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Chickpea displays a temporal growth response to *Mesorhizobium* strains under well-watered and drought conditions

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Abstract

The relative performance of rhizobial strains could depend on their resource allocation, environmental conditions, and host genotype. Here, we used a high-throughput shoot phenotyping to investigate the effects of *Mesorhizobium* strain on the growth dynamics, nodulation and bacteroid traits with four chickpea (*Cicer arietinum*) varieties grown under different water regimes in an experiment including four nitrogen sources (two *Mesorhizobium* strains, and two uninoculated controls: nitrogen fertilised and unfertilised) under well-watered and drought conditions. We asked three questions. Does the impact of rhizobial strains on chickpea growth change with well-watered versus drought conditions? Do *Mesorhizobium* strains differ in their ability to influence biomass and nodule traits of chickpea varieties under well-watered and drought conditions? Are bacteroid size and amount of polyhydroxybutyrate modified by *Mesorhizobium* strain, chickpea variety, water availability and their interactions? Under well-watered conditions, chickpea inoculated with CC1192 showed higher shoot growth rates than M075 and accumulated high plant biomass at harvest. Under drought conditions, however, the shoot growth rate was comparable between CC1192 and M075, with no significant difference in plant biomass and symbiotic effectiveness at harvest. Across sources of variation, plant biomass varied 3.0-fold, nodules per plant 3.9-fold, nodule dry weight 3.0-fold, symbiotic effectiveness 1.5-fold, bacteroid size 1.4-fold and bacteroid polyhydroxybutyrate 1.4-fold. Plant biomass was negatively correlated with both bacteroid size and allocation to polyhydroxybutyrate under well-watered conditions, suggesting a trade-off between plant and rhizobial fitness. This study demonstrates the need to reassess rhizobial strain effectiveness across diverse environments, recognising the dynamic nature of their interaction with host plants.

1 | INTRODUCTION

Most studies of legume growth responses to symbiotic rhizobia are based on a single biomass harvest, which may mask temporal responses to rhizobia. Measuring shoot growth over time may yield a deeper perspective on rhizobial effects. For example, if strains that promote early growth are not necessarily more beneficial later, then early harvest could give a misleading comparison among strains. We explore a more-comprehensive, high-throughput phenotyping approach, using chickpea (*Cicer arietinum*), one of the most important pulse crops in the world.

Chickpea growth is limited by different environmental stresses, including drought. Drought occurs in different periods of chickpea development in dry farming areas, leading to a significant reduction in growth and yield (Maqbool et al., 2017). Chickpea establishes symbiotic relationships with rhizobia and fix atmospheric nitrogen to enhance growth, nutrient acquisition and stress tolerance (López-Bellido et al., 2011, Greenlon et al., 2019). To the extent that N supply or the C cost of nodules is limiting under drought, a rhizobial strain that is more effective under drought would improve plant growth, nodulation, pod number and seed yield relative to other strains (Dimkpa et al., 2009). Plant growth responses vary with both plant variety and strain (Biabani et al., 2011; Zaw et al., 2021). Some *Mesorhizobium* and *Sinorhizobium* strains improved nodulation of chickpea and soybean under drought but did not always translate into increased shoot growth (Kibido et al., 2020; Abdela et al., 2020).

In nodules, symbiotic rhizobia differentiate into nitrogen-fixing bacteroids and may exhibit distinct morphological features relative to their kin in the soil (Oono and Denison, 2010). In some hosts, bacteroids undergo significant transformations, including swelling or branching and occasionally an amplification of the bacterial genome (Mergaert et al., 2006). Swollen bacteroids are bigger in size than free-living rhizobia and no longer divide normally (Montiel et al., 2016). Previous studies have investigated the underlying mechanisms that cause host-imposed swelling of the bacteroids (Van de Velde et al., 2010). The repeated evolution of host traits that lead to bacteroid swelling indicates that this phenomenon offers greater benefits to legume hosts (Sen and Weaver, 1984; Oono and Denison, 2010). One reason may be that non-swelling bacteroids hoard high-energy lipid poly-3-hydroxybutyrate (PHB) inside their cells for reproduction and survival in soil (Muller and Denison, 2024) at the expense of nitrogen fixation (Oono et al., 2010). Variation in strains for symbiotic qualities of swollen and non-swollen bacteroids has been explored in beans (*Phaseolus vulgaris*), cowpeas (*Vigna unguiculata*), peanuts (*Arachis hypogaea*), peas (*Pisum sativum*) and chickpea (*Cicer arietinum*) (Oono and Denison, 2010; Mandal and Sinharoy, 2019). The phenomenon of differentiation of size and shape of bacteroids is a plant-dependent trait (Van de Velde et al., 2010; Montiel et al., 2016). Bacteroids in chickpea nodules were previously reported to not be swollen (Oono et al., 2010), hence reproductive and potentially benefiting from hoarding PHB, but this was based on limited data.

In this paper, we designed an experiment combining four chickpea varieties, four sources of nitrogen supply (rhizobia or nitrogen fertiliser), and two water regimes to address the following questions. Does the relative impact of rhizobial strains on chickpea growth change over time and under well-watered versus drought conditions? We test the hypotheses that the effect of rhizobial strains on chickpea growth exhibits temporal variation and that the effect is also influenced by the water availability, as the growth of tomato, barley and *Medicago* change over time following inoculation of mycorrhizae (Watts–Williams et al., 2019).

Second, Do *Mesorhizobium* strains differ in their ability to influence biomass and nodule traits of chickpea varieties under well-watered and drought conditions? We hypothesise that some rhizobia strains are significantly more adapted to drought conditions.

Third, Are the bacteroid size and amount of polyhydroxybutyrate modified by *Mesorhizobium* strain, chickpea variety, water availability and their interactions? We hypothesised that rhizobial strain, plant variety, or water availability could influence the bacteroid morphology. A moderate increase in bacteroid size might increase rhizobia fitness, even if extreme swelling and genome duplication prevent rhizobia reproduction. We further hypothesised that differences in bacteroid morphology would influence plant growth. Differences in bacteroid size, in particular, were hypothesised to affect plant growth in two contrasting ways, which may not be mutually exclusive. If the greater N-per-C efficiency of host-imposed swollen bacteroids (Oono & Denison, 2010) is an inevitable consequence of greater size or surface area – it is not clear why this should be true – larger bacteroids should always be more beneficial. On the other hand, if greater bacteroid size in nodules of swelling-causing host plants is merely a side-effect of some other host-imposed change in bacteroid phenotype, then larger bacteroids might not always be more beneficial to hosts. In particular, the use of plant C to increase bacteroid size could come at the expense of using plant C to power N fixation. This would be analogous to lower N-per-C efficiency of PHB-hoarding bacteroids relative to an isogenic PHB-minus knockout (Oono et al., 2020).

2 | MATERIALS AND METHODS

2.1 | Plants and strains

We established an experiment combining four chickpea varieties, four sources of nitrogen supply (rhizobia or nitrogen fertiliser) and two water regimes in a glasshouse within the Australian Plant Phenomics Facility, University of Adelaide, Australia. We selected chickpea varieties that showed contrasting plant growth under drought: Kyabra, Boundary, Drummond and Pistol (Iqbal, Rahman et al., 2024). The seeds were obtained from Agriculture Victoria Research, Australia. Nitrogen sources were two *Mesorhizobium* strains, M075 and CC1192, and two uninoculated controls: a positive control, fertilised with 0.5 g KNO_3/L, and a negative control, with no fertilisation. Strain M075 was selected based on symbiotic performance with chickpea in previous experiments (Zaw, 2022; Iqbal, Zhou, et al., 2024), and CC1192 is a commercial strain used for inoculant manufacture in Australia (Zaw et al., 2021). *Mesorhizobium* strains were grown on broth culture (yeast mannitol) in a rotatory shaker at 120 rpm for 48 h before inoculation (Vincent, 1970).

2.2 | Plant growth and treatments

Seeds of chickpea were surface sterilised with 75% ethanol for 2 min and washed several times with distilled water before sowing. Three seeds were sown in non-draining pots (125 mm diameter, 137 mm height) filled with sandy soil (sand: 86%, silt: 6%, clay: 8%) with a pH ranging from 7.0 to 7.6. The soil collected from Loxton (34.3873° S, 140.7712° E) was confirmed to be free of chickpea rhizobia due to the absence of prior chickpea cultivation (Rathjen et al., 2024). Plants were thinned to one uniformly sized plant per pot and inoculated with 1 mL (approximately 10^9 cells ml $^{-1}$) of rhizobial broth at the base 7 days after planting (DAP). Plants were manually loaded onto the phenotyping cart system and imaged on a daily basis (Al-Tamimi et al., 2016). Plants were supplied with a nitrogen-free nutrient solution once a week and rainwater on a daily basis until 28 DAP. Positive controls were additionally supplied with 200 mL of KNO_3 (69.2 mg nitrogen per litre). After that time, two water regimes were established: well-watered control (80% pot capacity) and drought (1/3 of available soil water represents 33% pot capacity). Water levels were monitored and adjusted by the Scanalyzer 3D weighing and watering system (LemnaTec GmbH).

2.3 | Experimental design

The experiment occupied eight lanes in the southwest smart-house fitted with conveyor systems and imaging stations (LemnaTec Scanalyzer 3D) for the non-destructive, high-throughput phenotyping of leaf area. The experiment was arranged in three blocks that covered 4 lanes × 24 positions plus two blocks in 4 lanes × 16 positions. Each block occupied 4 lanes × 8 positions and contained one replicate of 32 carts (pots). A strip-unit design (or criss-cross design) was employed with the chickpea varieties randomised to the 4 lanes within each block, and the eight combinations from water regime-sources of nitrogen were randomized to the eight positions within a block. The design was constructed and randomized using dae (Brien, 2023b), a package for the R statistical computing environment (R Core Team, 2023).

2.4 | Plant growth analysis

Planting occurred on 19 August 2022, denoted 0 DAP. Imaging was carried out daily from 10 DAP to 53 DAP inclusive. The drought treatment (1/3 available soil water represents 33% pot capacity) for plants in the drought group commenced at 29 DAP. Thus, there were pre-drought and drought phases for this experiment. Based on the images obtained using RGB cameras, the projected shoot area (PSA kpixels) of each plant was calculated as the sum of the number of plant pixels from 3 camera views, comprising two side views and a view from above. The imaging data was prepared using the SET method described by Brien et al. (2021). The computations were carried out

using growthPheno (Brien, 2023c), a package for the R statistical computing environment (R Core Team, 2023). Following exploratory smoothing of the PSA using the traitSmooth function from growth-Pheno, smoothed PSA (sPSA) was produced by logarithmic smoothing of the PSA that employed P-splines with the smoothing penalty set to ten, and the relative growth rate for the sPSA (sPSA RGR per day) calculated by differencing consecutive values of the ln (sPSA) for a plant. After examination of the plots for the smoothed traits, it was decided to statistically examine sPSA on the following points: 10, 15, 20, 29, 32, 41 and 53 DAP and sPSA RGR for six intervals between these DAP points.

2.5 | End-point measurements

Plants were harvested at 54 DAP and dissected into shoots, roots and nodules. Shoot dry weight and nodule dry weight were measured after drying at 70°C for 48 h. Nodules per plant, symbiotic effectiveness, bacteroid size and polyhydroxybutyrate were also measured (see next section).

2.5.1 | Symbiotic effectiveness

We estimated net plant benefit from symbiosis as symbiotic effectiveness (LaRue and Patterson, 1981; Hardarson and Danso, 1993):

$$Symbiotic effectiveness (\%) = \frac{DW inoculated}{DW_{N-} \times 100},\quad (eq1)$$

where DW is shoot dry weight, and subscripts indicate plants inoculated with test strains (inoculated) and un-inoculated plants with no fertilisation (N-).

2.5.2 | Bacteroid morphology

We used flow cytometry to measure bacteroid size and polyhydroxybutyrate (PHB). We ground three nodules from each plant in phosphate-buffered saline (PBS) (pH ~ 7.4) with mortar and pestle and passed the homogenised material through a 70 μm filter to discard plant debris. Homogenate samples were centrifuged at 906 g for 10 min, and the supernatant was poured off. Homogenate was resuspended with 2 mL PBS and divided into two FACS tubes (1 mL per tube). We added 10 μL of Nile Red stock to cells in one tube (final concentration 100 μg ml^{-1}) and incubated at room temperature in the dark for 1 h to label polyhydroxybutyrate. Samples were centrifuged at 3000 g for 10 mins, and the supernatant was poured off, and pellets were resuspended in 1 mL PBS per tube. We analysed unstained cells for bacteroid size and Nile red-stained cells for polyhydroxybutyrate. Size/granularity was measured from forward and side scatter plots (Ratcliff et al., 2008). Nile Red fluorescence was measured in 'PE' (585/42 nm) channel off blue (488 nm) laser.

2.6 | Statistical analysis

To produce phenotypic estimated marginal means (EMMs) (Searle et al., 1980), each trait was analysed using the R packages ASReml-R (Butler et al., 2021) and asremlPlus (Brien, 2023a). The following linear mixed model was fitted to each trait:

$$y = 1\mu + X_t\tau + X_s\beta + Zu + e,$$

where y is the response vector of values for the trait being analysed; μ is the overall mean in this experiment for the response; τ is the vector of fixed effects of interest; β is the vector for fixed spatial effects; u is the vector of random spatial effects allowed for in the design; 1 is the vector of ones and the matrices X_t, X_s and Z are the design matrices for the corresponding effects; e is the vector of residual effects. The vector β of fixed spatial effects consists of the Block effects and the vector τ_t of fixed effects of interest is partitioned as $\left[\tau_V^\top \, \tau_W^\top \, \tau_R^\top \, \tau_{V:W}^\top \tau_{V:R}^\top \tau_{W:R}^\top \tau_{V:W:R}^\top\right]$, where the subvectors correspond to

the main effects of the treatment factors, namely plant variety (V), water regime (W) and nitrogen source (N); the two-way treatment interactions (V:W, V:N and W:N); and the three-way treatment interaction (V:W:N). The random vector u is partitioned as $\left[u_{B:L}^\top u_{B:P}^\top\right]$, where $u_{B:L}$ and $u_{B:P}$ allow for lane and position random variation within blocks.

The residual effects, e, reflect the random variation between individual plants in the experiment, and they are assumed to be normally distributed with the population mean equal to zero. Several models assessing individual plant variation were investigated for each trait, namely (1) a single variance, (2) variances that differed between water regimes and/or nitrogen source control and non-control treatments, and (3) a smooth spatial surface fitted using tensor-product splines, either natural cubic smoothing splines or P-splines. Firstly, variance models in (1) and (2) were compared, and the model with the smallest Akaike Information Criterion (AIC) was chosen. Secondly, each of the local spatial models in (3) was fitted and compared to the selected non-spatial model. The model with the smallest AIC was selected as the final model.
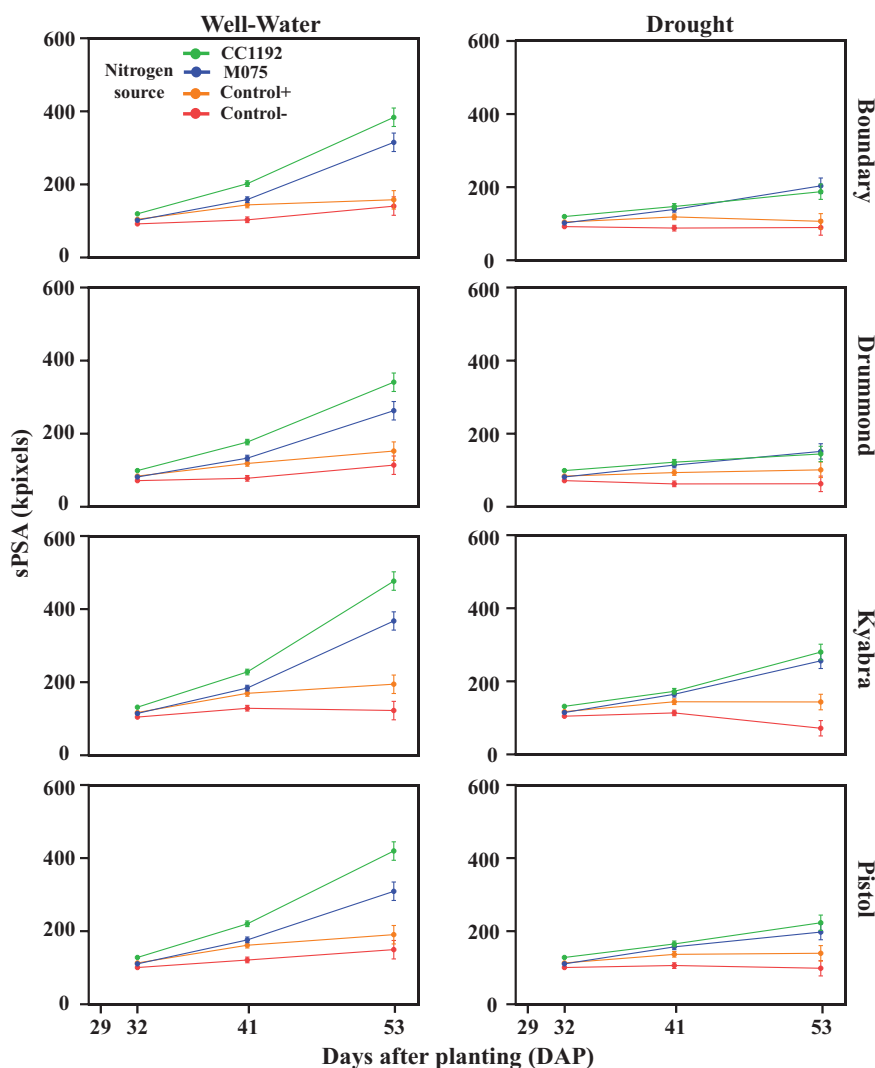

FIGURE 1 Projected Shoot Area (sPSA) of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192, M075, negative and positive control under well-watered and drought conditions. Error bars are an Estimated marginal mean ± half-LSD (5%). See Table S1 for statistical results. Two Estimated marginal means from the same panel and time-point are significantly different if their error bars do not overlap.

Residual-versus-fitted values plots and normal probability plots of the residuals were inspected to check that the assumptions underlying the analyses were met. All residual plots were satisfactory, indicating that the selected models appear to be appropriate.

Wald F-statistics were used to test the significance ($\alpha = 0.05$) of the plant variety, water regime and nitrogen source effects. Testing began with the three-way interaction. If this interaction was not significant, tests of the two-way interactions were conducted. Finally, tests were conducted to determine the main effects of any factors that did not occur in a significant two-factor interaction. Based on these tests, a chosen model was identified for each given trait.

EMMs that conform to the chosen model were obtained. Because watering treatments were not anticipated for any trait defined in the pre-drought phase (up to and including DAP 29), EMMs were generated (1) for each combination of plant variety and nitrogen source in the case of a pre-drought trait and (2) for each combination of plant variety, water regime and nitrogen source in the case of a drought-phase trait. The least significant differences for $\alpha = 0.05$ [LSD (5%)] were calculated to determine the significance of pairwise differences between the EMMs.

3 | RESULTS

3.1 | Plant growth over time

Under well-watered conditions, strain CC1192 supported significantly faster plant growth in all four cultivars relative to M075 (Figure 1). Growth was less under drought, and strain differences were insignificant. The pre-drought phase (10–29 DAP) showed variations in the projected shoot area and relative growth rates of the smoothed projected shoot area for plant varieties and nitrogen sources up to day 29 (Figures S1, S2 and Table S1). In the drought phase (32–53 DAP), the projected shoot area was influenced by water regime × nitrogen sources interaction at 41 and 53 DAP and plant variety × nitrogen sources interaction at 53 DAP (Table S1). Compared with water-stressed plants in the negative control, well-watered plants inoculated with CC1192 showed a projected shoot area of 123% at 41 DAP and 402% at 53 DAP (Figure 1). Kyabra inoculated with CC1192 produced a projected shoot area of 311% compared with Drummond as a negative control at 53 DAP (Figure 1).

The relative growth rate of the smoothed projected shoot area was influenced by the main effects of plant variety, water regime and
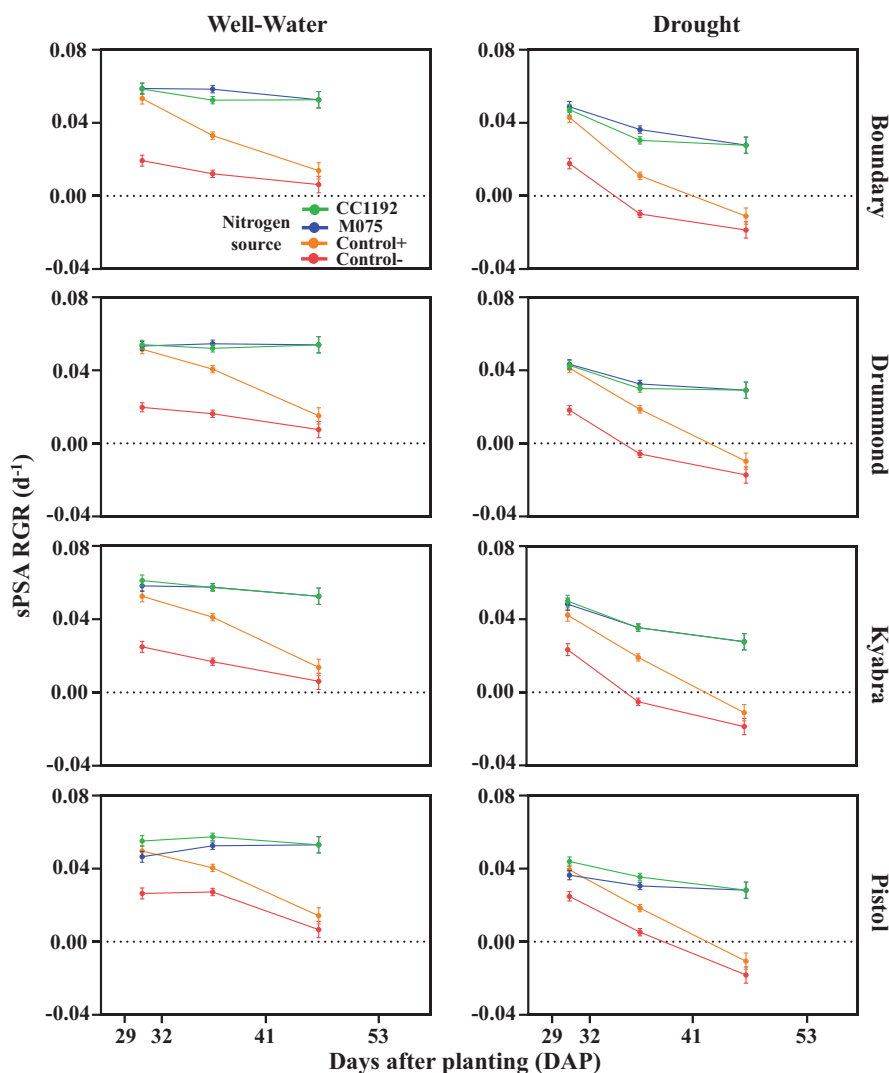

FIGURE 2 Relative Growth Rates of the smoothed Projected Shoot Area (sPSA RGR) of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192, M075, negative and positive control under well-watered and drought conditions. Error bars are an estimated marginal mean ± half-LSD (5%). See Table S1 for statistical results. Two estimated marginal means from the same panel and time-point are significantly different if their error bars do not overlap.
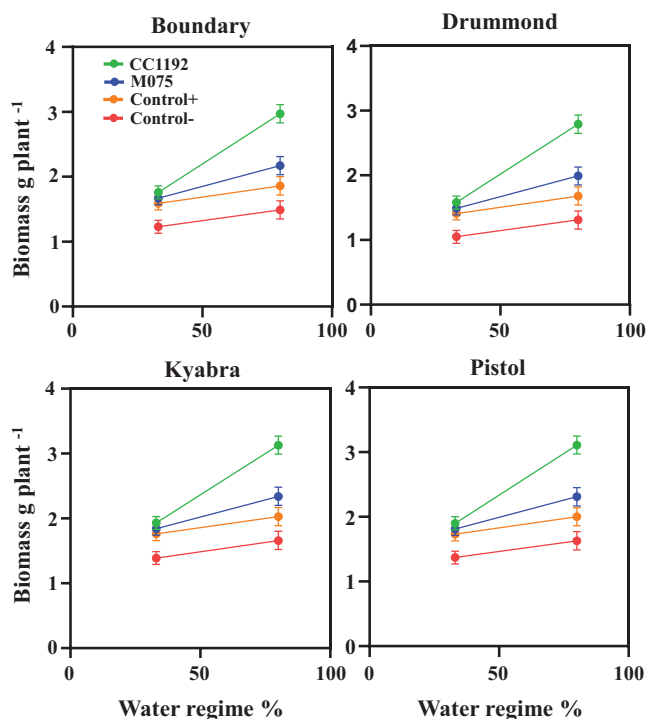
FIGURE 3   (a) Harvested plant biomass of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192, M075, negative and positive control under two water regimes: well-watered control (80% pot capacity) and drought (1/3 of available soil water represents 33% pot capacity). Error bars are an estimated marginal mean ± half-LSD (5%). See Table S1 for statistical results. Two estimated marginal means from the same panel are significantly different if their error bars do not overlap.

nitrogen source from 41 to 53 DAP (Table S1 and Figure 2). Well-watered plants with rhizobia maintained relative growth rates during the treatment phase, while RGR decreased in the drought and low-N treatments, perhaps reflecting increasing shortages of N or water relative to demand.

3.2 | Variation in total plant biomass at harvest

In all four cultivars, we observed less harvested plant biomass under drought stress in comparison to well-watered treatments (Figure 3). Plant biomass ranged from 1.2 g plant^{-1} in severe-stressed plants with no nitrogen source to 3.0 g plant $^{-1}$ in well-watered plants inoculated with CC1192 (Figure 3). The interaction of water regime and nitrogen source influenced the total plant biomass ($p < 0.001$) (Table S1).

3.3 | Variation in nodule traits and symbiotic effectiveness at harvest

The interaction of plant variety and nitrogen source altered nodules per plant ($p = 0.004$) and nodule dry weight ($p = 0.005$) (Table S1). Pistol inoculated with M075 had more nodules per

plant by 54% in comparison to Kyabra inoculated with CC1192 (Figure 4a). Drummond inoculated with CC1192 had 19% greater nodule dry weight in comparison to Boundary inoculated with M075 (Figure 4b).

The interaction of plant variety and water regime modified nodules per plant ($p < 0.001$) and nodule dry weight ($p < 0.001$) (Table S1). Well-watered Pistol had 143% greater nodules per plant in comparison to severe-stressed Drummond (Figure 4a). Well-watered Drummond had 155% greater nodule dry weight in comparison to severe-stressed Pistol (Figure 4b).

The interaction of water regime and nitrogen source influenced the symbiotic effectiveness ($p = 0.009$) (Table S1). Symbiotic effectiveness was reduced from 205% in well-watered plants inoculated with CC1192 to 141% in water-stressed plants inoculated with M075 (Figure 4c).

3.4 | Variation in bacteroid size and amount of polyhydroxybutyrate

Bacteroid size was larger compared to cultured cells under both drought and well-watered conditions (Figure S3). However, under drought conditions, bacteroid size was smaller across all four cultivars compared to the well-watered treatment (Figure 5a). The interaction of plant variety and water regime varied bacteroid size ($p = 0.012$) and amount of polyhydroxybutyrate ($p = 0.003$) (Table S1). Drummond had 10% greater bacteroid size in comparison to Boundary under well-watered treatment, and Pistol increased bacteroid size by 3% in comparison to Drummond under drought (Figure 5a). Drummond achieved a 23% increase in polyhydroxybutyrate size compared to Kyabra under well-watered conditions and a 22% increase under drought conditions (Figure 5b).

The bacteroid size and the amount of polyhydroxybutyrate were greater in the M075 strain (Figure 5a, b). The interaction of plant variety and nitrogen source altered bacteroid size ($p = 0.024$) (Table S1). Bacteroid size was increased in Kyabra inoculated with M075 by 16% in comparison to Boundary inoculated with CC1192 (Figure 5a). The interaction of water regime and nitrogen source influenced polyhydroxybutyrate accumulation ($p = 0.001$) (Table S1). Well-watered plants inoculated with CC1192 increased polyhydroxybutyrate accumulation by 22% in comparison to water-stressed plants inoculated with CC1192 (Figure 5b).

The association between bacteroid morphology and plant biomass in response to strain and water regimes showed that bacteroid size and the amount of polyhydroxybutyrate were correlated negatively with plant biomass under well-watered conditions (Figure 6 a, b and Table S2).

4 | DISCUSSION

4.1 | Chickpea growth responses to M*esorhizobium* strains differ over growth

The high-throughput phenotyping showed that the chickpea growth responses to *Mesorhizobium* strains are dynamic, varying
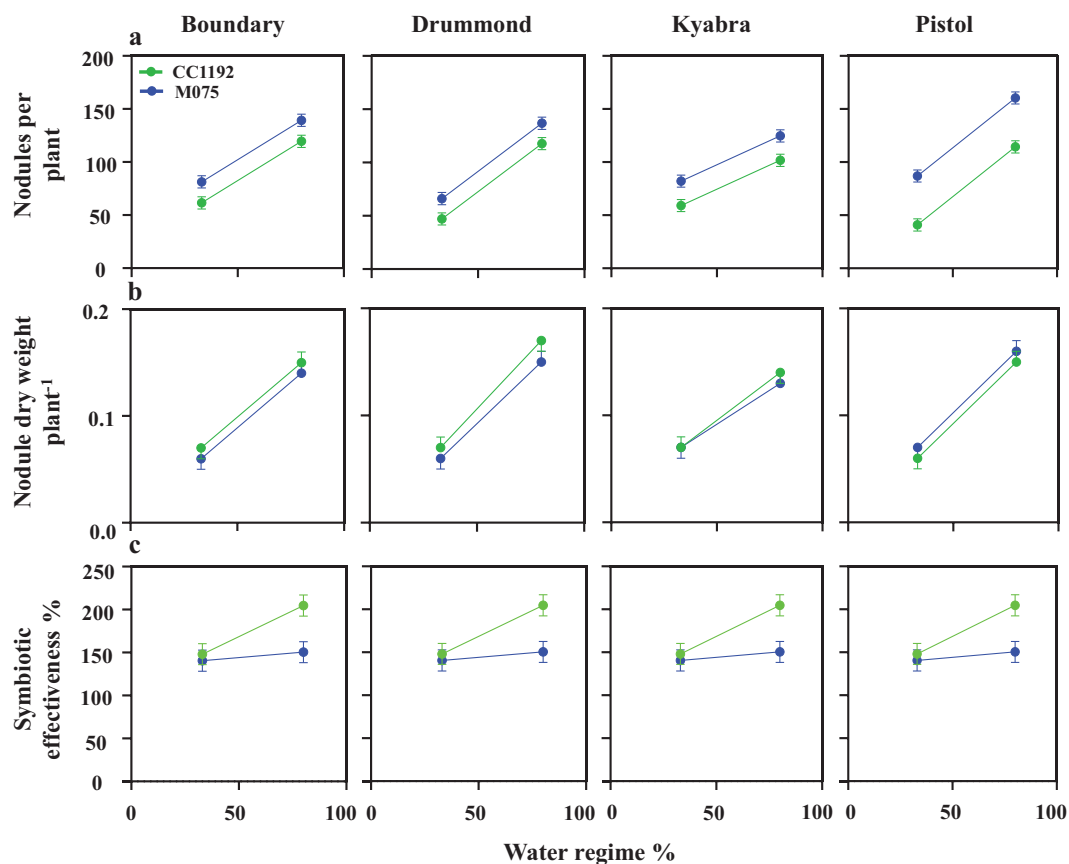
FIGURE 4 (a) Nodules per plant, (b) nodule dry weight and (c) symbiotic effectiveness of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192 and M075 under two water regimes: well-watered control (80% pot capacity) and drought (1/3 of available soil water represents 33% pot capacity). Error bars are an Estimated marginal mean ± half-LSD (5%). See Table S1 for statistical results. Estimated marginal means from the same panel are significantly different if their error bars do not overlap.

significantly over time. Yet CC1192-inoculated plants were larger throughout this period, as shown by both leaf-area projections (Figure 1) and harvested biomass (Figure 3). Thus, differences in plant growth between the two strains resulted mainly from early-growth differences prior to the drought treatment. Such a result might be expected if CC1192 nodulated faster, providing N earlier, but CC1192's fewer nodules per plant (Figure 4a) are somewhat inconsistent with that hypothesis. Furthermore, the relative contribution to leaf growth of N fixation (relative to seed N) would presumably be greater later in growth when absolute growth rates are greater, requiring more N per day. T An alternate hypothesis is that, although both strains were beneficial relative to uninoculated controls, extensive early nodulation can have costs to the plant that exceed its benefits relative to somewhat slower nodulation. That hypothesis is consistent with CC1192's smaller bacteroid size and less PHB (Figure 5), both of which could have reduced its costs to the plant, and with the negative correlation between those two traits and plant biomass, dominated by lower costs for CC1192 (Figure 6).

When subjected to drought, chickpea shoot growth rate was significantly less than the well-watered treatment and was comparable between both CC1192 and M075 up to 53 DAP, indicating no significant strain effect on plant biomass and symbiotic

efficiency at harvest (Figure 1, 3). This suggests either that 1) drought undermines differences between the rhizobia themselves, or 2) that drought reduces the demand for N, reducing the effects of still-persisting strain differences on plant growth, or 3) reduced photosynthesis under drought limits support for nitrogenase activity by either strain.

4.2 | *Mesorhizobium* strains differ in their ability to influence biomass and nodule traits of chickpea varieties under well-watered and drought conditions

Bacterial strains have been reported to contribute to symbiotic effectiveness under both well-watered and drought conditions (Mhadhbi et al., 2004, 2008; Tejera et al., 2004). In this study, however, strain differences were significant only under well-watered conditions (Figure 4 and 5). CC1192-inoculated plants showed a 1.6-fold increase in plant biomass, while their symbiotic effectiveness showed a 1.3-fold improvement compared to plants inoculated with M075 under well-watered conditions (Figure 3, 4c). These results confirm the potential of commercial strain CC1192 to fix a substantial quantity of nitrogen under well-watered conditions and are consistent with
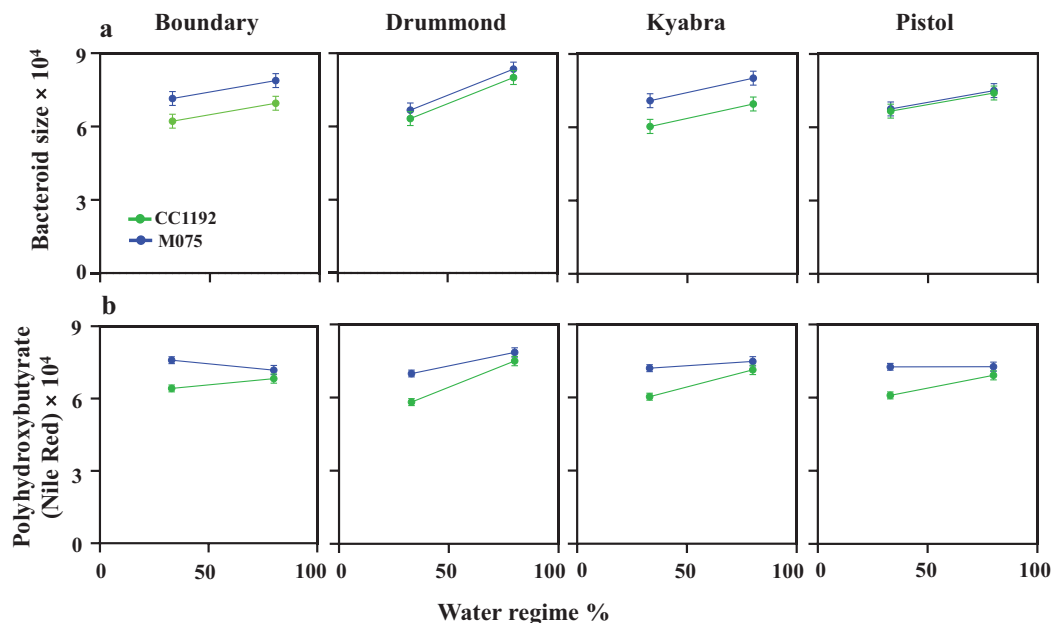
FIGURE 5 (a) Bacteroid size and (b) the amount of polyhydroxybutyrate of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192 and M075 under two water regimes: well-watered control (80% pot capacity) and drought (1/3 of available soil water represents 33% pot capacity). Error bars are an Estimated marginal mean ± half-LSD (5%). See Table S1 for statistical results. Estimated marginal means from the same panel are significantly different if their error bars do not overlap.
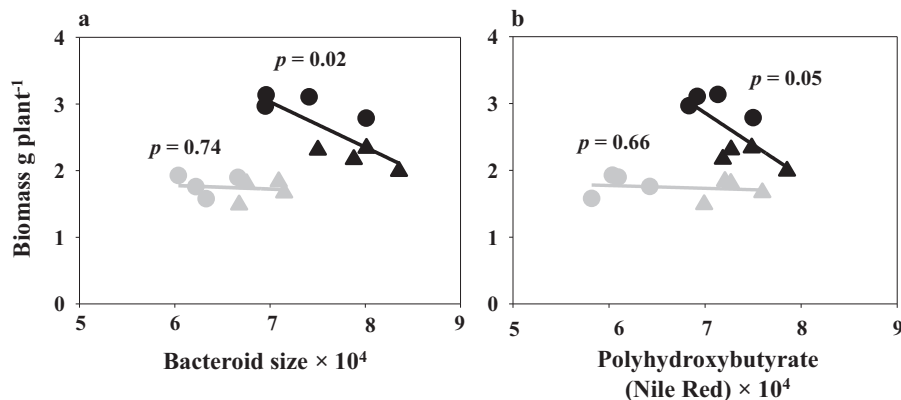
FIGURE 6 Relationship of plant biomass with (a) bacteroid size and (b) polyhydroxybutyrate accumulation of four chickpea varieties inoculated with CC1192 (circle) and M075 (triangle) in response to well-watered (black) and drought (grey).

studies on chickpea-rhizobia and *M. truncatula-S. meliloti* symbiosis under well-watered and lack of differences under drought (Mhadhbi et al., 2009; Esfahani and Mostajeran, 2011). Under drought conditions, the lack of a significant strain effect was likely due to limited substrate availability, reducing nitrogenase activity and trade-offs of carbon allocation (Mhadhbi et al., 2008; Kaschuk et al., 2010). For example, strain M075's higher nodule number likely required greater carbon investment, which could have diverted resources from shoot growth, diminishing its overall effectiveness compared to CC1192 (Figure 3, 4).

Rhizobial strains able to persist for longer periods in dry soils are important contributors to the plant variety-rhizobia symbioses (Mnasri et al., 2007). In this study, the interactions between plant varieties and strains suggest different infectivity potentials of rhizobia with host genotypes, as reported in common bean (Neila et al., 2014).

Our study showed that variety Pistol had 24% greater nodules per plant compared to Drummond inoculated with M075, and Pistol had a 17% increase in nodule dry weight compared to Boundary inoculated with M075 (Figure 4a, b). These findings suggest the effectiveness of *Mesorhizobium* strains and chickpea varieties, similar to the common bean-*Rhizobium* interaction, where an isolate of *Rhizobium* spp. induced the highest nodulation in one cultivar but not consistently across others (Argaw and Muleta 2018). It is worth mentioning that greater nodules per plant did not indicate the effectiveness of M075 and is consistent with the previous finding that improved nodule characteristics do not always translate into increased shoot growth (Kibido et al., 2020; Abdela et al., 2020).

CC1192's greater benefit to plants (under well-watered conditions, Figure 1) was apparently not due to greater nodulation, as it made significantly fewer nodules per plant (Figure 4). Nodule mass

per plant was similar for the two strains, so mass per nodule was greater for CC1192, consistent with host plants allocating more resources to nodules containing more-beneficial strains. One definition of benefit:cost efficiency for nitrogen fixation is shoot mass per nodule mass (a cost to the plant), both on a per-plant basis (Oono and Denison 2010, Figure 1). By that definition, Pistol inoculated with CC1192 grew 33 g dry weight per day per mg of nodule dry weight while Drummond inoculated with M075 grew 13 g dry weight per day per mg of nodule dry weight. Differences in plant biomass over time (Figure 1) were apparently due to early differences in growth, perhaps due to hypothetical faster nodulation by CC1192, since RGR did not differ much after day 30 (Figure 2).

4.3 | Bacteroid size and polyhydroxybutyrate accumulation vary in response to *Mesorhizobium* strains, chickpea variety, water availability and their interactions

Genetic and environmental factors shape bacteroid morphology (Oono et al., 2009; Rangarajan et al., 2020). In this study, we observed a 2.0-fold variation in bacteroid size in Drummond compared with a 1.2-fold variation in Kyabra in response to drought (Figure 5a). The amount of polyhydroxybutyrate varied 1.2-fold in Drummond compared with a 1.0-fold variation in Kyabra in response to drought (Figure 5b). These findings highlight the distinctive responses of different chickpea varieties to water availability and their potential to modulate bacteroid morphology and overall plant performance. It is likely that genetic variations inherent in each plant variety interact with water availability, influencing the physiology and metabolism of rhizobia within root nodules, as previously observed in *M. truncatula* (Larrainzar et al., 2009).

Previous studies have established that bacteroid differentiation into swollen and non-swollen states is regulated by both the bacteria and the plant species but not in chickpea (Oono et al., 2010). In this study, M075-bacteroids increased bacteroid size by 15% compared with CC1192-bacteroids in Kyabra (Figure 5). This is much smaller than the size difference between undifferentiated rhizobia and bacteroids in legume species that impose swelling. It is likely that the genetic differences between *Mesorhizobium* strains result in the production of signals that induce strain-specific responses from the host. This observation aligns with a previous study highlighting the role of host plants in driving bacteroid differentiation within *Mesorhizobium-Cicer arietinum* and *Bradyrhizobium-Aeschynomene* symbiosis (Montiel et al., 2016; Lamouche et al., 2019).

4.4 | Bacteroid size and polyhydroxybutyrate accumulation do not determine the symbiotic benefits in the chickpea-*Mesorhizobium* symbiosis

We observed a negative correlation between plant biomass and both bacteroid size and polyhydroxybutyrate accumulation under well-watered conditions (Figure 6a, b), in contrast with the positive (R2 = 0.4) correlation between cell size and "symbiotic effectiveness" reported previously (although one strain had a negative correlation between "efficiency" and "differentiation") in *Aeschynomene-Bradyrhizobium* Symbiosis (Lamouche et al., 2019). This phenomenon can be attributed to the substantial production of chickpea nodule-specific cysteine-rich (NCR) antimicrobial peptides that may impose bacteroid differentiation in Chickpea (Montiel et al., 2016). NCR peptides have multiple intracellular targets in bacteroids in the *Medicago-Sinorhizobium* symbiosis (Farkas et al., 2014), and water availability could influence the expression of NCR antimicrobial peptides (Kunert et al., 2016). Plants that initiate bacteroid differentiation into different sizes may allocate significant energy resources to maintain a large number of NCR genes in their genome, potentially compromising overall plant growth. The expression levels of genes related to the tricarboxylic acid cycle, a central metabolic pathway involved in cellular respiration and energy generation, were markedly higher in the swollen bacteroids of *Arachis hypogaea* compared to the non-swollen bacteroids of *Sophora flavescens* (Chen et al., 2023).

5 | CONCLUSION

Shoot growth rate responses to *Mesorhizobium* strains change over chickpea ontogeny, and the data collected at the harvest time point reflects the plant's growth patterns throughout its lifespan. Bacteroid size and polyhydroxybutyrate accumulation vary in response to *Mesorhizobium* strains, chickpea variety, and water availability but do not determine – in fact, may undermine, the symbiotic benefits in the chickpea-*Mesorhizobium* symbiosis. This information advances our understanding of the function of chickpea-*Mesorhizobium* symbiosis, which could lead to the development of targeted approaches to promote growth responses through rhizobia.

CONFLICT OF INTEREST STATEMENT

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available within article tables and in the supplementary material.

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