MgCl_2$, 0.1% BSA, 2 mM UDPGlc, 1.5 mM Ppi, 1 mM NADP, 2 mM 3-phosphoglycerate (3-PG), 8.0 U/mL G6PDH and 2.7 U/mL PGM in 100 mM TRIS–HCl buffer pH 8.0), and UDPGlc was omitted in control wells. The FK assay was done by adding extract into assay solution (5 mM $MgCl_2$,

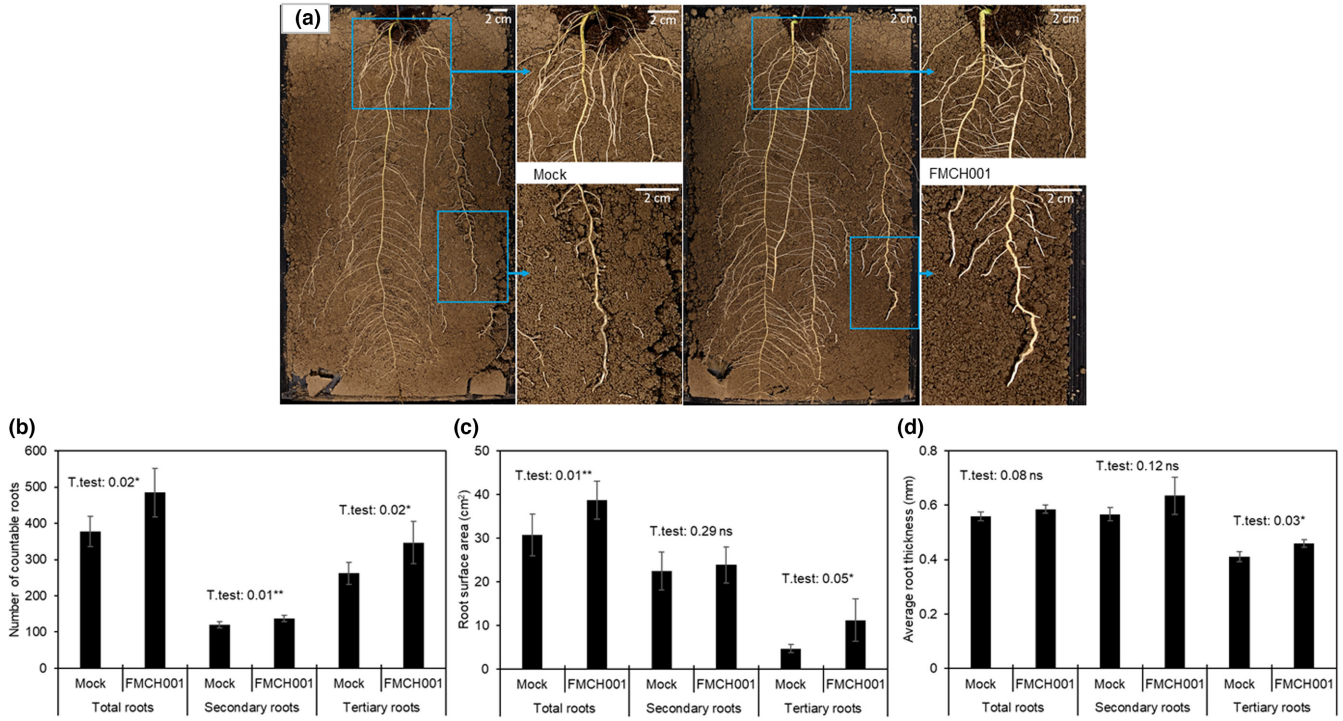


FIGURE 3 FMCH001 treatment changes root count and increases root surface area. (a) representative rhizobox picture of 29-day-old soybean roots which has been grown for 25 days with FMCH001 in well-watered conditions. (b) Number of countable roots, (c) surface area and (d) average root thickness quantified from rhizobox pictures by image analysis. * and ** indicate statistically significant difference as compared to mock based on the Student's *t*-test on four biological replicates at $p < 0.05$ and $p < 0.01$ respectively. Full bars indicate mock treatment and empty bars refer to FMCH001 treatment. Bars refer to standard deviation, $n = 4$.

5 mM fructose, 1 mM NAD, 2.5 mM ATP, 0.9 U/mL G6PDH and 5.0 U/mL PGI in 50 mM Bis-TRIS buffer pH 8.0), and fructose was omitted in control wells. For both UGPase and FK assays the increase in absorbance was measured at 340 nm (30°C, 40 min), and the extinction coefficient 6.22 mM^{-1} cm^{-1} was used for calculating enzyme activity.

Kinetic assays for glutathione reductase (GR) and catalase (CAT) were carried out according to (Fimognari et al., 2020). In short, GR was measured by adding the extract to the assay solution (0.2 mM NADPH, 0.6 mM oxidized glutathione/GSSG in 100 mM Tris HCl pH 7.8), in which GSSG was omitted from control wells. CAT assay was done by adding extract to assay solution (0.001% antifoam 204 and 100 mM H_2O_2 in 50 mM K_2HPO_4/KH_2PO_4 buffer pH 7.0), in which H_2O_2 was omitted from control wells. Decrease in absorbance was recorded at 340 nm (30°C, 40 min) for GR and at 240 nm (30°C, 40 min) for CAT. For calculation of enzyme activity, extinction coefficients of 6.22 mM^{-1} cm^{-1} and 43.6 mM^{-1} cm^{-1} were used for GR and CAT respectively. For all enzyme assays the spectrophotometric measurement was done in 96-well microtitre plates using 160 µL per well including 0.5–10 µL extract depending on assay and plant tissue type. The extract volumes per well were 10 µL for UGPase, 10 µL for FK, 5 µL for GR for both WW and drought-recovery samplings. For CAT, 5 µL of extract was used in WW conditions, 1 µL for the samples harvested during drought, and 0.5 µL the samples harvested after drought-recovery.

2.5 | Data analysis and statistics

Student's t-test was conducted for data collected at the first harvest in Microsoft Excel 2016 (Microsoft Corporation). For the data collected at the second and third harvest, two-way analysis of variance (ANOVA) was conducted to analyse the effects of inoculation and irrigation treatment on plant physiology, growth and WUE in SPSS 21 software package (Version 21.0; IBM SPSS). Graphs were plotted in Microsoft Excel 2016.

3 | RESULTS

3.1 | The *B. paralicheniformis* FMCH001 colonizes the root system and improves root growth and nutrient uptake in soybean plants

During V4 period, there was no significant difference in root dry mass, root length and root to shoot ratio (RSR) between mock and FMCH001 inoculated plants (Figure 2). And FMCH001 inoculation had no effect on root dry mass throughout the experiment (Figure 2a). During the V7 period, after recovery from drought, the root dry mass was significantly depressed compared to that of well-watered plants (Figure 2a). Accordingly, at the same time point root length was increased by 9.4% after recovery from drought in the inoculated treatment as compared to mock inoculation (Figure 2b). During V5

TABLE 1 The concentrations of ions in xylem sap of soybean at 4 nodes on the main stem beginning with the unifoliate node (V4) stage (before drought), 5 nodes on the main stem beginning with the unifoliate node (V5) stage (drought stress exposed) and 7 nodes on the main stem beginning with the unifoliate node (V7) stage (recovery from drought).

	Cl^-	NO_3^-	PO_4^{3-}	SO_4^{2-}	Na^+	K^+	Ca^{2+}	Mg^{2+}
	$mg\,L^{-1}$							
V4								
Mock, W	10.20	389.41	189.40	33.05b	5.64	233.01b	238.09b	29.83b
FMCH001, W	8.98	672.20	196.54	64.22a	4.31	356.51a	342.90a	44.72a
T test	*0.39*	*0.08*	*0.20*	*0.01***	*0.21*	*0.03**	*0.05**	*0.05**
V5								
Mock, W	15.12	328.28	203.71b	85.65	9.75	139.74	437.68	53.57
FMCH001, W	15.65	287.68	368.89a	65.94	9.07	119.32	456.44	41.81
Mock, D	28.34	764.40	193.60b	98.91	5.13	321.51	626.43	75.78
FMCH001, D	34.99	800.03	203.71b	61.60	10.03	280.93	608.00	68.18
Two-way ANOVA								
Inoculation	*0.421*	*0.98*	*<0.001****	*0.03*	*0.375*	*0.44*	*0.99*	*0.28*
Water	*0.003**	*<0.001****	*0.01***	*0.70*	*0.439*	*0.001****	*<0.001****	*0.02**
Interaction	*0.490*	*0.66*	*0.01***	*0.45*	*0.247*	*0.80*	*0.50*	*0.81*
V7								
Mock, W	26.62	106.03	774.66	177.81	5.14	184.61	624.18a	135.77
FMCH001, W	28.62	132.13	850.74	146.04	27.95	117.63	608.21a	117.44
Mock, DR	23.29	211.38	402.11	109.98	11.04	103.45	417.16c	73.21
FMCH001, DR	25.94	271.46	475.76	110.15	13.08	90.99	491.91b	75.22
Two-way ANOVA								
Inoculation	*0.459*	*0.34*	*0.10*	*0.28*	*0.119*	*0.04**	*0.19*	*0.20*
Water	*0.336*	*0.02**	*<0.001****	*0.01***	*0.556*	*0.01*	*<0.001****	*<0.001****
Interaction	*0.911*	*0.70*	*0.98*	*0.28*	*0.186*	*0.14*	*0.05**	*0.12*

Note: *, **and *** refer to statistically significant differences as compared to control at $p < 0.05$, $p < 0.01$ and $p < 0.001$ based on the Student's *t*-test or two-way ANOVA (italic values) on four biological replicates. D, drought plants; Dap, days after planting; DR, drought-recovery; W, well-watered treatment.

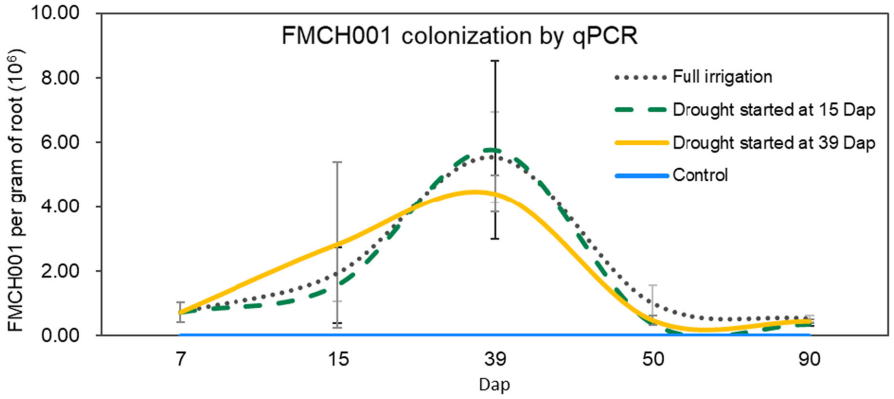


FIGURE 4 qPCR quantification of FMCH001 colonization of soybean roots in full irrigation and drought treatments. Black dotted line represents FMCH001 growth in full irrigation conditions. Green dashed line refers to drought treatment during the 15–35 Dap period and the yellow line indicates drought treatment during the 39–58 Dap period. In control (mock) treated plants, FMCH001 was not detected at any harvest points (blue full line). FMCH001 quantification was performed via a standard curve correlating CT value to bacteria count in the soil matrix. Dap: days after planting. Bars depict the standard error and each point represents the average of three biological replicates.

period, RSR was significantly decreased by drought stress, and the negative effect faded away after recovery from drought. At V7, RSR of FMCH001 inoculated plants was 14.09% higher than that of the mock (Figure 2c). Thought plant height of FMCH001-treated plants was higher than that of Mock at V4, total dry mass, plant height and specific leaf area did not differ significantly between control and

the inoculation treatment at other harvest points (Supplementary Table 1).

To visualize these changes in root size/architecture, we established a rhizobox platform where the soybean roots grew against a plexiglass slide at a 45° angle for 25 days. Pictures were taken at the end of the experiment and root area was quantified by means of image analysis (Figure 3a). FMCH001-treated plants showed an increased total, secondary and tertiary roots count (Figure 3b, $p < .05$), an increase in total and tertiary roots surface area (Figure 3c, $p < .05$) and thicker tertiary roots (Figure 3d, $p < .05$). Collectively, these data suggest that the FMCH001 treatment influences root growth and RSR at later stages of vegetative growth.

The size and architecture of the root system are critical for the access of water and nutrients in the soil. Given the effect of FMCH001 to stimulate root biomass and root length, we tested the elemental concentrations in the xylem sap of soybean treated or untreated with FMCH001 (Table 1). During the first harvest at 23 Dap, FMCH001-treated plants showed higher levels of SO_4^{2-} (+94.31%), K^+ (+53.00%), Ca^{2+} (+44.02%) and Mg^{2+} (+49.94%) than Mock ones. During V5 stage, PO_4^{3-} levels were 81.08% and 12.94% higher in the FMCH001 as compared to control during the well-watered and drought stress treatment respectively. In the last harvest at V7 stage, FMCH001-treated plants that were exposed to the drought-recovery treatment showed a 17.92% higher Ca^{2+} as compared to control. These results imply a higher uptake of nutrients in the plants treated with FMCH001, especially at early developmental stages.

Having noticed the effects of the bacteria in stimulating root growth and nutrient acquisition, we performed two parallel experiments aimed at quantifying and visualizing FMCH001 colonization. In the first experiment (Figure 4), qPCR was used to quantify bacterial colonization over time in the root system (closely associated rhizosphere soil and roots). FMCH001 growth peaked around 39 Dap to a concentration of $4.41–5.76 \times 10^6$ bacteria/gram of root. Bacterial growth was independent from the watering treatment

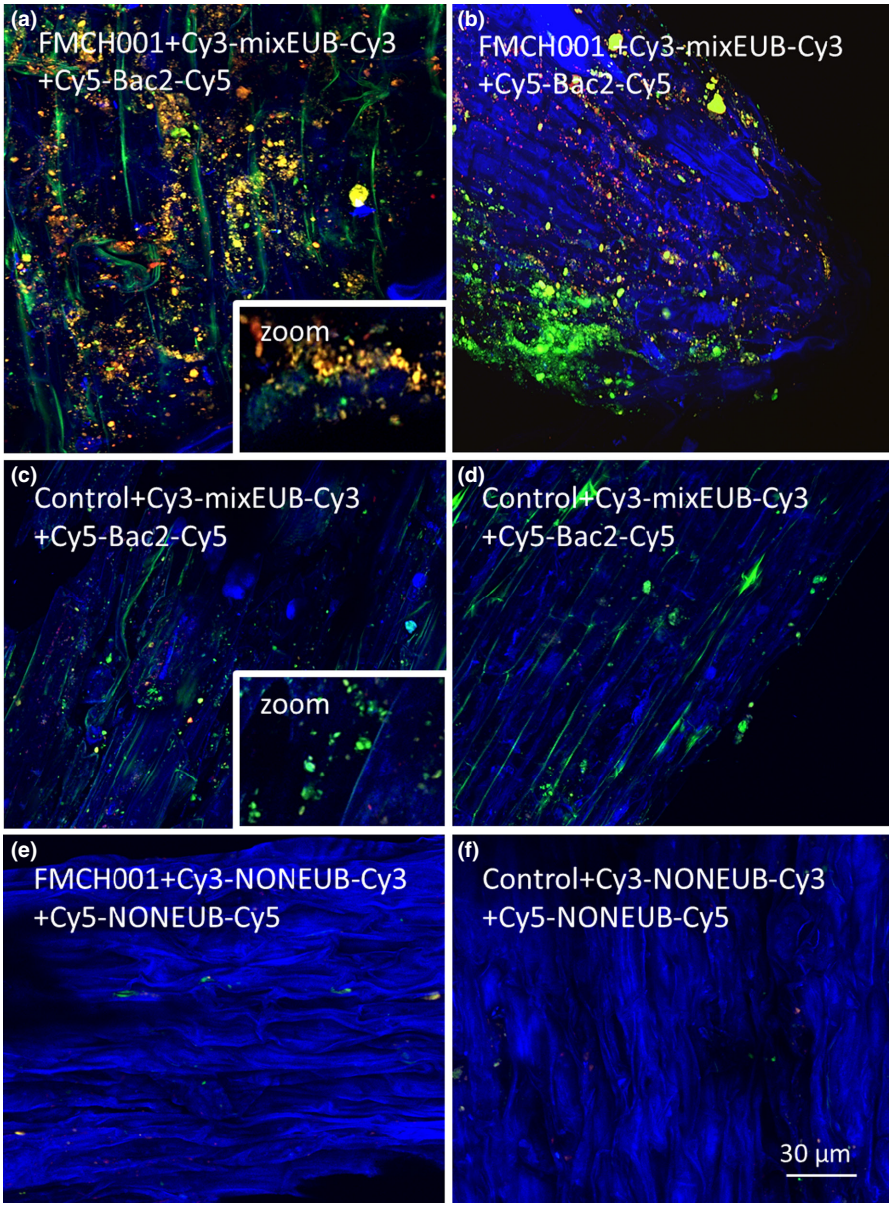


FIGURE 5 Fluorescence in situ hybridization (FISH) of FMCH001 colonization. The pictures above depict soybean roots 23 Dap of seeds treated with FMCH001. (a) Root surface colonized by FMCH001. FMCH001 appears in orange/reddish and other bacteria as green fluorescent using mixEUB and Bac2 probes as well as negative probe (NONEUB) coupled with Cy3 and Cy5. Cy3 signal was assigned as green and Cy5 as red colour. (b) root tip colonized by FMCH001 in presence of other bacteria. (c and d) The presence of bacteria on the root surface of uninoculated control plants. Note that some orange signals were recorded but only a few compared to roots from seeds inoculated with FMCH001. (E and F) Detection of a few native autofluorescent microorganisms on the root surfaces of samples subjected to NONEUB probes. Representative images of 15 biological replicates.

TABLE 2 The change in photosynthetic activity, WU and WUE at 4 nodes on the main stem beginning with the unifoliate node (V4) stage (before drought), 5 nodes on the main stem beginning with the unifoliate node (V5) stage (drought stress exposed) and 7 nodes on the main stem beginning with the unifoliate node (V7) stage (recovery from drought). A_n: photosynthetic rate, g_s: stomatal conductance, T_r: transpiration rate, WUE_i: intrinsic water use efficiency, WUE_T: instantaneous water use efficiency, WU: water use, WUE: water use efficiency at the plant level.

	A_n	g_s	T_r	WUE_i	WUE_T	WU	WUE
	$\mu mol\, m^{-2}\, s^{-1}$	$mol\, m^{-2}\, s^{-1}$	$mmol\, m^{-2}\, s^{-1}$	$\mu mol\, mol^{-1}$	$\mu mol\, mmol^{-1}$	ml	$kg\, m^{-3}$
V4							
Mock, W	12.94b	0.21b	4.37b	61.34	2.99	2258	1.47
FMCH001, W	14.96a	0.26a	5.20a	58.76	2.89	1902	2.18
T test	*0.02**	*0.02**	*0.01***	*0.14*	*0.18*	0.07	0.03*
V5							
Mock, W	14.69	0.32	6.75	47.60	2.26	4062	1.87
FMCH001, W	17.22	0.39	7.83	49.10	2.31	3528	2.21
Mock, D	8.76	0.14	3.22	70.12	2.61	2834	2.32
FMCH001, D	10.64	0.17	3.94	68.11	2.54	2184	2.98
Two-way ANOVA							
Inoculation	*0.01***	*0.03**	*0.02**	*0.94*	*0.89*	*0.04**	*0.01***
Water	*<0.001****	*<0.001****	*<0.001****	*<0.001****	*0.01***	*<0.001****	*0.01***
Interaction	*0.68*	*0.39*	*0.62*	*0.61*	*0.57*	*0.82*	*0.34*
V7							
Mock, W	17.55	0.41	8.01	44.45	2.23	8571	2.53
FMCH001, W	18.51	0.40	8.22	47.58	2.27	8422	2.50
Mock, DR	16.05	0.31	7.08	54.10	2.31	5466	2.29
FMCH001, DR	17.19	0.32	7.18	56.01	2.43	5216	2.49
Two-way ANOVA							
Inoculation	*0.01***	*0.90*	*0.51*	*0.19*	*0.11*	*0.54*	*0.47*
Water	*<0.001****	*<0.001****	*<0.001****	*<0.001****	*0.02**	*<0.001****	*0.29*
Interaction	*0.81*	*0.55*	*0.81*	*0.75*	*0.52*	*0.88*	*0.36*

Note: *, **and *** refer to statistically significant differences as compared to control at $p < 0.05$, $p < 0.01$ and $p < 0.001$ based on the Student's *t*-test or two-way ANOVA (italic values) on four biological replicates. D, drought plants; Dap, days after planting; DR, drought-recovery; W, well-watered treatment.

suggesting that drought did not delay or restrict FMCH001 root colonization (Figure 4). During the peak of FMCH001 colonization at 39 Dap, highest bacterial counts were found in the middle depth part of the root (30–60 cm depth, Supplementary Table 2).

In a second experiment, we visualized FMCH001 colonization on soybean roots by means of *fluorescence* in situ *hybridization* (FISH) microscopy. Soybean seeds were inoculated with FMCH001 and grown in nonsterile soil conditions for 23 days. FMCH001 was visible on the surface of the roots after 23 days of growth among other bacteria, especially at root hairs zones (Figure 5a) and root tips (Figure 5b). In comparison, only a small amount of signal from probes targeting *Bacillus paralicheniformis* and other closely related *Bacillus* spp. was recorded on the root surfaces of uninoculated control plants (Figure 5c,d). Only a few native autofluorescent microbes were detected using negative probes (Figure 5e,f).

These results demonstrate that the FMCH001 is a good soybean root colonizer and its growth is detectable up to a period of 90 days after inoculation. The FMCH001 colonization peaked at 39 Dap irrespective of drought conditions and it colonized the root surface, preferentially the root tips and in the vicinity of root hairs.

3.2 | FMCH001 promotes a systemic response, influencing photosynthesis rate, carbon and oxygen isotope composition, plant water relations and C:N partitioning

In order to investigate whether the beneficial bacteria can influence the metabolism of plant tissues beyond roots, we measured photosynthetic activity and water relations in the shoot and at the whole plant level. For FMCH001-inoculated plants in both well-watered and drought conditions, we measured increased photosynthesis rate (A_n) at 23 Dap, 28 Dap and 36 Dap when plants were at vegetative stages (V4, V5 and V7 respectively); and improved stomatal conductance (g_s) and transpiration rate (T_r) at V4 and V5 stages (Table 2). At

TABLE 3 The change in $\delta^{13}C$ and $\delta^{18}O$ levels in stem, leaf and root at 4 nodes on the main stem beginning with the unifoliate node (V4) stage (before drought), 5 nodes on the main stem beginning with the unifoliate node (V5) stage (drought stress exposed) and 7 nodes on the main stem beginning with the unifoliate node (V7) stage (recovery from drought).

	$[\delta^{13}C]_{stem}$	$[\delta^{18}O]_{stem}$	$[\delta^{13}C]_{leaf}$	$[\delta^{18}O]_{leaf}$	$[\delta^{13}C]_{root}$	$[\delta^{18}O]_{root}$
	‰					
V4						
Mock, W	−29.97	23.09	−30.19	28.68	−29.54	20.14
FMCH001, W	−29.78	22.94	−29.74	29.17	−29.35	19.93
T test	*0.54*	*0.63*	*0.16*	*0.25*	*0.55*	*0.49*
V5						
Mock, W	−30.50	23.35	−30.44	28.16	−29.47	19.46
FMCH001, W	−30.62	23.05	−30.49	28.35	−29.70	19.62
Mock, D	−28.90	23.27	−29.20	28.87	−28.39	19.50
FMCH001, D	−29.01	23.69	−29.21	29.15	−28.32	19.69
Two-way ANOVA						
Inoculation	*0.37*	*0.63*	*0.83*	*0.27*	*0.61*	*0.54*
Water	*<0.001***	*0.04**	*<0.001****	*0.01***	*<0.001****	*0.85*
Interaction	*0.94*	*0.01***	*0.90*	*0.82*	*0.35*	*0.95*
V7						
Mock, W	−29.91	23.30	−29.92	27.93	−29.05	19.72
FMCH001, W	−30.34	23.05	−30.05	27.86	−29.48	19.39
Mock, DR	−29.20	23.30	−29.44	28.31	−28.17	19.73
FMCH001, DR	−29.07	23.81	−29.28	28.67	−28.15	19.51
Two-way ANOVA						
Inoculation	*0.26*	*0.48*	*0.87*	*0.36*	*0.09*	*0.04**
Water	*<0.001****	*0.04**	*<0.001****	*0.01***	*<0.001****	*0.60*
Interaction	*0.05**	*0.04**	*0.17*	*0.19*	*0.07*	*0.64*

Note: *, **and *** refer to statistically significant differences as compared to control at $p < 0.05$, $p < 0.01$ and $p < 0.001$ based on the Student's *t*-test or two-way ANOVA (italic values) on four biological replicates. D, drought plants; Dap, days after planting; DR, drought-recovery; W, well-watered treatment.

all measuring periods, the well-watered plants possessed higher A_n, g_s and T_r than the drought-stressed or the recovered from drought plants. WUE_i and WUE_T were solely affected by water, and plants that experienced drought stress possessed higher WUE_i and WUE_T than the well-watered ones.

In relation to Mock, FMCH001 inoculation significantly reduced plant water use (WU) during the V4–V5 stage, consequently, significantly improved water use efficiency (WUE) by 28.29% and 18.35% under well-watered and drought-stressed conditions respectively.

Previous studies (Wang et al., 2018) demonstrated a correlation between carbon and oxygen isotope composition ($\delta^{13}C$ and $\delta^{18}O$) levels and g_s, T_r, A_n and WUE. Given the significant improvement of the FMCH001 on WUE and A_n, as well as the increases in g_s and T_r, we tested whether $\delta^{13}C$ and $\delta^{18}O$ levels were differently regulated in the FMCH001 plants (Table 3). FMCH001 inoculation only had effect on $[\delta^{18}O]_{root}$ at V7 stage that $[\delta^{18}O]_{root}$ was decreased by FMCH001. $[\delta^{13}C]$ in stem, leaf and root of plants experienced drought stress at V5 stage or recovered from drought at V7 stage was significantly improved compared to those of well-watered plants. Moreover, $\delta^{18}O$ in stem and leaf was significantly affected by water at V5 and V7 stages. In relation to well-watered plants, plant experienced drought had increased $\delta^{18}O$ in leaf for both Mock and FMCH001-treated plants, and the increase of $\delta^{18}O$ in stem under drought stress or recovery from drought was only observed in FMCH001 plants.

WUE, g_s, T_r and photosynthetic efficiency are all affected by the water status within the plant. To shed light on the physiological causes of improved WUE and photosynthetic efficiency in FMCH001-treated plants, we measured leaf and root water potential (Ψ_l and Ψ_r), osmotic potential (O_s), turgor pressure (Ψ_p) and relative water content (RWC) as seen in Table 4. At V4 stage, we noticed higher Ψ_l, Ψ_p and RWC in the FMCH001-inoculated plants compared to the mock plants, while, Ψ_l and O_s were lower in the FMCH001-inoculated plants at V5 stage, and inoculation had no effect on plant water status at V7 stage. Ψ_l, O_s, Ψ_p and Ψ_r were significantly decreased by drought at V5 stage, and after the recovery from drought (V7), there was no significant difference in O_s, Ψ_p and Ψ_r between well-watered and drought-stressed plants.

Having measured improved A_n and mineral uptake in FMCH001-treated plants, we investigated the partitioning of the chemical elements C, H and N in the stem, leaves and roots by means of mass

TABLE 4 Plant water relations are influenced by the FMCH001 at early developmental stages. Ψ_l: leaf water potential, Os: osmotic potential, Ψ_p: turgor pressure, Ψr: root water potential, RWC: relative water content.

	Ψ_l MPa	*Os* MPa	Ψ_p MPa	Ψ_r MPa	RWC
V4					
Mock, W	−0.30b	−0.67	0.37b	−0.04	0.85b
FMCH001, W	−0.14a	−0.73	0.60a	−0.03	0.88a
T test	*0.05**	*0.29*	*0.05**	*0.20*	*0.001***
V5					
Mock, W	−0.46	−0.92	0.46	−0.06	0.80
FMCH001, W	−0.70	−1.00	0.30	−0.06	0.81
Mock, D	−1.31	−1.26	0.04	−0.61	0.79
FMCH001, D	−1.44	−1.36	0.07	−0.70	0.77
Two-way ANOVA					
Inoculation	*0.05**	*0.05**	*0.36*	*0.17*	*0.72*
Water	*<0.001****	*<0.001****	*0.001***	*<0.001****	*0.08*
Interaction	*0.50*	*0.96*	*0.19*	*0.22*	*0.40*
V7					
Mock, W	−0.80	−1.12	0.32	−0.10	0.83
FMCH001, W	−0.75	−1.06	0.31	−0.12	0.85
Mock, DR	−0.91	−1.01	0.10	−0.11	0.84
FMCH001, DR	−0.97	−1.12	0.15	−0.14	0.86
Two-way ANOVA					
Inoculation	*0.97*	*0.67*	*0.85*	*0.07*	*0.30*
Water	*0.04**	*0.64*	*0.07*	*0.17*	*0.62*
Interaction	*0.61*	*0.11*	*0.80*	*0.52*	*0.85*

Note: *, **and *** refer statistically significant differences as compared to control at $p < 0.05$, $p < 0.01$ and $p < 0.001$ based on the Student's *t*-test or two-way ANOVA (italic values) on four biological replicates. D, drought plants; Dap, days after planting; DR, drought-recovery; W, well-watered treatment.

spectrometry (Supplementary Figure 1). C:N ratio is a particular important parameter to observe since it describes how much carbon the plant is able to incorporate with a given amount of nitrogen and represent the nitrogen use efficiency in N-limiting conditions (Zhang, He, et al., 2020; Zhang, Wang, et al., 2020). As shown in Figure 6, C:N ratio were higher in the FMCH001-inoculated plants as compared to control at early stages of vegetative soybean growth. At the V4 stage, FMCH001-inoculated plants had 7.23% higher C:N ratio in the stem than mock plants (Figure 6a). At the V5 stage, FMCH001-inoculated plants had 7.36% and 15.60% higher C:N ratio in the roots of well-watered and drought plants, respectively, as compared to control (Figure 6b).

3.3 | FMCH001 inoculation had no effect on ABA levels in the leaf and xylem sap

FMCH001 inoculation had no effect on concentrations of ABA in leaf and xylem at V4, V5 or V7 stage (Figure 7a,b). As expected under drought conditions at V5, ABA levels spiked to 3–10-fold higher as compared to well-watered conditions. After recovery from drought at V7, ABA dropped to levels comparable to plants that were not subjected to drought.

3.4 | FMCH001 inoculation modulates key enzymatic activities at V4 stage

Because we observed changes in photosynthesis and C:N ratio in FMCH001 treated plants, we decided to measure the activity of UDP-Glc pyrophosphorylase (UGPase, Figure 8a,b) and fructokinase (FK, Supplementary Figure 2), which are the enzymes involved in central sugar metabolism. The activity of UGPase in the roots was regulated by FMCH001 inoculation in the first harvest at V4, that FMCH001 significantly lowered UGPase activity in the roots by 47.63% (Figure 8b). Another important factor of plant health and growth is a balanced control of reactive oxygen species (ROS), which accumulate during photosynthesis and stress such as drought. A fine-tuning of ROS levels is important, as too high levels results in oxidative cell damage; however, ROS are still required to some extent as they function as important signalling molecules regulating growth (Huang et al., 2019; Kasote et al., 2015). We measured

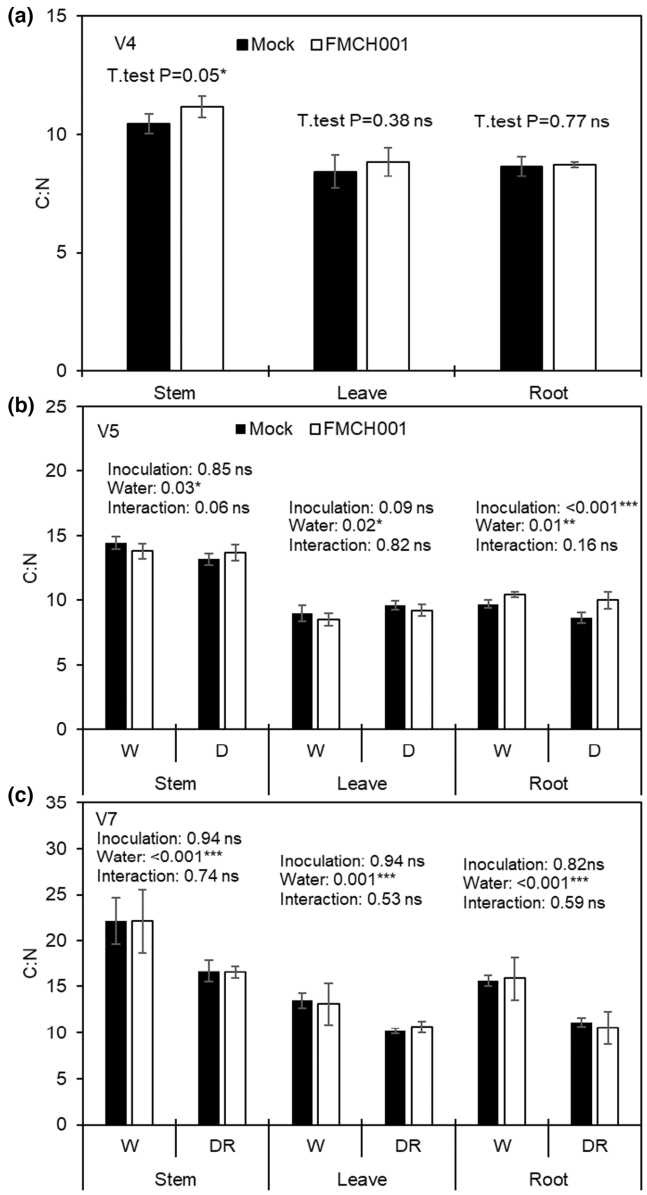


FIGURE 6 FMCH001 treatment increases the C:N ratio at early vegetative stages. *, ** and *** indicate statistically significant difference based on the Student's *t*-test or the two-way ANOVA on four biological replicates at $p < 0.05$, $p < 0.01$ and $p < 0.001$ respectively. Dap: days after planting. D, drought plants; DR, drought-recovery plants; W, well-watered plants. Bars refer to standard deviation, $n = 4$.

the activity of the ROS-scavenging enzymes glutathione reductase (GR, Figure 8c,d) and catalase (CAT, Supplementary Figure 2). In our study, at any harvest point, GR was not affected by bacteria inoculation or water. There was no difference in catalase and fructokinase activities in leaves or roots (Supplementary Figure 2), highlighting the specificity of the FMCH001 to alter UGPase activities.

4 | DISCUSSION

Soybean is one of the most widely cultivated crops due to its excellent nutritional values, making it a high source of proteins, oils and minerals (Engels et al., 2017). Finding strategies for improving soybean productivity under conditions of drought stress is one of the key aspects to achieve high yield. To this end, we explored the potential of a commercial PGPR, *Bacillus paralicheniformis* FMCH001, to alter water relations and other key physiological and biochemical parameters in soybean exposed to drought and well-watered conditions. Desirable plant traits include the stimulation of photosynthetic rate (A_n), nutrient acquisition, root growth, nitrogen and water use efficiency. The ability of this strain to influence these key survival parameters is discussed below.

4.1 | The *Bacillus* FMCH001 promotes root growth which is a key feature in drought escape

Broadly speaking, plants have three main mechanisms that allow them to sustain drought stress in the field: (1) escaping drought, (2) dehydration postponement and (3) drought tolerance (Turner et al., 2001). Drought escape is achieved by minimizing the possibility of encountering drought (Mayla et al., 2021), for example, by the selection of cultivars that are early flowering (Subbarao et al., 1995). However, in climates where rainfall is unpredictable, this strategy may not be sufficient, and early flowering may put the plant in danger in the season, encountering frost damage in certain parts of the world (Srinivasan et al., 1998). Another well-known strategy to escape drought is by maximizing root growth and reaching water in deeper soil. It has been previously shown that a longer and larger root system is a feature associated with the presence of beneficial *Bacillus* spp. and confers water stress resistance (Jochum et al., 2019). Our results suggest that FMCH001 inoculation increases root surface area, root count and root thickness (Figure 3), as well as root to shoot ratio (RSR, Figure 2c) at V7, therefore, eliciting a beneficial plant trait that can alleviate stress in water-limiting conditions. In the literature, a myriad of pathways controlling root growth and RSR are described. For example, a connection between UGPase levels and root growth has been identified in *Arabidopsis* as the double knockout mutant *ugp1 ugp2* showed elongated hypocotyl and roots when grown in dark conditions (Meng et al., 2009). Overexpression of *PdUGPase2* in poplar, which is the native gene coding for UGPase, resulted in decreased shoot and root growth (Payyavula et al., 2014). Although the mechanism remains unclear, there seems to be a negative correlation between UGPase levels and root growth. In our study we found reduced UGPase activity in the roots of FMCH001 inoculated soybean at V4 and V7 in well-watered conditions which may be involved in the larger root system that we noticed after bacteria inoculation. Overall, FMCH001 inoculation increased root growth in soybean; this may partly be due to the alteration of key enzymatic activities in the roots like the UGPase, thereby conferring a competitive advantage to the plant.

4.2 | The *Bacillus* FMCH001 promotes leaf gas exchange rates and influences elemental concentration which may promote dehydration postponement in soybean

The second survival mechanism for plants suffering drought is dehydration postponement, which is achieved by maintaining turgor

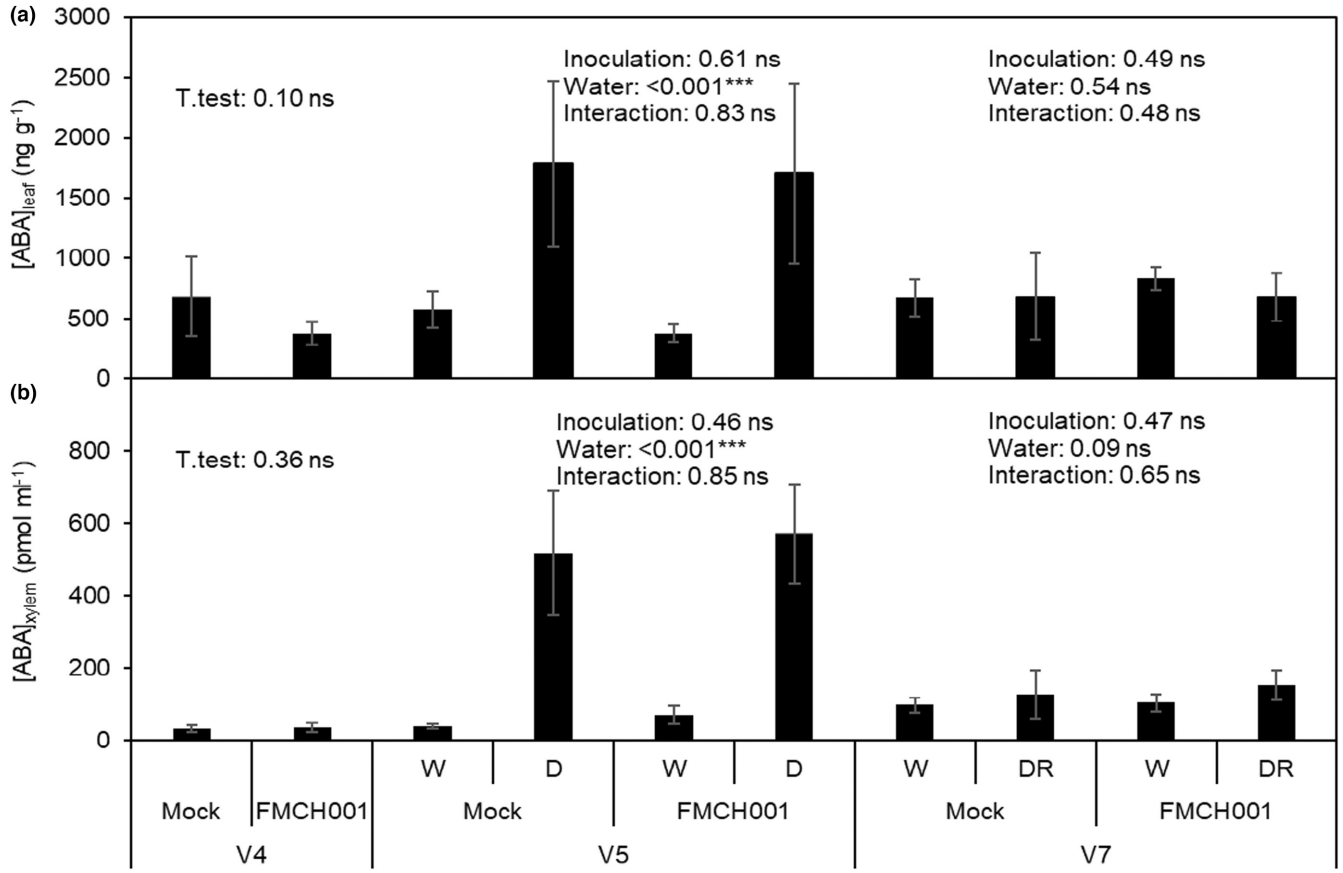
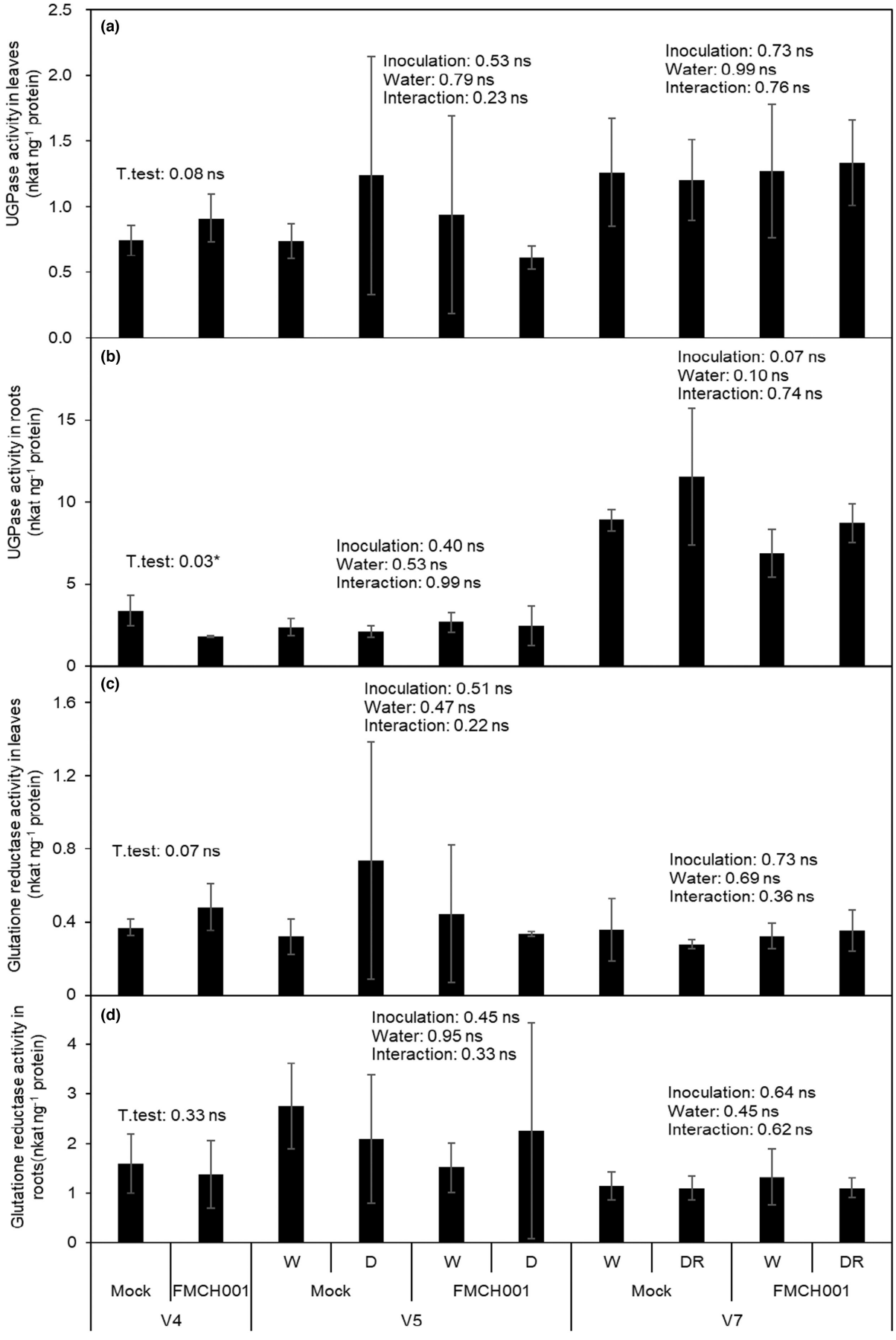


FIGURE 7 Concentrations of ABA in leaves and xylem sap at stage when 4 nodes on the main stem beginning with the unifoliate node (V4, before drought), 5 nodes on the main stem beginning with the unifoliate node (V5, drought stress exposed) and 7 nodes on the main stem beginning with the unifoliate node (V7, recovery from drought). (a) ABA concentration in leaves ($[ABA]_{leaf}$) and (b) ABA concentration in xylem sap ($[ABA]_{xylem}$). *** indicates statistically significant differences based on the Student's *t*-test or the two-way ANOVA on four biological replicates at $p < 0.001$. D, drought plants; Dap, days after planting; W, well-watered plants; DR, drought-recovery plants. Bars refer to standard deviation, $n = 4$.

in conditions of water limitation. This can occur through increased water uptake and limiting water evaporation through cuticles and stomata. Abscisic acid (ABA) is synthesized primarily in the roots and it is then translocated to the leaves through the xylem sap to elicit stomata closing and drought responses due to a reprogramming of the transcriptome and activation of either ABA-dependent or independent pathways (Yamaguchi-Shinozaki & Shinozaki, 1994). ABA can then be recirculated from the leaves back to roots via the phloem and a part of it can be funnelled back to the xylem sap (Hartung et al., 2002). As expected, in our experiments we detected a spike in ABA levels in both leaves and xylem sap during drought stress (Figure 7). It has previously been shown that ABA translocated from the leaves to the roots has an impact in promoting root growth and inhibiting lateral root development in well-watered conditions (McAdam et al., 2016). This translocation may, therefore, contribute to the higher RSR that we noticed in the FMCH001 treatment during V7 in well-watered conditions, but it is in contrast to the increased lateral root formation that we noticed in rhizoboxes. Furthermore, during nondry conditions, it is desirable to have high stomatal conductance (g_s) and transpiration rates (T_r) in order to facilitate high rates of photosynthesis, and ABA is a dominant negative regulator of these processes (Munemasa et al., 2015). In the present study, increases in g_s, T_r and A_n were observed in FMCH001-treated plants in comparison with Mock plants at 28 Dap (Table 2), and similar results were reported in potato plants by Liu et al. (2022), while the difference in $[ABA]_{leaf}$ or $[ABA]_{xylem}$ induced by FMCH001 inoculation was not significant (Figure 7). Strains of *Bacillus* spp. have previously been shown to be able to synthesize ABA (Shahzad et al., 2017), hence we cannot exclude that the slight increase in $[ABA]_{xylem}$ of FMCH001-treated plants may have originated from the bacterium

FIGURE 8 Quantification of UGPase and GR enzymatic activities in leaves and roots. (a) UGPase activity in leaves, (b) UGPase activity in roots, (c) Glutathione reductase activity in leaves, (d) Glutathione reductase activity in roots. * refers to statistically significant differences as compared to control ($p < 0.05$) based on the Student's *t*-test. Enzymatic activity is given in nkat activity normalized by total amount of protein in the sample and is an average of four biological replicates. D, drought plants; Dap, days after planting; DR, drought-recovery plants; W, well-watered plants. Bars refer to standard deviation, $n = 4$.



even though it cannot induce the change of g_s. This could indicate FMCH001 inoculation affected soybean leaf gas exchange rates under both well-watered and drought-stress situations by non-ABA pathway. Furthermore, it should be noticed that the ABA levels of drought-stressed plants were very high (Figure 7; Liu et al., 2006; Liu et al., 2019), possibly masking an effect of the bacterium, and we cannot exclude that there might have been differences between FMCH001 and control if we sampled at earlier drought stages.

Osmotic adjustment is a very important factor for increasing plant water potential, thereby allowing more water to flow into the roots and to be retained within the leaves (Turner et al., 2001). Elemental analysis in the xylem sap point at a higher concentration of minerals in the xylem sap of FMCH001-treated soybean as compared to control, especially at V4 stage (Table 1). Besides being an indicator of better nutrient acquisition, element concentrations and especially high K^+ has an impact on the osmotic potential (Os) and the plant–water relationships due to its crucial role in stomata opening (Cochrane & Cochrane, 2009). We did not observe changes in Os after FMCH001 treatment, but ψ_l, ψ_p as well as RWC were significantly higher in the FMCH001 at V4 stage (Table 4). These data suggest better water status in the FMCH001 treatment, especially at early developmental stages.

4.3 | The Bacillus FMCH001 increases WUE and photosynthetic rate which are key parameters for plant productivity, especially in water stress conditions

The third drought survival mechanism is drought tolerance, which includes a physiological adaptation that allows the plant to operate at low leaf water status. Membrane integrity is often disrupted at high dehydration levels, so the ability of plants to prevent electrolyte leakage and/or reconstruct membranes after extreme dehydration is crucial for tolerating drought and is ubiquitous in resurrection plants (Turner et al., 2001). During drought conditions, the photosynthetic apparatus is under particular stress. The closure of stomata and consequent limitation of intracellular CO_2 concentration promotes photoreduction of oxygen and the generation of reactive oxygen species (ROS) (Inzé & Montagu, 1995). When ROS are present in high amounts, they can damage the lipids and proteins, impacting especially photosystem II (Kale et al., 2017) and leading to a decrease in plant growth and crop productivity (Zhang, He, et al., 2020; Zhang, Wang, et al., 2020). Plants make use of both enzyme dependent and independent pathways to suppress excessive levels of ROS and maintain a balanced oxidative status to preserve the proper functioning of the photosynthetic machinery. Microbial inoculation could confer drought resistance to plants, for example, Rocha et al. (2019) observed a general positive effect of PGPR via seed coating on crop productivity under moderated water stress. In our experiments, we observed higher A_n in the FMCH001 treatment in well-watered conditions (Table 2). A_n is critical for plant productivity and may be in part explained by the higher mineral uptake in FMCH001-treated plants. Higher g_s increases CO_2 uptake and thereby stimulates photosynthesis; in fact, we did observe higher g_s at early developmental stages in well-watered or drought-stressed FMCH001 plants than that in the Mock ones. Although they trended higher, a statistically significant increase in g_s during the recovery period for the FMCH001 treatment was not observed (Table 2).

C:N partitioning in plants is an important parameter describing the amount of C that can be incorporated in the plant at a given N content (Liu et al., 2019). A meta-analysis (Luo et al., 2006) found increased C:N ratio in the shoots and roots of plants grown at elevated CO_2. A higher C:N ratio was also observed in conditions of increased water availability for the plant (Luo et al., 2017). Our experiments showed an increase in the C:N ratio in stem of FMCH001-treated plants at early developmental stages (Figure 6a). These results may suggest that the higher C:N ratio in the FMCH001 treatment may be an early marker for vigorous plant growth after bacteria treatment. In relation to Mock plants, during the V4–V5 period, WUE increased by 28.29% and 18.35% in the FMCH001-treated plants under well-watered and drought stress conditions, respectively (Table 2). In a previous study, higher WUE and root growth were observed when maize seeds were coated with FMCH001 (Akhtar et al., 2020), indicating the ability of the FMCH001 to benefit different crops. There are several molecular mechanisms behind the improved WUE which have been suggested to involve control of the transpiration from leaves, the growth pattern of the crop, the water exchange at the stomata and the uptake of water from the root system (Blankenagel et al., 2018; Hatfield & Dold, 2019). FMCH001 may impact several of these mechanisms, given the observed increased root growth and reduced plant water use.

5 | CONCLUSION

Bacillus paralicheniformis FMCH001 is able to improve water use efficiency, nutrient uptake, root growth, photosynthesis rate, C:N ratio and plant–water relations in soybean. These features make FMCH001 a potential candidate for sustaining plant growth in the field, especially where water supply may not be constant throughout the growing season. In this context, FMCH001-treated plants used 22.94% less water during drought as compared to control plants without a cost to biomass yield. It is well established that plants secrete sugars through the root exudates to promote symbiosis with PGPR that in turn increase plant survival and growth. We have demonstrated that FMCH001 colonizes soybean roots and reaches a peak in population at 39 Dap, and potentially promotes plant growth. This raises the hypothesis if the mechanism of root colonization and growth promoting of certain types of PGPR are conserved among monocotyledon and dicotyledon plants. In the coming decades, agriculture productivity will be challenged more than ever from an increasing world population and climate change. Strain FMCH001 and other PGPR may become increasingly important tools for sustaining crop vigour.

AUTHOR CONTRIBUTIONS

Jie Liu: Data curation; formal analysis; investigation; software; writing – original draft; writing – review and editing. **Lorenzo Fimognari:** Data curation; formal analysis; investigation; methodology; writing – original draft; writing – review and editing. **Jaqueline de Almeida:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Camilla Niketa Gadomska Jensen:** Data curation; formal analysis; investigation; methodology; writing – review and editing. **Stephane Compant:** Data curation; formal analysis; investigation; methodology; software; writing – review and editing. **Tiago Oliveira:** Investigation; methodology; writing – review and editing. **Jacob Baelum:** Formal analysis; writing – review and editing. **Milica Pastar:** Investigation; methodology; writing – review and editing. **Angela Sessitsch:** Formal analysis; writing – review and editing. **Lars Moelbak:** Formal analysis; funding acquisition; methodology; project administration; supervision; writing – review and editing. **Fulai Liu:** Formal analysis; funding acquisition; methodology; project administration; supervision; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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

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