Genetic and Environmental Modulation of the Chickpea – *Mesorhizobium* Symbiosis

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Submitted by

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TABLE OF CONTENTS

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ABSTRACT

Chickpea (*Cicer arietinum*) is a valuable crop grown worldwide. It fixes atmospheric nitrogen by establishing a symbiotic relationship with rhizobia and secretes organic acids from its roots and green tissues that play a role in interactions with beneficial microbes, insect herbivores, and pathogens. As chickpea is nodulated with diverse *Mesorhizobium* species, genetic factors related to both rhizobia and host plant, and environmental conditions are likely to influence plant growth and defence, and chickpea-*Mesorhizobium* symbiosis. The primary objective of this thesis is to investigate the effects of genetic and environmental factors, and their interactions, on plant traits related to growth, nitrogen fixation and responses to herbivory.

A quantitative review was conducted using published data to investigate the effect of environmental factors, particularly drought, on legume growth, nitrogen fixation and its related traits in the light of a hierarchy of phenotypic plasticity. Drought reduced total nitrogen fixation and average nodule mass more severely than plant shoot mass and elicited a hierarchy of plasticities whereby number of nodules per plant varied substantially, and average nodule mass and nitrogen fixation per unit nodule mass were relatively conserved.

Four experiments were carried out in a glasshouse, with specific objectives converging to the primary objective. The objective of experiment 1 was to investigate the interaction between plant variety, *Mesorhizobium* strain, and environment, and their effects on plant growth and nitrogen fixation and its related traits, from the perspective of phenotypic plasticity. Experiment 2 investigated the effects of rhizobia on the growth dynamic of chickpea varieties, nodulation and bacteroid morphology under different water regimes. Experiment 3 and 4 investigated the effects of drought and herbivory on chickpea growth and defence and explored the trade-offs between exudation of organic acids and growth.

Experiment 1. The phenotypic plasticity of chickpea varieties and mesorhizobia strains was quantified in an experiment combining factorially five chickpea varieties, seven *Mesorhizobium* strains and three photothermal regimes. Phenotypic plasticity was quantified for shoot dry weight, nodule dry weight, nodules per plant, nodule colour, symbiotic efficiency, and nitrogen cost. The working hypotheses were that there is a hierarchy of plasticities between plant growth and nitrogen fixation traits, and the plasticity of these traits depends on the genetic variation in both partners. Phenotypic plasticity of nodules per plant was higher than the plasticity of shoot dry weight, verifying a hierarchy of plasticities between these traits. The variation in phenotypic plasticity for shoot dry weight and nitrogen fixing traits was larger among strains than among varieties.

Experiment 2. The effects of rhizobia on the growth of chickpea varieties, nodulation and bacteroid morphology were investigated in a factorial combining four varieties, four nitrogen sources including two *Mesorhizobium* strains, and two un-inoculated controls (nitrogen fertilised and un-fertilised), and two water regimes, well-watered and droughted. Shoot growth rate showed temporal variations in response to strain CC1192 under well-watered and drought conditions. Across sources of variation, leaf exudates varied 3.4-fold, total plant biomass 3.0-fold, nodules per plant 3.9-fold, nodule dry weight 3.0-fold, symbiotic efficiency 1.5-fold, bacteroid size 1.4-fold and amount of polyhydroxybutyrate 1.4-fold. Plant biomass was negatively correlated with bacteroid size and the amount of polyhydroxybutyrate under well-watered conditions. Symbiotic efficiency was negatively correlated with both bacteroid size and the amount of polyhydroxybutyrate under drought.

Experiment 3 and 4. Experiment 3 was a factorial including twelve chickpea varieties and three water regimes to investigate the effects of variety and drought, and their interaction, on plant growth and exudation of organic acids. In experiment 4, six chickpea varieties, two water regimes, and two herbivory treatments were used, with plants challenged with

Helicoverpa armigera larvae or as untreated controls. Drought decreased, and herbivory increased, the amount of leaf exudates. Water regime modulated the response of leaf exudates to herbivory, potentially leading to change in chickpea-herbivore interaction. Leaf damage, and survival and size of *H. armigera* larvae were larger in water-stressed plants and correlated negatively with the leaf exudates in both water regimes. There was no trade-off between exudates and growth traits in most cases; a weak trade-off was apparent under water stress, suggesting a trade-off between growth and defence contingent on water availability.

All experiments were conducted meticulously under controlled conditions using small pots. Therefore, the results cannot be extrapolated to field environments but may provide some fruitful avenues of research to pursue. This study, therefore, lays the groundwork for future research, which is imperative in the improvement of chickpea productivity for sustainable agriculture.

DECLARATION

I certify that this work contains no material which has been accepted for the award of any other degree or diploma in my name, in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. In addition, I certify that no part of this work will, in the future, be used in a submission in my name, for any other degree or diploma in any university or other tertiary institution without the prior approval of the University of Adelaide and where applicable, any partner institution responsible for the joint award of this degree.

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Nasir Iqbal

30/06/2023

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30/06/2023

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CHAPTER 1: Introduction

1.1 Introduction

Chickpea (*Cicer arietinum*) is cultivated extensively and is recognised as a valuable crop worldwide. Chickpea fixes atmospheric nitrogen into available form by establishing symbiotic relationship with several *Mesorhizobium* species (Giller 2001). It responds to beneficial microbes, pests and pathogens by secreting organic acids from roots (Veneklaas et al. 2003) and green tissues (Toker et al., 2004).

Genetic factors associated with both rhizobia and host plant, as well as environmental conditions and their interactions, can affect plant growth and defence, and the chickpea - *Mesorhizobium* symbiosis. The ability of rhizobial strains to nodulate and fix atmospheric nitrogen can vary with chickpea variety (Gunnabo et al., 2020). Similarly, plant growth, nodulation and symbiotic effectiveness can vary among chickpea varieties inoculated with a single strain, and between rhizobia species on the same plant variety (Biabani et al., 2011). The genetic variation in both rhizobial strain and plant variety influence the ability of host–rhizobia symbiosis to fix nitrogen, improve plant growth, and potentially influence the plant's defence.

Environmental factors such as drought, temperature, light and herbivory have both direct or indirect effects on plant growth, plant defence, nodulation and nitrogen fixation (Molina et al., 2008; Devi et al., 2013; Ashrafi et al., 2018; Chrigui et al., 2020; Nandanwar et al., 2020). Drought poses a challenge to chickpea in dry farming areas that affect different stages of development, leading to a significant reduction in growth (Maqbool et al., 2017). Several rhizobial strains have potential to improve plant growth and productivity under drought (Dimkpa et al., 2009). Pod borer (*Helicoverpa armigera*) is a well-known polyphagous insect that causes an estimated loss of US $328 million in chickpea (Sharma, 2005). In response to

the threat posed by aphids (*Aphis craccivora* and *Acyrthosiphon pisum*), semilooper (*Autographa nigrisigna*), leaf miner (*Liriomyza cicerina*) and pod borer (*Helicoverpa armigera*), chickpea secretes organic acids from leaves, leaflets, stems, and pods as a mechanism of defence (Cıkman et al., 2008; Toker et al., 2010; Manjunatha et al., 2022).

Genetic factors and prevailing environmental conditions limit nodulation and nitrogen fixation (outlined in chapters 2, 3 and 4) and impact plant growth and defence (outlined in chapter 5). This thesis focuses on genetic and environmental modulation of growth, defence and nitrogen fixation in chickpea.

1.2. Aims and hypotheses

Previous research has predominantly focused on investigating the influence of variety, strain and environmental factors, and their interaction on plant growth, plant defence, nitrogen fixation and its related traits in cowpea, chickpea, common bean and soybean (Yusuf et al. 2008; Zhang et al., 2014; Agoyi et al., 2017; Argaw and Muleta, 2018). Despite this, there are gaps in our current understanding of interaction between variety, M*esorhizobium* strain, and environmental factors in relation to phenotypic plasticity, growth dynamics, bacteroid morphology, plant defence, and the trade-off between growth and defence in chickpea. These gaps need to be addressed to provide better insights into the effects of genetic and environmental factors to improve the growth, defence and chickpea – M*esorhizobium* symbiosis. Therefore, this thesis aims to investigate the genetic and environmental factors that modulate plant growth and defence, and the chickpea-*Mesorhizobium* symbiosis. The working hypotheses that guide this study are developed and formulated in the subsequent chapters.

Each chapter in this thesis has an individual focus aimed at contributing to the overall objective of investigating how genetic and environmental factors modulate plant growth and defence, and the chickpea – *Mesorhizobium* symbiosis (Fig.1).

- Chapter 2: A meta-analysis of published data investigating the hierarchy of plasticities in legume growth and nitrogen fixation traits under drought, providing context for the experimental studies conducted as part of this thesis.

- Chapter 3: Investigates the interactions between chickpea variety, *Mesorhizobium* strain, and environment, on plant growth and nitrogen fixation from the perspective of phenotypic plasticity.

- Chapter 4: Identifies the effects of rhizobia on the growth dynamic of chickpea varieties, nodulation and bacteroid morphology under different water regimes.

- Chapter 5: Investigates the effects of drought and herbivory on chickpea growth and defence, and explore the trade-off between exudation of organic acids and growth.

- Chapter 6: Provides a discussion of the findings of this study, and directions for future research.
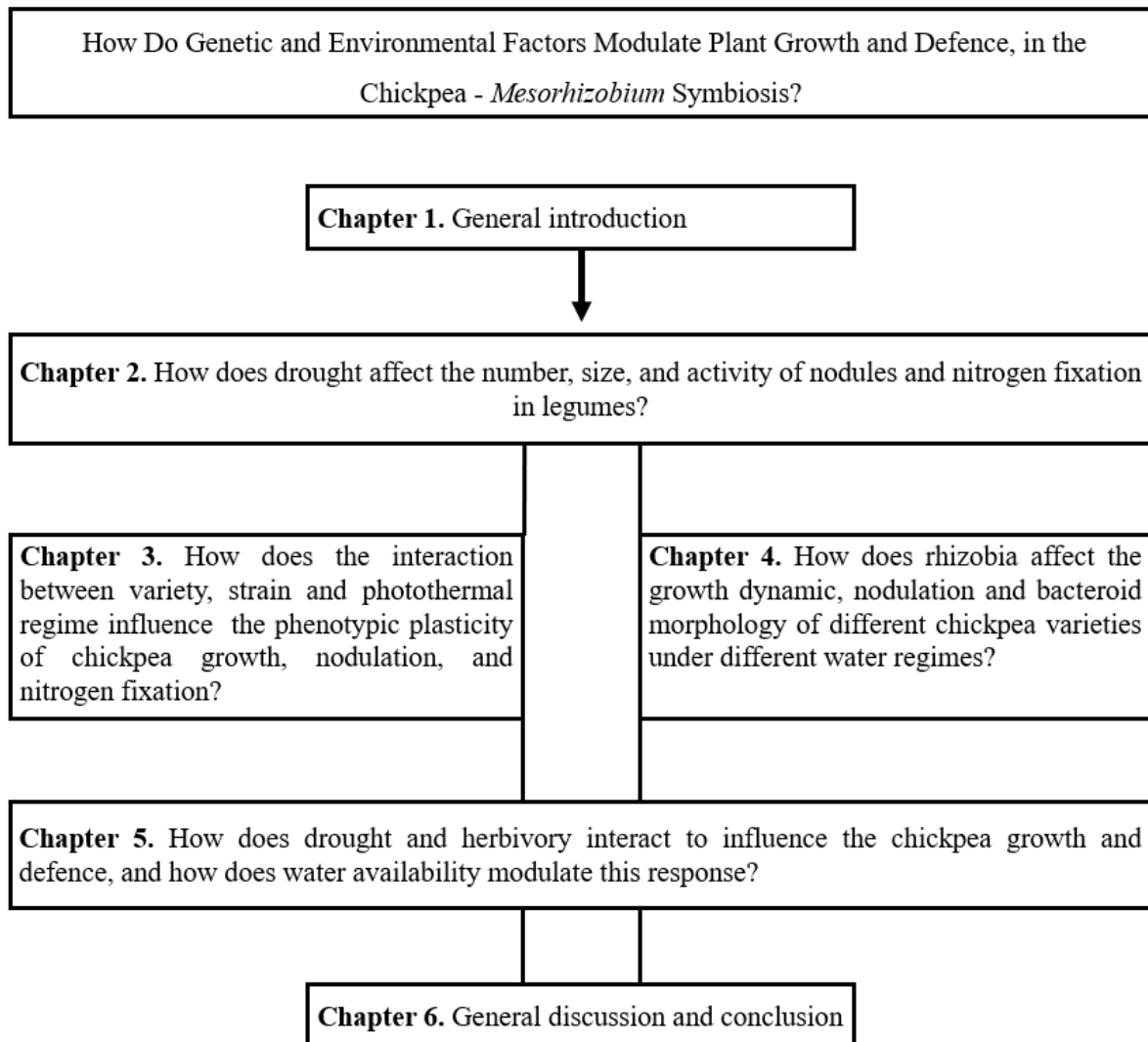
Fig. 1. Thesis structure illustrating the connection among research questions explored in the experimental chapters.

References

Agoyi, E.E., Odong, T.L., Tumuhairwe, J.B., Chigeza, G., Diers, B.W., Tukamuhabwa, P.

(2017). Genotype by environment effects on promiscuous nodulation in soybean (*Glycine*

max L. Merrill). *Agriculture & Food Security*, 6, 1-14.

Argaw, A., Muleta, D. (2018). Effect of genotypes-Rhizobium-environment interaction on

nodulation and productivity of common bean (*Phaseolus vulgaris* L.) in eastern

Ethiopia. *Environmental Systems Research*, 1-16.

Ashrafi, M., Azimi-Moqadam, M. R., Moradi, P., MohseniFard, E., Shekari, F., & Kompany-Zareh, M. (2018). Effect of drought stress on metabolite adjustments in drought tolerant and sensitive thyme. *Plant Physiology and Biochemistry*, 132, 391-399.

Biabani, A., Carpenter-Boggs, L., Coyne, C. J., Taylor, L., Smith, J. L., Higgins, S. (2011). Nitrogen fixation potential in global chickpea mini-core collection. *Biology and Fertility of Soils* 47, 679-685.

Chrigui, N., Sari, D., Sari, H., Eker, T., Cengiz, M. F., Ikten, C., & Toker, C. (2020). Introgression of Resistance to Leaf miner (*Liriomyza cicerina* Rondani) from *Cicer reticulatum* Ladiz. to *C. arietinum* L. and Relationships between Potential Biochemical Selection Criteria. *Agronomy*, 11, 57.

Cıkman, E., Civelek, H. S., & Weintraub, P. G. (2008). The parasitoid complex of Liriomyza cicerina on chickpea (*Cicer arietinum*). *Phytoparasitica*, 36, 211-216.

Devi, M.J., Sinclair, T.R., Beebe, S.E., Rao, I.M. (2013). Comparison of common bean (*Phaseolus vulgaris* L.) varieties for nitrogen fixation tolerance to soil drying. *Plant and Soil*, 364, 29–37.

Dimkpa, C., Weinand, T., & Asch, F. (2009). Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant, cell & environment*, 32, 1682-1694.

Giller, K. (2001). Nitrogen fixation in tropical cropping systems 2nd Edition.

Gunnabo, A. H., van Heerwaarden, J., Geurts, R., Wolde-meskel, E., Degefu, T., Giller, K. E. (2020). Symbiotic interactions between chickpea (*Cicer arietinum* L.) genotypes and Mesorhizobium strains. *Symbiosis,* 82, 235-248.

Manjunatha, L., Puyam, A., Prema, G. U., Sanjay Bandi, M., Kumar, R., Keerthi, M. C., ... &

Kavitha, T. R. (2022). Chickpea Biotic Stresses. *In Genomic Designing for Biotic Stress*

Resistant Pulse Crops (pp. 117-159). Cham: Springer International Publishing.

Maqbool, M. A., Aslam, M., & Ali, H. (2017). Breeding for improved drought tolerance in

Chickpea (*Cicer arietinum* L.). *Plant Breeding*, 136, 300-318.

Molina, C., Rotter, B., Horres, R. et al. (2008). Super SAGE: the drought stress-responsive

transcriptome of chickpea roots. *BMC Genomics*, 9, 553–581.

Nandanwar, S., Yele, Y., Dixit, A., Goss-Souza, D., Singh, R., Shanware, A., Kharbikar, L.

(2020). Effects of pesticides, temperature, light, and chemical constituents of soil on nitrogen

fixation. *Nitrogen fixation*, 1-8.

Sharma, H.C. (Ed.), (2005). Heliothis/Helicoverpa Management: Emerging Trends and

Strategies for Future Research. Oxford and IBH Publishing, New Delhi, India, 469 pp.

Toker, C., Karhan, M., & Ulger, S. (2004). Endogenous organic acid variations in different

chickpea (*Cicer arietinum* L.) genotypes. *Acta Agriculturae Scandinavica, Section B-Soil &*

Plant Science, 54, 42-44.

Toker, C., Erler, F., Ceylan, F. O., & Canci, H. (2010). Severity of leaf miner *[Liriomyza*

*cicerin*a (Rondani, 1875) (Diptera: Agromyzidae)] damage in relation to leaf type in

chickpea. *Turkish Journal of Entomology*, 34, 211-225.

Veneklaas, E. J., Stevens, J., Cawthray, G. R., Turner, S., Grigg, A. M., & Lambers, H.

(2003). Chickpea and white lupin rhizosphere carboxylates vary with soil properties and

enhance phosphorus uptake. *Plant and Soil*, *248*, 187-197.

Yusuf, A.A., Abaidoo, R.C., Iwuafor, E.N.O., Olufajo, O.O. (2008). Genotype effects of cowpea and soybean on nodulation, N2-fixation and N balance in the Northern Guinea savanna of Nigeria. *Journal of Agronomy*, 3, 258-264.

Zhang, J. J., Yu, T., Lou, K., Mao, P. H., Wang, E. T., Chen, W. F., & Chen, W. X. (2014). Genotypic alteration and competitive nodulation of *Mesorhizobium* muleiense against exotic chickpea rhizobia in alkaline soils. *Systematic and applied microbiology*, 37, 520-524.

CHAPTER 2: Review of the literature

Clade-dependent effects of drought on nitrogen fixation and its components–Number, size, and activity of nodules in legumes

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Overall percentage (%)	80%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
Signature		Date	19/06/2023

Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

  i.   the candidate's stated contribution to the publication is accurate (as detailed above);
  ii.   permission is granted for the candidate in include the publication in the thesis; and
  iii. the sum of all co-author contributions is equal to 100% less the candidate's stated contribution.

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Clade-dependent effects of drought on nitrogen fixation and its components – Number, size, and activity of nodules in legumes

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ABSTRACT

Drought affects the growth of legumes directly, and indirectly, by reducing total nitrogen fixation. Here, we compiled published data to compare the sensitivity to water deficit on plant growth and total nitrogen fixation traits, i.e., the number of nodules per plant, average nodule mass, and nitrogen fixation per unit nodule mass. Hierarchies of phenotypic plasticity have been established for seeds and organelles, whereby variation in number associates with conserved size. By analogy, our first hypothesis is that there is a hierarchy of plasticities between nitrogen fixation traits. Our second hypothesis is that determinate nodules are more sensitive to water deficit than their indeterminate counterparts, because the latter can reactivate meristems when water becomes available. In our sample, onset of stress treatment averaged 28 d after sowing; median duration of stress was 12 d; and intensity of stress (ratio of shoot biomass between stressed and control) averaged 0.65. These drought conditions (i) reduced total nitrogen fixation and average nodule mass more severely than plant shoot mass, (ii) elicited a hierarchy of plasticities whereby number of nodules per plant varied substantially, and average nodule mass and nitrogen fixation per unit nodule mass were relatively conserved, and (iii) affected more severely Milletioids (determinate, ureide exporting nodules) than their IRLC counterparts (indeterminate, amide exporting nodules).

1. Introduction

Borrowing from Milne Edwards, Darwin (1859) wrote "nature is prodigal in variety, but niggard in innovation". Diazotrophy, the capacity to fix atmospheric nitrogen, evolved in prokaryotes around 1.5 to 2.2 Bya and plants tapped profusely on this innovation, with legume-rhizobia symbiosis appearing 55 to 60 Mya (Coba de la Peña et al., 2018). Legumes (Leguminosae) have spread to become the third largest flowering plant family, with ecological and economic importance particularly at higher latitudes (Sprent et al., 2017). Agronomically, grain and forage legumes contribute nitrogen fixation in association with rhizobia, diversify crop rotations, and are an important source of protein for animals and humans (Voisin et al., 2014; Andrews and Andrews, 2017). Despite its importance, nitrogen fixation is the most uncertain component of the nitrogen balance in agroecosystems (Zhang et al., 2021). Drought is a major constraint of crop yield worldwide (Stewart and Lal, 2018), and is projected to increase in frequency and severity with climate change in some regions (Shukla et al., 2019); hence, we focus on the effects of water deficit on nitrogen fixation in this paper.

The effects of drought on nitrogen fixation vary (at least quantitatively) with plant genotype, rhizobial genotype, and their interaction (Brockwell and Hely, 1966; Israel et al., 1986; Hafeez et al., 2000; Esfahani and Mostajeran, 2011; Duc et al., 2015). Other factors such as nutrient availability and temperature further modulate the responses of nodulation and nitrogen fixation to water deficit (Bonetti et al., 1984; Wahab and Abd-Alla, 1995; Sangakkara et al., 1996; Frechilla et al., 2000; Younis, 2010; Sadras et al., 2016; Jemo et al., 2017; Pérez-Fernández et al., 2019).

Nitrogen fixation can be insufficient to sustain high grain yield in soybean and common bean (Martinez-Romero, 2003; Salvagiotti et al., 2009; Cafaro et al., 2020), partly because it is energetically costly (Kaschuk et al., 2009; Tamagno et al., 2018). As an indication of this cost, nitrogen-fertilised soybean out-yielded their nitrogen-fixing counterparts by 8% under irrigation and by 17% under water deficit (Ray et al., 2006). Direct selection for nitrogen fixation increased yield of water-stressed soybean but a trade-off between nitrogen fixation and yield was apparent under high yielding conditions (Sinclair et al., 2007).

A number of plant processes underlie the response of nitrogen fixation to water deficit, including nodule formation and senescence, oxygen diffusion in the nodule, activity of key plant enzymes such as phosphoenolpyrovate carboxylase and malate dehydrogenase, carbon limitation and nitrogen-feedback (Durand et al., 1987; Larrainzar et al., 2009; Sulieman et al., 2010; Arrese-Igor et al., 2011; Castañeda et al., 2018; Nazari et al., 2019). Furthermore, rhizobia vary in efficiency, i.e., the amount of N fixed per plant-supplied C consumed. Hoarding of C by rhizobia to support their future reproduction can contribute to these differences (Oono et al., 2020).

Total nitrogen fixed per plant (TNF) up to time of sampling, $t = s$, is the cumulative product of three traits:

$$TNF = \sum_{t=0}^{t=s} NP \bullet NM \bullet NF \qquad (1)$$

where NP is number of nodules per plant, NM is average nodule mass, and NF is average nitrogen fixation per unit nodule mass. The relative variation of these traits can be interpreted in the light of a hierarchy of phenotypic plasticities, as predicted by Bradshaw (1965): ".in the evolution of processes maximizing fitness a variety of different solutions may be developed in different plants. The essential common character of all such solutions will be that some characters of the plant will for various reasons be held constant, whereas others will be permitted to vary, and therefore show high plasticity. Characters which are held constant can be properly said to show homeostasis or canalization. For such constancy can be considered to be at least in part the outcome of the plasticity of the other characters."

The interplay between variable seed number and conserved seed size in annuals is a prime example of such hierarchy of plasticities (Bonaparte and Brawn, 1975; Sadras and Slafer, 2012). Conserved seed size results from mother-offspring conflict and stabilising natural selection reinforced by agronomic selection (Smith and Fretwell, 1974; De-Jong et al., 2005; Sadras, 2007, 2021; Sadras and Denison, 2009). Similarly, the number of mitochondria and chloroplasts scale with cell size, whereas the average size of organelles is conserved (Okie et al., 2016). By analogy, here we advance hypothesis 1: drought would elicit a hierarchy between nitrogen fixation traits (Eq. 1). However, the actual hierarchy would depend on the timing, intensity and duration of water stress, and the relative speed of response of each trait. For example, early drought could reduce nodule initiation and final nodule number. Once nodule number has peaked, responses to drought are constrained to mass per nodule and fixation per unit nodule mass, except for severe drought that can hasten nodule senescence and further reduce nodule number. Further, slow processes underly variation in nodule number and size relative to modulating the activity of an existing nodule.

Inherent variation in efficiency among rhizobial strains has additional implications for plastic responses to drought or other factors. A plant's first chance to assess a nodule's N-fixation rate or efficiency comes a week or more after the nodule first forms. A cohort of nodules, initiated over a few days, will later be found to differ in N fixation per nodule mass or per C consumed. Plants then tend to discriminate among those nodules, growing more-beneficial nodules faster (Kiers et al., 2006). If a plant initiates a new cohort of nodules, some may turn out to be more efficient than some of the older nodules, but the plant cannot instantly grow those more-efficient nodules large enough to meet its entire nitrogen demand. There must also be some constraints on increasing the size of established nodules or their rate of nitrogen fixation per mass.

As drought develops, however, the plant may benefit from decreasing the total C cost of nitrogen fixation by senescing the least-efficient nodules. The more nodules a plant has, the greater chance of variation in N-fixation efficiency. Thus, early over-production of nodules increases flexibility later. A model of soil-N effects on evolution of legume-rhizobia symbiosis predicted that, as soil N supply increases, plants would tend to senesce more nodules: those with a higher C-per-N

cost than soil N uptake by roots. However, an earlier tendency to make fewer nodules in high-N soil could exactly balance this by giving the plant less nodule variation to choose among, but this model did not explore timing issues in detail (West et al., 2002). Later cohorts of nodules and senescence of less-beneficial nodules under drought could raise some doubts about the number-then-size-then-activity proposition above.

Determinate nodules usually export reduced nitrogen as ureides (*Glycine max*, *Vigna unguiculata*, *Phaseolus vulgaris*, *Lablab purpureus*) and indeterminate nodules usually export reduced nitrogen as amides (*Medicago sativa*, *Cicer arietinum*, *Vicia faba*, *Pisum sativum*, *Medicago truncatula*) (Tegeder and Masclaux-Daubresse, 2018). *Arachis hypogaea* and *Stylosanthes hamata* are exceptions with determinate nodules that export amides. These two related species also differ from other species with determinate nodules in that they cause rhizobia cells to swell as they differentiate into the N-fixing, bacteroid form (Oono et al., 2010), making the bacteroids non-reproductive but increasing their N-per-C efficiency (Oono and Denison, 2010). This greater efficiency could favour plants that continue supporting nodules during drought, relative to nodules with less-efficient reproductive bacteroids. Our hypothesis 2 is that determinate nodules are more sensitive to water deficit than their indeterminate counterparts because the persistent meristem may allow for re-activation of nodule function after the release of stress (Engin and Sprent, 1973; Serraj et al., 1999). However, the lack of a persistent meristem did not preclude the recovery of mature soybean nodules after the release of salt stress (James et al., 1993). Direct comparisons are lacking to assess the sensitivity to drought of different nodule types.

Using data from the literature, our aims were to (1) probe for hierarchies in phenotypic plasticity of plant growth, nitrogen fixation and its components (Eq. 1) in response to water deficit, and (2) compare the response of nitrogen fixation to water deficit in plants with different nodule types, namely amide exporters with indeterminate nodules and ureide exporters with determinate nodules.

2. Method

2.1. Data sources

We searched articles published between January 2000 and December 2021 using Web of Science and Scopus. Search keywords included (legume) AND (symbio* OR nodul*) AND (drought OR water deficit OR water stress). Data were extracted from tables or digitized from figures with WebPlot Digitizer (https://automeris.io/WebPlotDigitizer/). We focused on four traits that are relevant to plants and rhizobia, including shoot dry biomass, nodule dry biomass, nodule number per plant and total nitrogen fixation. Nitrogen fixation was estimated with different methods, most commonly acetylene reduction assay, apparent nitrogenase activity through H_2 evolution, total N difference as % N in fixing and non-fixing plants, and ^{15}N substrate by isotope dilution. Soper et al. (2021) reviewed the technical advantages and disadvantages of these methods, protocols to capture the temporal and spatial variation in biological nitrogen fixation, and the scaling from point measurement to ecologically relevant scale.

We excluded studies where plants were not inoculated with specific symbiotic rhizobia. We retrieved 53 papers that satisfied our inclusion criteria (Table 1). Some studies reported problems with acetylene reduction assay related to an acetylene-induced decline in nitrogenase activity over the time (Minchin et al., 1983). Our data sample showed no bias in total nitrogen fixation or drought effect associated with method to measure nitrogen fixation, incubation period, or follow systems in the acetylene reduction assay (Fig S1).

2.2. Phylogenetic analysis

Molecular sequences of genes previously used to construct legume phylogenies, *matK* and 5.8 S rRNA (Wojciechowski et al., 2004; Oono

Table 1

Legume clade and species; nodule type; rhizobia species, strain, and bacteroid type; onset (days after sowing) and duration (days) of water deficit; number of observations (N); and traits analyzed. Nodule type: I, indeterminate; D, determinate; U, ureide exporter; A, amide exporter. Bacteroid is S, swollen or N, non-swollen. Traits are SM, shoot dry mass; NM, total nodule dry mass; NP, number of nodules per plant; TNF, total nitrogen fixation. Methods to measure TNF are (1) acetylene reduction assay; (2) apparent nitrogenase activity measured as H_2 evolution; (3) total N difference as % N in the fixing and non-fixing plants, and (4) ^{15}N substrate by isotope dilution method.

Clade and species	Nodule type I/D	Nodule type U/A	Rhizobia species	Strain	Bacteroid	Onset	Dur.	N	Trait	Reference
Millettioids										
Phaseolus vulgaris	D	U	*Rhizobium. etli*	CE3 wt	N	21	15	2	SM, TNF[1]	Chiozzotto et al. (2018)
	D	U	*R. leguminosarum*	Biovar phaseoli		28	18	6	SM, NM, TNF[1]	Fenta et al. (2014)
	D	U	unreported	unreported		15	45	2	SM, NM, NP, TNF[2]	Ashraf and Iram (2005)
	D	U	*Rhizobium sp.*	NGR234		25	20	1	SM, TNF[1]	Zacarías et al. (2004)
	D	U	*R. tropici, Ensifer meliloti & R. etli*	CIAT899, 4H41 & Ma1A32		45	8	3	SM, NP, TNF[1]	Mhadhbi et al. (2011)
	D	U	*R. tropici*	CIAT 899		18	12	2	SM, NM	Sassi et al. (2010)
	D	U	*R. tropici & P. polymyxa*	CIAT 899 & DSM 36		30	15	4	SM, NM, NP	Figueiredo et al. (2008)
	D	U	*R. leguminosarum & R. tropici*	BR 353 & BR 322		24	8	4	SM, NP, TNF[1]	Ramos et al. (2003)
	D	U	*R. leguminosarum*	ISP 14		28	7	8	SM, NM, TNF[2]	Coleto et al. (2014)
	D	U	*R. tropici & R. etli*	CIAT899 & 12a3		–	20	4	SM, NM, NP	Tajini et al. (2012)
Vigna unguiculata	D	U	*Bradyrhizobium sp.*	BR-3256	N	28	4	1	SM, NM, TNF[2]	Silveira et al. (2001)
Lablab purpureus	D	U	*Rhizobium sp.*	Strain 14	N	21	21	8	NM, NP, TNF[1]	Abdel-Wahab et al. (2002)
Glycine max	D	U	*B. japonicum*	USDA110	N	17	10	2	SM, NM, TNF[1]	Silvente et al. (2012)
	D	U	*Sinorhizobium fredii & B. diazoefficiens*	SMH12 & WB74–1		21	9	4	SM, NM, NP	Kibido et al. (2020)
	D	U	*B. japonicum*	WB74–1		21	18	3	SM, NM, NP, TNF[1]	Fenta et al. (2012)
	D	U	*B. japonicum*	USDA 110		35	10	1	SM, NM, TNF[1]	Porcel et al. (2003)
	D	U	*B. japonicum*	USDA 110		35	10	1	SM, NM, TNF[1]	Ruiz-Lozano et al. (2001)
	D	U	*B. japonicum*	USDA 438		56	28	1	SM, NM, TNF[1]	Streeter (2003)
	D	U	*B. japonicum*	WB 74		42	4	3	NM, NP	Cilliers et al. (2018)
	D	U	*B. japonicum*	UPM792		42	6	2	TNF[2]	Ladrera et al. (2007)
	D	U	*B. japonicum*	USDA110		42	8	3	TNF[1]	Clement et al. (2008)
	D	U	*B. japonicum*	UPM752		42	7	3	TNF[2]	Gil-Quintana et al. (2013)
	D	U	*B. japonicum*	532 C		7	15	1	NM, NP, TNF[3]	Prudent et al. (2016a)
	D	U	*B. japonicum*	532 C		7	16	1	SM, NM, NP, TNF[3]	Prudent et al. (2015)
IRLC										
Medicago sativa	I	A	*E. meliloti*	102F34	S	62	15	2	SM, NM, TNF[3]	Soba et al. (2019)
	I	A	*S. meliloti*	102F78		66	8	1	SM, NP	Molero et al. (2019)
	I	A	*S. meliloti*	Rm1021		10	28	6	SM, NM, NP	Mouradi et al. (2016)
	I	A	*E. meliloti*	1021 & RD64		28	6	2	SM, TNF[1]	Defez et al. (2017)
Cicer arietinum	I	A	*Mesorhizobium ciceri*	C-15 & CP-36	N	28	15	6	SM, NM, NP, TNF[2]	Esfahani and Mostajeran (2011)
	I	A	*M. ciceri*	Ch–191		30	3	1	NM, TNF[2]	(Nasr-Esfahani et al., 2014)
	I	A	*B. japonicum*	TAL 620, Thal-8 & Dulawala		21	7	3	SM, NP	Bano et al. (2010)
	I	A	*M. mediterraneum*	UPM-Ca36		30	60	2	SM, NP	Oliveira et al. (2017)
	I	A	*M. ciceri*	CP41		50	7	4	SM, NM, NP	Abdela et al. (2020)
Vicia faba	I	A	*R. leguminosarum*	Fb41	S	20	30	3	SM, NM	Kabbadj et al. (2017)
	I	A	*R. leguminosarum*	WSM1455		10	60	2	SM, NM, NP, TNF[4]	Parvin et al. (2020)
	I	A	unreported	unreported		14	28	2	SM, NP, TNF[2]	Al-Amri (2019)
Medicago truncatula	I	A	*E. meliloti*	ICMP 19861	S	30	14	1	SM, TNF[1]	Dhanushkodi et al. (2018)
	I	A	*E. meliloti*	2011		70	6	1	TNF[2]	Larrainzar et al. (2014)
	I	A	*S. meliloti*	2011		70	6	1	TNF[2]	Larrainzar et al. (2007)
	I	A	*M. loti & S. meliloti*	MAFF303099 & Rm1021		56	6	2	TNF[1]	Sańko-Sawczenko et al. (2019)
	I	A	*S. meliloti*	1021		7	7	5	NM, NP, TNF[1]	Li et al. (2018)
	I	A	*S. meliloti*	RCR2011 & TII7		7	–	6	SM, NM, NP, TNF[1]	Mhadhbi et al. (2009)
Pisum sativum	I	A	*R. leguminosarum*	NLV 8	S	21	9	1	SM, NM, TNF[2]	González et al. (2001)
	I	A	*R. leguminosarum*	NLV8		28	7	1	TNF[2]	Irar et al. (2014)
	I	A	*R. leguminosarum*	NLV8		28	12	1	TNF[2]	Marino et al. (2007)
	I	A	*R. leguminosarum*	NLV8		32	4	2	SM, NM, TNF[2]	Marino et al. (2006)
	I	A	*R. leguminosarum*	NLV8		28	4	4	TNF[2]	Aldasoro et al. (2019)
	I	A	*R. leguminosarum*	P221		14	14	6		Prudent et al. (2016b)

20

Table 1 (*continued*)

Clade and species	Nodule type		Rhizobia species	Strain	Bacteroid	Onset	Dur.	N	Trait	Reference
	I/D	U/A								
	I	A	*R. leguminosarum*	P221		14	13	2	SM, NM, NP, TNF[3] / SM, NM, NP, TNF[4]	Couchoud et al. (2020)
	I	A	*R. leguminosarum*	1066 S		3	45	2	SM, NP, TNF[4]	Belimov et al. (2019)
Dalbergioids s. l.										
Arachis hypogaea	D	A	*Bradyrhizobium* sp.	SEMIA6144	S	30	14	2	SM, NM, NP, TNF[4]	Furlan et al. (2017)
	D	A	*Bradyrhizobium* sp.	SEMIA6144		30	14	1	TNF[1]	Furlan et al. (2014)
Stylosanthes hamata	D	A	*Bradyrhizobium* sp.	ISRA 97 & ISRA 674	S	40	14	4	TNF[4]	Woldeyohannes et al. (2007)

and Denison, 2010), were downloaded from NCBI database. Sequences were concatenated and aligned in MEGA 7 software. The maximum likelihood method based on the substitution model General Time Reversible with Gamma Distribution was used to construct phylogenetic tree. The robustness of tree topology was calculated from bootstrap analysis with 1000 replications and genes similarity was estimated using Kimura 2 model.

2.3. Test of hypotheses

Traits were normalised as the ratio between actual and maximum in the experiment to account for differences among species and other sources of variation (Divito and Sadras, 2014). Hierarchies of phenotypic plasticities (hypothesis 1) were visualized in pair-wise scatterplots of traits against the $y = x$ line representing the null-hypothesis of no difference in the response to water deficit between traits, and assessed statistically with *t*-tests (Divito and Sadras, 2014). To compare the ureide transporters with determinate nodules and amide transporters with indeterminate nodules (hypothesis 2), we calculated the $\log_e$ of the response ratio as the effect size for each group (Hedges et al., 1999; Shakoor et al., 2021) with "OpenMEE meta-analysis" software. The whole data set was used to test hypothesis 1 across species. *Arachis*
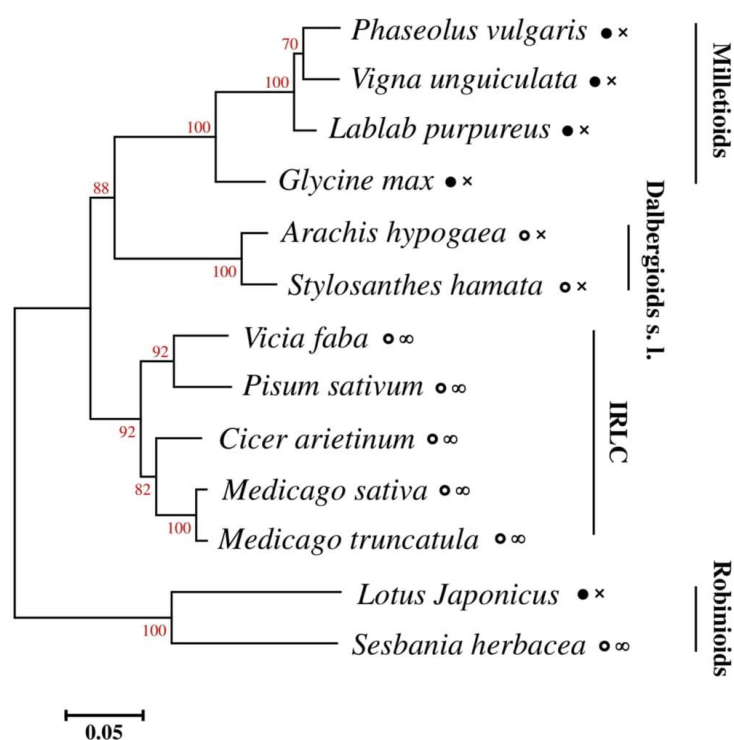

Fig. 1. Phylogeny of legume species sampled in this study, based on *matK* and 5.8 S rRNA gene sequences. Clades are Milletioids, Dalbergioids s. l, IRLC and Robinioids. Symbols are: indeterminate nodule (infinity symbol); determinate nodule (cross); amide exporter (open circle); ureide exporter (closed circle).

4

hypogaea and *Stylosanthes hamata* were excluded from the test of hypothesis 2 for two reasons: they comprised a small sample and are amide exporters with determinate nodules (Table 1, Fig. 1).

We report *p* as a continuous quantity, and Shannon information transform [*s* = -log₂ (*p*)] as a measure of the information against the tested hypothesis (Greenland, 2019). Although *s* is a function of *p*, the additional information provided is not redundant. With base-2 log, the units for measuring this information are bits (binary digits). For example, the chance of seeing all heads in 4 tosses of a fair coin is $1/2^4 = 0.0625$. Thus, $p = 0.05$ conveys only $s = -\log_2(0.05) = 4.3$ bits of information, "which is hardly more surprising than seeing all heads in 4 fair tosses" (Greenland, 2019).

3. Results

3.1. Overview

Table 1 and Fig. 1 summarise the studies retrieved in our search. Three clades IRLC, Milletioids and Dalbergioids s.l. represented 48%, 47% and 5% of the sample, respectively. The main species were common bean, *Phaseolus vulgaris* (25%); soybean, *Glycine max* (17%); and pea, *Pisum sativum* (13%). Other species sampled were cowpea, *Vigna unguiculata;* lablab bean, *Lablab purpureus;* alfalfa, *Medicago sativa;*

chickpea, *Cicer arietinum;* faba bean, *Vicia faba;* barrel clover, *Medicago truncatula;* peanut, *Arachis hypogaea;* and Caribbean stylo, *Stylosanthes hamata.* The main rhizobia species were *Rhizobium* spp. (45%) and *Bradyrhizobium* spp. (22%). The main methods used to measure nitrogen fixation were acetylene reduction assay (36%) and apparent nitrogenase activity (25%). The effects of drought depend on the timing, duration and intensity of stress. In most cases (89%), water deficit was applied at vegetative stages, with an average onset of treatment at 28 days after sowing. The median duration of stress treatment was 12 d. The intensity of stress, quantified as trait ratio between stressed and unstressed controls, averaged 0.65 for shoot biomass and 0.51 for total nitrogen fixation (next section).

3.2. Hypothesis 1: a hierarchy of plasticities in plant and nodule traits in response to water deficit

Total nodule mass and total nitrogen fixation were more sensitive to water deficit than shoot mass (Fig. 2). The average (±s.e.) stress-to-control ratio of 0.65 ± 0.02 for shoot mass compared with 0.57 ± 0.02 for total nodule mass and 0.51 ± 0.03 for total nitrogen fixation. Nodule number per plant, with average ratio of 0.63 ± 0.02, was more sensitive to water deficit than both average nodule mass with average ratio 0.98 ± 0.05, and nitrogen fixation per unit nodule mass with
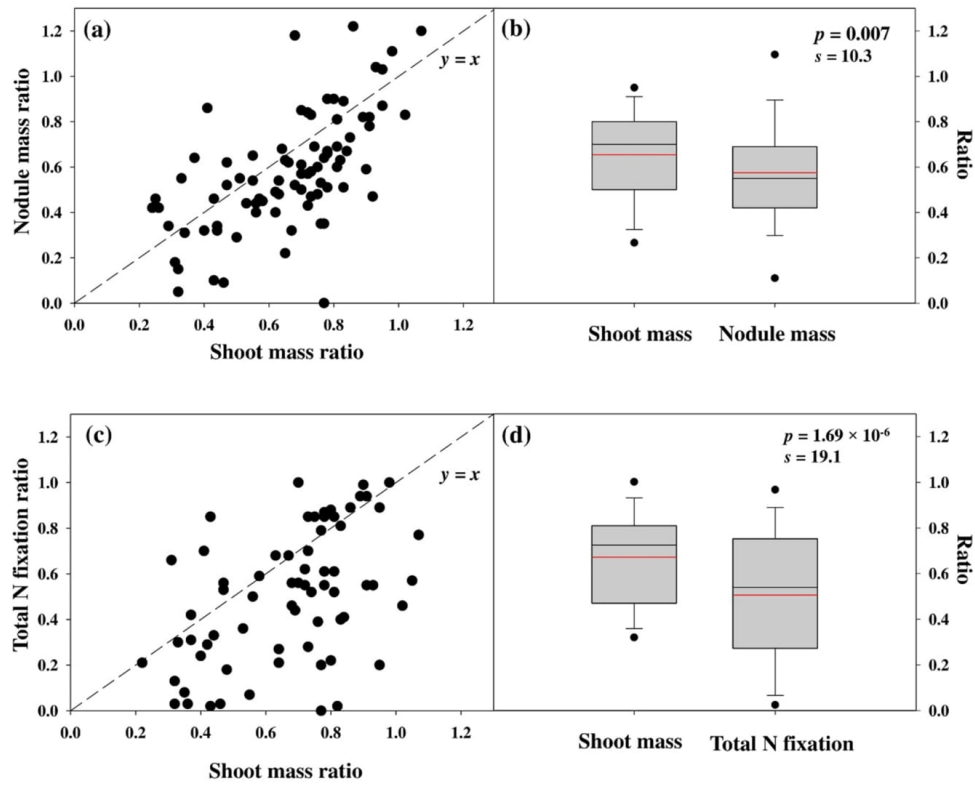

Fig. 2. Comparison of (a, b) nodule mass and shoot mass (n = 83), and (c, d) nitrogen fixation and shoot mass (n = 68) in response to water deficit. Traits are expressed as ratios of water deficit and well-watered treatments. In (a, c), the $y = x$ line is the null hypothesis of traits with similar response to water deficit. In (b, d), ratios close to 1 indicate less sensitivity to water deficit; *p* and *s* are from paired *t-test*. In box plots, red lines are means, lines are 25th, 50th and 75th percentiles, whiskers are 10th and 90th percentiles and dots are 5th and 95th percentiles.

average ratio 0.88 ± 0.04 (Fig. 3a-d). Average nodule mass (ratio = 0.86 ± 0.04) and nitrogen fixation per unit nodule mass (ratio = 0.90 ± 0.05) had similar sensitivity to water deficit (Fig. 3e-f).

3.3. Hypothesis 2: clade-dependent effects of water deficit on shoot mass, total nitrogen fixation and its components

In our sample, ureide transporters with determinate nodules were all in the Millettioid clade, and amide transporters with indeterminate nodules were all in the IRLC clade (Fig. 1). We thus refer to the clades to
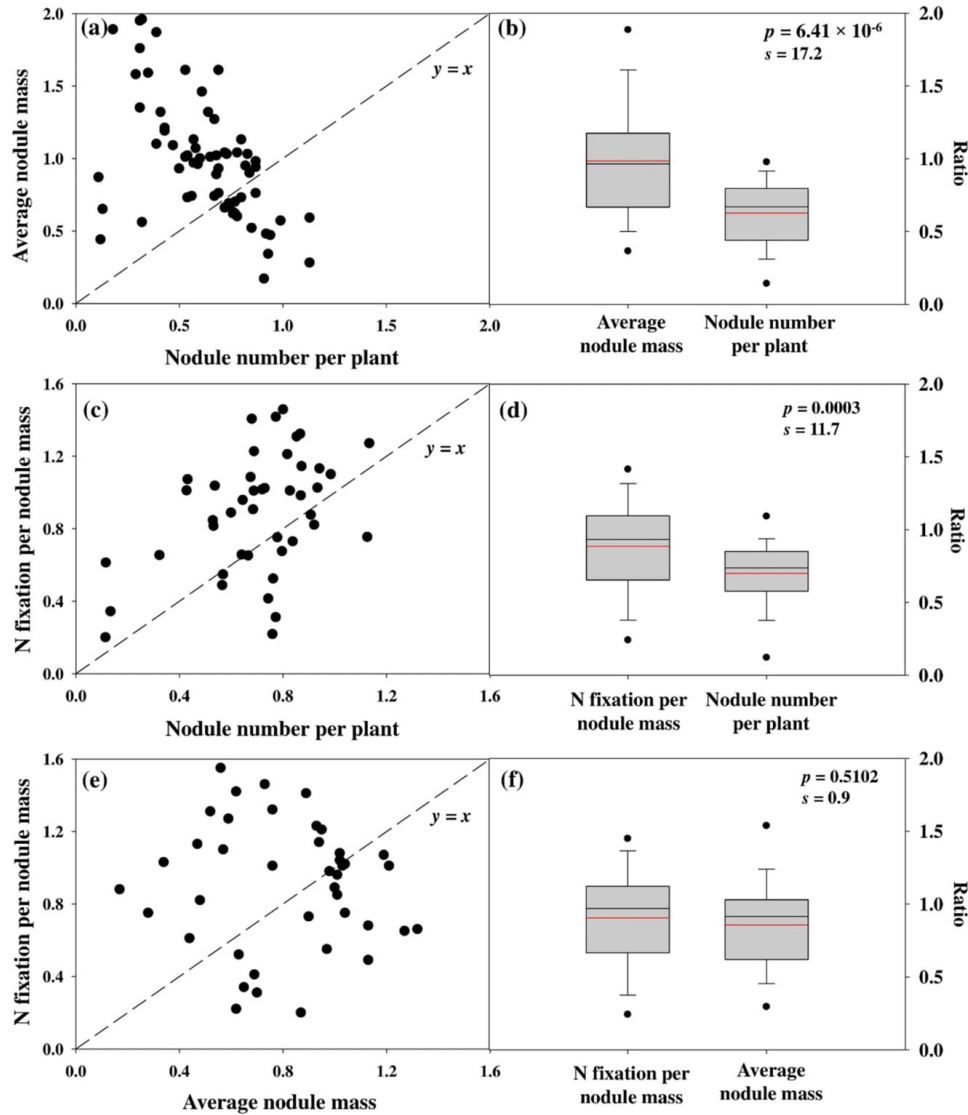

Fig. 3. Comparison of (a, b) average nodule mass and nodule number per plant (n = 64), (c, d) nitrogen fixation per nodule mass and nodule number per plant (n = 44), and (e, f) nitrogen fixation per nodule mass and average nodule mass (n = 44) in response to water deficit. Traits are expressed as ratios of water deficit and well-watered treatments. In (a, c), the $y = x$ line is the null hypothesis of traits with similar response to water deficit. In (b, d), ratios close to 1 indicate less sensitivity to water deficit; *p* and *s* are from paired *t-test*. In box plots, red lines are means, lines are 25th, 50th and 75th percentiles, whiskers are 10th and 90th percentiles and dots are 5th and 95th percentiles.

account for nodule type and metabolism in the following analysis. Fig. 4 compares the clade-dependent effect of water deficit on shoot mass, total nitrogen fixation and its components. Water deficit reduced shoot mass by 46% in the Millettioids compared with 32% in their IRLC counterparts ($p < 0.05$, $s > 4.3$). Water deficit reduced total nitrogen fixation by 62% in the Millettioids compared with 34% in their IRLC counterparts ($p < 0.001$, $s > 9.9$). The difference in total nitrogen fixation between clades was primarily related to the number of nodules per plant under stress, which was 57% of controls in Millettioids and 25% of controls in IRLC ($p < 0.001$, $s > 9.9$). The effect of water deficit on average nodule mass did not vary between clades ($p = 0.132$, $s = 2.9$). Water deficit slightly depressed nitrogen fixation per unit nodule mass in Millettioids and slightly increased nitrogen fixation per unit nodule mass in IRLC ($p < 0.001$, $s > 9.9$).

4. Discussion

4.1. A hierarchy of plasticities between plant growth and nodule traits in response to water deficit

Water deficit reduced total nitrogen fixation and nodule mass more severely than shoot mass. A similar hierarchy of plasticities was found for deficit of phosphorus, sulfur or potassium that reduced nodule mass more severely than shoot mass in grain and forage legumes (Divito and Sadras, 2014). This differential sensitivity is consistent with higher antioxidant enzymatic activity (Aranjuelo et al., 2014; Mouradi et al., 2018) and higher accumulation of sucrose and amino acids (Soba et al., 2019) in the nodules compared with leaves in response to water deficit. Nutrient availability can modulate the response of shoot growth and nodulation to water deficit (Wahab and Abd-Alla, 1995; Frechilla et al., 2000). Water stress reduced nodulation and nitrogen fixation of

Fig. 4. Clade-dependent effects of water deficit on plant shoot mass and nitrogen fixation traits. Clades are Millettioids, ureide transporters with determinate nodules (closed symbols) and IRLC, amide transporters with indeterminate nodules (open symbols). Effect is the response ratio between the trait measured under water deficit and well-watered controls. The number of observations is indicated in parentheses. Error bars are two standard errors. Statistics for the comparisons (p and s) are shown in text.

common bean and lablab more severely in potassium deficient plants (Sangakkara et al., 1996; Younis, 2010). Phosphorus addition increased shoot growth and decreased nodulation under water deficit but slightly improved nitrogen fixation in cowpea, common bean and shrubby legumes (Bonetti et al., 1984; Jemo et al., 2017; Pérez-Fernández et al., 2019). A comparison between alfalfa and tall fescue (*Festuca arundinacea* Schreb.) showed a drought-driven nitrogen deficit partially accounted for the effect of water deficit on shoot growth, irrespective of the source of nitrogen, i.e., biological fixation in alfalfa and mineral soil nitrogen in tall fescue (Kunrath et al., 2018). Irrespective of the physiological mechanisms, legumes seem to halt energetically expensive nitrogen fixation in response to water deficit (Ray et al., 2006; Sadras et al., 2016; Jemo et al., 2017) and nutrient deficit (Divito and Sadras, 2014). This is the opposite to the notion that maintenance of nitrogen fixation enhances crop yield under drought (Sinclair et al., 2007). Identifying the drought scenarios where each strategy is superior solves this conflict (Jordan and Miller, 1980; Schwinning and Ehleringer, 2001; Tardieu, 2012); for example, maintenance of nitrogen fixation might be superior with intermittent drought, and accelerated nodule senescence may be superior under terminal drought.

4.2. A hierarchy of plasticities between nodule number, and nodule mass and activity in response to water deficit

Water deficit elicited a hierarchy in nitrogen fixation traits: water stressed plants had fewer nodules than their well-watered counterparts and largely maintained nodule mass and nitrogen fixation per unit nodule mass. Fewer nodules in water-stressed plants may arise from reduced initiation and development of nodules, increasing nodule senescence or a combination, depending on the timing of stress. Most of the studies in our sample established stress during early vegetative growth, hence nodule initiation and development were more likely sources of variation in nodule number. Our sample does not allow us to evaluate the hypothesis that late-season drought enhances nodule senescence more than cessation of nodule growth or reduced activity.

In comparison to the large change in nodule number per plant, average nodule mass and nitrogen fixation per unit nodule mass were conserved. In soybean inoculated with *B. japonicum* grown in a factorial with two levels of radiation and two levels of soil nitrogen, nodule number per plant was 0.02 at low light, high nitrogen and 22.6 at high light, low nitrogen whereas average nodule mass varied from 1.3 to 5.1 mg (Lau et al., 2012). Although both traits were responsive, the 1128-fold variation in number compared with a 3.8-fold variation in mass, consistent with our finding for responses to drought. Lau et al. (2012) concluded that plants may reduce investment in symbionts as the net benefit of association decrease, potentially limiting how far this interaction shifts toward parasitism in low-light, high nutrient environments. More broadly, the nature of the interaction between macro and micro symbiont in the continuum from mutualism to parasitism is context-dependent, that is, it depends on resource availability and the presence of third organisms (Denison and Kiers, 2004; Lau et al., 2012; Rogalski et al., 2021; Ueno et al., 2021). Interpretation of this continuum with a model based on the traded resources, i.e., carbon and nitrogen in the legume-rhizobia system (Lau et al., 2012) is useful but insufficient to capture the effects of drought where the notion of a single limiting resource does not apply (Kunrath et al., 2018); this carbon-nitrogen model fails to capture, for example, the direct effect of nodule dehydration, independent of photosynthesis, on nodule nitrogenase activity (Albrecht et al., 1984).

The apparent constancy of nitrogen fixation per unit nodule mass could reflect a true adaptation, or be an artefact of the methods of measuring nitrogen fixation. Another source of bias in our analysis is that total nitrogen fixation at the time of sampling is the *cumulative* product of the number of nodules per plant, average nodule mass, and average nitrogen fixation per unit nodule mass (Eq. (1)). However, we have taken the number of nodules and average nodule mass as a
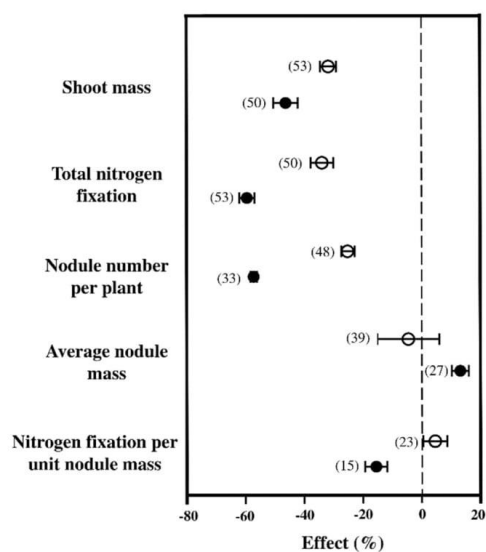
snapshot at sampling time that overlooks the evolution of these components with plant ontogeny; few studies have focused on these dynamics (Gerson et al., 1978; Danso et al., 1990). Further bias could arise from the use of averages if frequency distributions of nitrogen fixation traits depart from normal.

With these caveats, our pair-wise comparisons of traits and meta-analysis provide preliminary support of a hierarchy between nodule number and size. This is a new number-size hierarchy in a list that includes seed yield components in annuals, whereby the plant accommodates environmental variation through seed number and seed size is conserved (Bradshaw, 1965; Sadras, 2007, 2021; Gambín and Borrás, 2010; Sadras and Slafer, 2012) and the scaling of the number of organelles with cell size compared to the relatively conserved organelle size (Okie et al., 2016). The proximal causal link is particular for each of the three systems, but a common theme is the preservation of size that ensures functionality of the unit concerned – seed, organelle or nodule. The legume-rhizobia symbiosome features organelle-like traits such as the composite origin and differentiation of its membrane, the retargeting of host cell proteins, and the host's control of microsymbiont proliferation, but lacks other traits needed to fully conform to the definition of organelle, chiefly rhizobial genome reduction and gene transfer to the nucleus of the host (Coba de la Peña et al., 2018). Adjustment of seed number and of nodule number require plants to forecast growing conditions in a process of pre-emptive acclimation (Aphalo and Sadras, 2022). For example, wheat regulates floret mortality and seed number at flowering using photoperiod as a cue for seed fill conditions (Ghiglione et al., 2008). In comparison to untreated controls, supplemental UVB radiation reduced needle senescence and improved the ratio of variable to maximum fluorescence (Fv/Fn), the apparent photon yield for oxygen evolution and the photosynthetic capacity at 5% CO_2 in field-grown droughted pine plants (Petropoulou et al., 1995). After this early observation, empirical evidence reinforced the link between UV radiation and adaptation to drought including morphological acclimation, improved control of gas exchange and hydraulic sufficiency (Robson et al., 2015). In *Medicago truncatula*, pre-exposure to solar UVB + UVA2 radiation suppressed the expression upon soil drying of most genes annotated as stress-related that were expressed in controls no pre-exposed to solar UVB + UVA2 radiation (Yan, 2021). On this basis, a model has been advanced that supports the role of UV-radiation-induced modulation of ABA signalling influencing plant readiness to acclimate to drought (Aphalo and Sadras, 2022). This model leads to the testable hypothesis that legumes may use UV-radiation to modulate the component traits of nitrogen fixation. UV-radiation influences the production of plant flavonoids, which in turn modulate nodulation (Shiozaki et al., 1999). Associations between UV-radiation and nitrogen fixation in diverse systems have mostly focused on harmful intensity of UV-radiation (Solheim et al., 2002), in contrast to the photo-morphogenetic effect typical of more realistic settings with natural sunlight (Yan et al., 2019).

4.3. Clade-dependent effect of water deficit on nitrogen fixation and its components

Legumes predominantly export either amides or ureides from nodules, and this trait is linked to nodule morphology (Table 1, Fig. 1). Indeterminate nodules have persistent, elongated meristems and spherical determinate nodules lack a persistent meristem. Peanut, Caribbean stylo and *Lotus* spp. (Sprent and Platzmann, 2001) are exceptions, with determinate nodules that export amides. Legumes with determinate nodules are predominately tropical and subtropical while indeterminate nodules are more common in temperate species (Gage, 2004; Sprent et al., 2017).

Separation of nodule morphology and metabolism is not feasible in our data set. Shoot mass, number of nodules and total nitrogen fixation were more responsive to water deficit in the Millettioids (ureide exporters, determinate nodules) than in IRLC legumes (amide exporters,

indeterminate nodules). Plants have an abundance of reduced carbon under water deficit (Muller et al., 2011), and this differential sensitivity may correlate with more consumption of carbon for bacteroids in the determinate nodules where bacteroids typically retain the ability to reproduce, as opposed to those in indeterminate nodules (Denison, 2000). The indeterminate nodules of white clover (*Trifolium repens*) recovered nodule activity on re-watering with both a renewed growth from the indeterminate meristem and rehydration of the existing nitrogen fixing tissue (Engin and Sprent, 1973). In contrast, determinate nodules had limited meristematic activity and appeared more sensitive to soil drying, with low capacity for recovery (Venkateswarlu et al., 1990; Serraj et al., 1999). The nitrogen-feedback inhibition of nodule activity may differ between ureide and amide groups (Serraj et al., 2001). Nitrogen from leaves is translocated within plants and reaches nodules quickly in both the ureide transporter, common bean, and in the amide-transporter, barrel clover (Fischinger et al., 2006; Sulieman et al., 2010). Downregulation of nitrogenase activity associated with transport of ^{15}N from leaf to nodule was apparent in common bean (Fischinger et al., 2006). However, the regulation of nodule function by negative N-feedback mechanisms was absent in barrel clover, thereby challenging the role of the phloem mobile N-signal in the amide group (Sulieman et al., 2020).

Established mechanisms converge to allow greater homeostasis of nodule establishment, growth and function in the amide/indeterminate group. A complex root-to-shoot and shoot-to-root signalling loop autoregulates nodulation (Ferguson et al., 2019). Under this regulatory system, the plant monitors nodulation, and balances nodule formation with overall growth and development (Ferguson et al., 2019). Autoregulation reduces the speed of cortical cell divisions to restrict nodulation at the early stages, depending on nodule type. This mechanism inhibits nodulation in the determinate nodules through a major control point after initiation of cortical cell division clusters (Mathews et al., 1989). However, in indeterminate nodules, autoregulation acts at an earlier stage such that nodulation-inhibited root zones are void of cortical cell divisions (Caetano-Anollés and Gresshoff, 1991; Sagan and Gresshoff, 1996). Water deficit induces ethylene in the rhizosphere and the plant evolves a complex mechanism that prevents the use of photo-assimilates for nodule development under stress (Ferguson et al., 2010). Ethylene also plays a role in the switch between indeterminate and determinate nodules in *Sesbania rostrata* (Fernández-López et al., 1998).

5. Conclusion and directions for further research

Water deficit, primarily during vegetative growth, reduced total nitrogen fixation and nodule mass more severely than shoot mass, and elicited a hierarchy in nitrogen fixation traits, whereby nodule number was highly plastic and associated with conserved average nodule mass and nitrogen fixation per unit nodule mass. Millettioids (ureide exporters, determinate nodules) were more sensitive to water deficit than in IRLC legumes (amide exporters, indeterminate nodules). Work is needed to (a) quantify the dynamics of nitrogen fixation traits with plant ontogeny in well-watered and droughted plants; (b) determine clade-dependent frequency distribution of nitrogen fixation traits; averages are a source of bias in putatively skewed distributions; (c) untangle the influence of nodule morphology and mode of nitrogen exportation in response to drought; intraspecific variation in host plant for swelling/non-swelling bacteroids also needs attention as the trait is plant-dependent; (d) explore pre-emptive acclimation to drought influencing nitrogen fixation traits; the functional connections between UV-radiation, flavonoids, ABA and ethylene are of interest; (e) advance theory on the number-size hierarchy across biological systems to account for observations in nodules, seeds and organelles.

CRediT authorship contribution statement

Victor O. Sadras, Yi Zhou, Matthew D. Denton: Conceptualization. **Nasir Iqbal, Victor O. Sadras**: Data collection, Analysis, Writing – original draft. **Nasir Iqbal, Victor O. Sadras, R. Ford Denison, Yi Zhou, Matthew D. Denton**: Writing – review & editing. All authors read and approved the submitted version.

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.fcr.2022.108586.

References

Abdel-Wahab, A., Shabeb, M., Younis, M., 2002. Studies on the effect of salinity, drought stress and soil type on nodule activities of *Lablab purpureus* (L.) sweet (Kashrangeeg). J. Arid. Environ. 51, 587–602. https://doi.org/10.1006/jare.2002.0974.

Abdela, A.A., Barka, G.D., Degefu, T., 2020. Co-inoculation effect of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* on physiological and biochemical responses of Kabuli chickpea (*Cicer arietinum* L.) during drought stress. Plant Physiol. Rep. 25, 359–369. https://doi.org/10.1007/s40502-020-00511-x.

Al-Amri, S., 2019. Differential response of faba bean (*Vicia faba* l.) plants to water deficit and waterlogging stresses. Appl. Ecol. Environ. Res. 17, 6287–6298. https://doi.org/10.15666/aeer/1703_62876298.

Albrecht, S., Bennett, J., Boote, K., 1984. Relationship of nitrogenase activity to plant water stress in field-grown soybeans. Field Crops Res. 8, 61–71. https://doi.org/10.1016/0378-4290(84)90052-2.

Aldasoro, J., Larrainzar, E., Arrese-Igor, C., 2019. Application of anti-transpirants temporarily alleviates the inhibition of symbiotic nitrogen fixation in drought-stressed pea plants. Agric. Water Manag. 213, 193–199. https://doi.org/10.1016/j.agwat.2018.10.014.

Andrews, M., Andrews, M.E., 2017. Specificity in legume-rhizobia symbioses. Int. J. Mol. Sci. 18, 705. https://doi.org/10.3390/ijms18040705.

Aphalo, P.J., Sadras, V.O., 2022. Explaining preemptive acclimation by linking information to plant phenotype. J. Exp. Bot. erab537. https://doi.org/10.1093/jxb/erab537.

Aranjuelo, I., Arrese-Igor, C., Molero, G., 2014. Nodule performance within a changing environmental context. J. Plant Physiol. 171, 1076–1090. https://doi.org/10.1016/j.jplph.2014.04.002.

Arrese-Igor, S.C., Gonzále, G.E., Marino, B.D., Ladrera, F.R., Larrainzar, R.E., Gil, Q.E., 2011. Physiological responses of legume nodules to drought. Glob. Sci. Books. Plant Stress 5 (Special issue 1), 24–31.

Ashraf, M., Iram, A., 2005. Drought stress induced changes in some organic substances in nodules and other plant parts of two potential legumes differing in salt tolerance. Flora 200, 535–546. https://doi.org/10.1016/j.flora.2005.06.005.

Bano, A., Batool, R., Dazzo, F., 2010. Adaptation of chickpea to desiccation stress is enhanced by symbiotic rhizobia. Symbiosis 50, 129–133. https://doi.org/10.1007/s13199-010-0051-9.

Belimov, A.A., Zinovkina, N.Y., Safronova, V.I., Litvinsky, V.A., Nosikov, V.V., Zavalin, A.A., Tikhonovich, I.A., 2019. Rhizobial ACC deaminase contributes to efficient symbiosis with pea (*Pisum sativum* L.) under single and combined cadmium and water deficit stress. Environ. Exp. Bot. 167, 103859 https://doi.org/10.1016/j.envexpbot.2019.103859.

Bonaparte, E., Brawn, R., 1975. The effect of intraspecific competition on the phenotypic plasticity of morphological and agronomic characters of four maize hybrids. Ann. Bot. 39, 863–869. https://doi.org/10.1093/oxfordjournals.aob.a085003.

Bonetti, R., Montanheiro, M.N.S., Saito, S.M., 1984. The effects of phosphate and soil moisture on the nodulation and growth of *Phaseolus vulgaris*. J. Agric. Sci. 103, 95–102. https://doi.org/10.1017/S0021859600043367.

Bradshaw, A.D., 1965. Evolutionary significance of phenotypic plasticity in plants. Adv. Genet 13, 115–155. https://doi.org/10.1016/S0065-2660(08)60048-6.

Brockwell, J., Hely, F., 1966. Symbiotic characteristics of Rhizobium meliloti: and appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. Aust. J. Agric. Res. 17, 885–899. https://doi.org/10.1071/AR9660885.

Caetano-Anollés, G., Gresshoff, P.M., 1991. Plant genetic control of nodulation. Ann. Rev. Microbiol. 45, 345–382. https://doi.org/10.1146/annurev.mi.45.100191.002021.

Cafaro, L.M.N., Monzon, J.P., Lindquist, J.L., Arkebauer, T.J., Knops, J.M.H., Unkovich, M., Specht, J.E., Grassini, P., 2020. Insufficient nitrogen supply from symbiotic fixation reduces seasonal crop growth and nitrogen mobilization to seed in highly productive soybean crops. Plant Cell Environ. 43, 1958–1972. https://doi.org/10.1111/pce.13804.

Castañeda, V., Gil-Quintana, E., Echeverria, A., González, E., 2018. Legume nitrogen utilization under drought stress. Engineering Nitrogen Utilization in Crop Plants. Springer,, Cham, pp. 173–184. https://doi.org/10.1007/978-3-319-92958-3_10.

Chiozzotto, R., Ramírez, M., Talbi, C., Cominelli, E., Girard, L., Sparvoli, F., Hernández, G., 2018. Characterization of the symbiotic nitrogen-fixing common bean Low *phytic acid* (1pa1) mutant response to water stress. Genes 9, 99. https://doi.org/10.3390/genes9020099.

Cilliers, M., Van-Wyk, S.G., Van-Heerden, P.D.R., Kunert, K.J., Vorster, B.J., 2018. Identification and changes of the drought-induced cysteine protease transcriptome in soybean (*Glycine max*) root nodules. Environ. Exp. Bot. 148, 59–69. https://doi.org/10.1016/j.envexpbot.2017.12.005.

Clement, M., Lambert, A., Herouart, D., Boncompagni, E., 2008. Identification of new up-regulated genes under drought stress in soybean nodules. Gene 426, 15–22. https://doi.org/10.1016/j.gene.2008.08.016.

Coba de la Peña, T., Fedorova, E., Pueyo, J.J., Lucas, M.M., 2018. The symbiosome: legume and rhizobia co-evolution toward a nitrogen-fixing organelle? Front. Plant Sci. 8, 2229. https://doi.org/10.3389/fpls.2017.02229.

Coleto, I., Pineda, M., Rodiño, A., De-Ron, A., Alamillo, J., 2014. Comparison of inhibition of N2 fixation and ureide accumulation under water deficit in four common bean genotypes of contrasting drought tolerance. Ann. Bot. 113, 1071–1082. https://doi.org/10.1093/aob/mcu029.

Couchoud, M., Salon, C., Girodet, S., Jeudy, C., Vernoud, V., Prudent, M., 2020. Pea efficiency of post-drought recovery relies on the strategy to fine-tune nitrogen nutrition. Front. Plant Sci. 11, 204. https://doi.org/10.3389/fpls.2020.00204.

Danso, S., Kapuya, J., Hardarson, G., 1990. Nitrogen fixation and growth of soybean as influenced by varying the methods of inoculation with *Bradyrhizobium japonicum*. Plant Soil 125, 81–86. https://doi.org/10.1007/BF00010747.

Darwin, C., 1859. On the Origin of Species by Means of Natural Selection, or the Preservation of Favoured Races in the Struggle for Life. Routledge,, London. https://doi.org/10.4324/9780203509104.

De-Jong, T., Van-Dijk, H., Klinkhamer, P., 2005. Hamilton's rule, imprinting and parent–offspring conflict over seed mass in partially selfing plants. J. Evol. Biol. 18, 676–682. https://doi.org/10.1111/j.1420-9101.2004.00856.x.

Defez, R., Andreozzi, A., Dickinson, M., Charlton, A., Tadini, L., Pesaresi, P., Bianco, C., 2017. Improved drought stress response in alfalfa plants nodulated by an IAA over-producing *Rhizobium* strain. Front. Microbiol. 8, 2466. https://doi.org/10.3389/fmicb.2017.02466.

Denison, R.F., 2000. Legume sanctions and the evolution of symbiotic cooperation by rhizobia. Am. Nat. 156, 567–576. https://doi.org/10.1086/316994.

Denison, R.F., Kiers, E.T., 2004. Lifestyle alternatives for rhizobia: mutualism, parasitism, and forgoing symbiosis. FEMS Microbiol. Lett. 237, 187–193. https://doi.org/10.1111/j.1574-6968.2004.tb09695.x.

Dhanushkodi, R., Matthew, C., McManus, M.T., Dijkwel, P.P., 2018. Drought-induced senescence of *Medicago truncatula* nodules involves serpin and ferritin to control proteolytic activity and iron levels. N. Phytol. 220, 196–208. https://doi.org/10.1111/nph.15298.

Divito, G.A., Sadras, V.O., 2014. How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? A meta-analysis. Field Crops Res 156, 161–171. https://doi.org/10.1016/j.fcr.2013.11.004.

Duc, G., Agrama, H., Bao, S., Berger, J., Bourion, V., De-Ron, A.M., Gowda, C.L., Mikic, A., Millot, D., Singh, K.B., 2015. Breeding annual grain legumes for sustainable agriculture: new methods to approach complex traits and target new cultivar ideotypes. Crit. Rev. Plant Sci. 34, 381–411. https://doi.org/10.1080/07352689.2014.898469.

Durand, J.L., Sheehy, J., Minchin, F., 1987. Nitrogenase activity, photosynthesis and nodule water potential in soyabean plants experiencing water deprivation. J. Exp. Bot. 38, 311–321. https://doi.org/10.1093/jxb/38.2.311.

Engin, M., Sprent, J.I., 1973. Effects of water stress on growth and nitrogen-fixing activity of *Trifolium repens*. N. Phytol. 72, 117–126. https://doi.org/10.1111/j.1469-8137.1973.tb02016.x.

Esfahani, M.N., Mostajeran, A., 2011. Rhizobial strain involvement in symbiosis efficiency of chickpea–rhizobia under drought stress: plant growth, nitrogen fixation and antioxidant enzyme activities. Acta Physiol. Plant 33, 1075–1083. https://doi.org/10.1007/s11738-010-0635-2.

Fenta, B.A., Beebe, S.E., Rao, I.M., Kunert, K.J., 2014. Association of nodule performance traits with shoot performance traits of common bean under drought stress. J. Crop Improv 28, 418–435. https://doi.org/10.1080/15427528.2014.910287.

Fenta, B.A., Driscoll, S., Kunert, K.J., Foyer, C.H., 2012. Characterization of drought-tolerance traits in nodulated soya beans: The importance of maintaining photosynthesis and shoot biomass under drought-induced limitations on nitrogen metabolism. J. Agron. Crop Sci. 198, 92–103. https://doi.org/10.1111/j.1439-037X.2011.00491.x.

Ferguson, B.J., Indrasumunar, A., Hayashi, S., Lin, M.H., Lin, Y.H., Reid, D.E., Gresshoff, P.M., 2010. Molecular analysis of legume nodule development and autoregulation. J. Integr. Plant Biol. 52, 61–76. https://doi.org/10.1111/j.1744-7909.2010.00899.x.

Ferguson, B.J., Mens, C., Hastwell, A.H., et al., 2019. Legume nodulation: The host controls the party. Plant Cell Environ. 42, 41–51. https://doi.org/10.1111/pce.13348.

Fernández-López, M., Goormachtig, S., Gao, M., D'Haeze, W., Van-Montagu, M., Holsters, M., 1998. Ethylene-mediated phenotypic plasticity in root nodule development on *Sesbania rostrata*. Proc. Natl. Acad. Sci. 95, 12724–12728. https://doi.org/10.1073/pnas.95.21.12724.

Figueiredo, M.V., Burity, H.A., Martinez, C.R., Chanway, C.P., 2008. Alleviation of drought stress in the common bean (*Phaseolus vulgaris* L.) by co-inoculation with *Paenibacillus polymyxa* and *Rhizobium tropici*. Appl. Soil Ecol. 40, 182–188. https://doi.org/10.1016/j.apsoil.2008.04.005.

Fischinger, S.A., Drevon, J.J., Claassen, N., Schulze, J., 2006. Nitrogen from senescing lower leaves of common bean is re-translocated to nodules and might be involved in a N-feedback regulation of nitrogen fixation. J. Plant Physiol. 163, 987–995. https://doi.org/10.1016/j.jplph.2006.03.017.

Frechilla, S., González, E.M., Royuela, M., Minchin, F.R., Aparicio-Tejo, P.M., Arrese-Igor, C., 2000. Source of nitrogen nutrition (nitrogen fixation or nitrate assimilation) is a major factor involved in pea response to moderate water stress. J. Plant Physiol. 157, 609–617. https://doi.org/10.1016/S0176-1617(00)80003-6.

Furlan, A.L., Bianucci, E., Castro, S., Dietz, K.J., 2017. Metabolic features involved in drought stress tolerance mechanisms in peanut nodules and their contribution to biological nitrogen fixation. Plant Sci. 263, 12–22. https://doi.org/10.1016/j.plantsci.2017.06.009.

Furlan, A.L., Bianucci, E., del Carmen, T.M., Castro, S., Dietz, K.J., 2014. Antioxidant enzyme activities and gene expression patterns in peanut nodules during a drought and rehydration cycle. Funct. Plant Biol. 41, 704–713. https://doi.org/10.1071/FP13311.

Gage, D.J., 2004. Infection and invasion of roots by symbiotic, nitrogen-fixing rhizobia during nodulation of temperate legumes. Microbiol. Mol. Biol. Rev. 68, 280–300. https://doi.org/10.1128/MMBR.68.2.280-300.2004.

Gambín, B., Borrás, L., 2010. Resource distribution and the trade-off between seed number and seed weight: a comparison across crop species. Ann. Appl. Biol. 156, 91–102. https://doi.org/10.1111/J.1744-7348.2009.00367.X.

Gerson, T., Patel, J., Wong, M., 1978. The Effects of Age, Darkness and Nitrate on Poly-β-hydroxybutyrate Levels and Nitrogen-Fixing Ability of *Rhizobium* in *Lupinus angustifolius*. Physiol. Plant 42, 420–424. https://doi.org/10.1111/j.1399-3054.1978.tb04107.x.

Ghiglione, H.O., Gonzalez, F.G., Serrago, R., Maldonado, S.B., Chilcott, C., Curá, J.A., Miralles, D.J., Zhu, T., Casal, J.J., 2008. Autophagy regulated by day length determines the number of fertile florets in wheat. Plant J. 55, 1010–1024. https://doi.org/10.1111/j.1365-313X.2008.03570.x.

Gil-Quintana, E., Larrainzar, E., Arrese-Igor, C., González, E.M., 2013. Is N-feedback involved in the inhibition of nitrogen fixation in drought-stressed *Medicago truncatula*? J. Exp. Bot. 64, 281–292. https://doi.org/10.1093/jxb/ers334.

González, E., Gálvez, L., Royuela, M., Aparicio-Tejo, P., Arrese-Igor, C., 2001. Insights into the regulation of nitrogen fixation in pea nodules: lessons from drought, abscisic acid and increased photoassimilate availability. Agronomie 21, 607–613. https://doi.org/10.1051/agro:2001151.

Greenland, S., 2019. Valid p-values behave exactly as they should: Some misleading criticisms of p-values and their resolution with s-values. Am. Stat. 73, 106–114. https://doi.org/10.1080/00031305.2018.1529625.

Hafeez, F., Shah, N., Malik, K., 2000. Field evaluation of lentil cultivars inoculated with *Rhizobium leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution. Biol. Fertil. Soils 31, 65–69. https://doi.org/10.1007/s003740050625.

Hedges, L.V., Gurevitch, J., Curtis, P.S., 1999. The meta-analysis of response ratios in experimental ecology. Ecology 80, 1150–1156. https://doi.org/10.2307/177062.

Irar, S., González, E.M., Arrese-Igor, C., Marino, D., 2014. A proteomic approach reveals new actors of nodule response to drought in split-root grown pea plants. Physiol. Plant 152, 634–645. https://doi.org/10.1111/ppl.12214.

Israel, D.W., Mathis, J.N., Barbour, W.M., Elkan, G.H., 1986. Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. Appl. Environ. Microbiol. 51, 898–903. https://doi.org/10.1128/aem.51.5.898-903.1986.

James, E.K., Sprent, J.I., Hay, G.T., Minchin, F.R., 1993. The effect of irradiance on the recovery of soybean nodules from sodium chloride-induced senescence. J. Exp. Bot. 44, 997–1005.

Jemo, M., Sulieman, S., Bekkaoui, F., Olomide, O.A., et al., 2017. Comparative analysis of the combined effects of different water and phosphate levels on growth and biological nitrogen fixation of nine cowpea varieties. Front. Plant. Sci. 8, 2111. https://doi.org/10.3389/fpls.2017.02111.

Jordan, W.R., Miller, F.R., 1980. Genetic variability in sorghum root system: Implications for drought tolerance. Adaptation of plants to water and high temperature stress. Wiley Interscience,, New York, pp. 383–399.

Kabbadj, A., Makoudi, B., Mouradi, M., Pauly, N., Frendo, P., Ghoulam, C., 2017. Physiological and biochemical responses involved in water deficit tolerance of nitrogen-fixing *Vicia faba*. PloS One 12, e0190284. https://doi.org/10.1371/journal.pone.0190284.

Kaschuk, G., Kuyper, T.W., Leffelaar, P.A., Hungria, M., Giller, K.E., 2009. Are the rates of photosynthesis stimulated by the carbon sink strength of rhizobial and arbuscular mycorrhizal symbioses? Soil Biol. Biochem. 41, 1233–1244. https://doi.org/10.1016/j.soilbio.2009.03.005.

Kibido, T., Kunert, K., Makgopa, M., Greve, M., Vorster, J., 2020. Improvement of rhizobium-soybean symbiosis and nitrogen fixation under drought. Food Energy Secur 9, e177. https://doi.org/10.1002/fes3.177.

Kiers, E.T., Rousseau, R.A., Denison, R.F., 2006. Measured sanctions: legume hosts detect quantitative variation in rhizobium cooperation and punish accordingly. Evol. Ecol. Res. 8, 1077–1086.

Kunrath, T.R., Lemaire, G., Sadras, V.O., Gastal, F., 2018. Water use efficiency in perennial forage species: Interactions between nitrogen nutrition and water deficit. Field Crops Res 222, 1–11. https://doi.org/10.1016/j.fcr.2018.02.031.

Ladrera, R., Marino, D., Larrainzar, E., González, E.M., Arrese-Igor, C., 2007. Reduced carbon availability to bacteroids and elevated ureides in nodules, but not in shoots, are involved in the nitrogen fixation response to early drought in soybean. Plant Physiol. 145, 539–546. https://doi.org/10.1104/pp.107.102491.

Larrainzar, E., Molenaar, J.A., Wienkoop, S., GIL-QUINTANA, E., et al., 2014. Drought stress provokes the down-regulation of methionine and ethylene biosynthesis pathways in *Medicago truncatula* roots and nodules. Plant Cell Environ. 37, 2051–2063. https://doi.org/10.1111/pce.12285.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., et al., 2009. Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. Mol. Plant Microbe Inter. 22, 1565–1576. https://doi.org/10.1094/MPMI-22-12-1565.

Larrainzar, E., Wienkoop, S., Weckwerth, W., Ladrera, R., Arrese-Igor, C., González, E.M., 2007. *Medicago truncatula* root nodule proteome analysis reveals differential plant and bacteroid responses to drought stress. Plant Physiol. 144, 1495–1507. https://doi.org/10.1104/pp.107.101618.

Lau, J.A., Bowling, E.J., Gentry, L.E., Glasser, P.A., et al., 2012. Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. Acta Oecol 39, 80–86. https://doi.org/10.1016/j.actao.2012.01.004.

Li, X., Feng, H., Wen, J., Dong, J., Wang, T., 2018. MtCAS31 aids symbiotic nitrogen fixation by protecting the leghemoglobin MtLb120-1 under drought stress in *Medicago truncatula*. Front. Plant Sci. 9, 633. https://doi.org/10.3389/fpls.2018.00633.

Marino, D., Frendo, P., Ladrera, R., Zabalza, A., Puppo, A., Arrese-Igor, C., González, E.M., 2007. Nitrogen fixation control under drought stress. Local. Or. Syst. ? Plant Physiol. 143, 1968–1974. https://doi.org/10.1104/pp.106.097139.

Marino, D., González, E.M., Arrese-Igor, C., 2006. Drought effects on carbon and nitrogen metabolism of pea nodules can be mimicked by paraquat: evidence for the occurrence of two regulation pathways under oxidative stresses. J. Exp. Bot. 57, 665–673. https://doi.org/10.1093/jxb/erj056.

Martinez-Romero, E., 2003. Diversity of Rhizobium-Phaseolus vulgaris symbiosis: overview and perspectives. Plant Soil 252, 11–23. https://doi.org/10.1023/A:1024199013926.

Mathews, A., Carroll, B., Gresshoff, P., 1989. Development of *Bradyrhizobium* infections in supernodulating and non-nodulating mutants of soybean (*Glycine max* [L.] Merrill). Protoplasma 150, 40–47. https://doi.org/10.1007/BF01352919.

Mhadhbi, H., Chihaoui, S., Mhamdi, R., Mnasri, B., Jebara, M., 2011. A highly osmotolerant rhizobial strain confers a better tolerance of nitrogen fixation and enhances protective activities to nodules of *Phaseolus vulgaris* under drought stress. Afr. J. Biotechnol. 10, 4555–4563. https://doi.org/10.5897/AJB10.1991.

Mhadhbi, H., Fotopoulos, V., Djebali, N., Polidoros, A., Aouani, M., 2009. Behaviours of *Medicago truncatula–Sinorhizobium meliloti* symbioses under osmotic stress in relation with the symbiotic partner input: effects on nodule functioning and protection. J. Agron. Crop Sci. 195, 225–231. https://doi.org/10.1111/j.1439-037X.2009.00361.x.

Minchin, F.R., Witty, J.F., Sheehy, J.E., Müller, M., 1983. A major error in the acetylene reduction assay: decreases in nodular nitrogenase activity under assay conditions. J. Exp. Bot. 34, 641–649.

Molero, G., Tcherkez, G., Roca, R., Mauve, C., Cabrera-Bosquet, L., Araus, J.L., Nogués, S., Aranjuelo, I., 2019. Do metabolic changes underpin physiological responses to water limitation in alfalfa (*Medicago sativa*) plants during a regrowth period? Agric. Water Manag 212, 1–11. https://doi.org/10.1016/j.agwat.2018.08.021.

Mouradi, M., Bouizgaren, A., Farissi, M., Latrach, L., Qaddoury, A., Ghoulam, C., 2016. Seed osmopriming improves plant growth, nodulation, chlorophyll fluorescence and nutrient uptake in alfalfa (*Medicago sativa* L.)–rhizobia symbiosis under drought stress. Sci. Hortic. 213, 232–242. https://doi.org/10.1016/j.scienta.2016.11.002.

Mouradi, M., Farissi, M., Bouizgaren, A., Qaddoury, A., Ghoulam, C., 2018. *Medicago sativa*-rhizobia symbiosis under water deficit: Physiological, antioxidant and nutritional responses in nodules and leaves. J. Plant Nutr. 41, 384–395. https://doi.org/10.1080/01904167.2017.1385805.

Muller, B., Pantin, F., Génard, M., Turc, O., Freixes, S., Piques, M., Gibon, Y., 2011. Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. J. Exp. Bot. 62, 1715–1729. https://doi.org/10.1093/jxb/erq438.

Nasr-Esfahani, M., Sulieman, S., Schulze, J., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.S.P., 2014. Mechanisms of physiological adjustment of N2 fixation in *Cicer arietinum* L.(chickpea) during early stages of water deficit: single or multi-factor controls. Plant J. 79, 964–980. https://doi.org/10.1111/tpj.12599.

27

Nazari, M., Mostajeran, A., Zarinkamar, F., 2019. Strong effect of recovery period between hypoxia events on roots of chickpea (*Cicer arietinum* L). Rhizosphere 11, 100163. https://doi.org/10.1016/j.rhisph.2019.100163.

Okie, J.G., Smith, V.H., Martin-Cereceda, M., 2016. Major evolutionary transitions of life, metabolic scaling and the number and size of mitochondria and chloroplasts. Proc. R. Soc. B 283, 20160611. https://doi.org/10.1098/rspb.2016.0611.

Oliveira, R.S., Carvalho, P., Marques, G., Ferreira, L., et al., 2017. Increased protein content of chickpea (*Cicer arietinum* L.) inoculated with *arbuscular mycorrhizal* fungi and nitrogen-fixing bacteria under water deficit conditions. J. Sci. Food Agric. 97, 4379–4385. https://doi.org/10.1002/jsfa.8201.

Oono, R., Denison, R.F., 2010. Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. Plant Physiol. 154, 1541–1548. https://doi.org/10.1104/pp.110.163436.

Oono, R., Muller, K.E., Ho, R., Jimenez, S.A., Denison, R.F., 2020. How do less-expensive nitrogen alternatives affect legume sanctions on rhizobia? Ecol. Evol. 10, 10645–10656. https://doi.org/10.1002/ece3.6718.

Oono, R., Schmitt, I., Sprent, J.I., Denison, R.F., 2010. Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. N. Phytol. 187, 508–520. https://doi.org/10.1111/j.1469-8137.2010.03261.x.

Parvin, S., Uddin, S., Tausz-Posch, S., Armstrong, R., Tausz, M., 2020. Carbon sink strength of nodules but not other organs modulates photosynthesis of faba bean (*Vicia faba*) grown under elevated [CO_2] and different water supply. N. Phytol. 227, 132–145. https://doi.org/10.1111/nph.16520.

Pérez-Fernández, M., Míguez-Montero, Á., Valentine, A., 2019. Phosphorus and nitrogen modulate plant performance in shrubby legumes from the Iberian Peninsula. Plants 8, 334. https://doi.org/10.3390/plants8090334.

Petropoulou, Y., Kyparissis, A., Nikolopoulos, D., Manetas, Y., 1995. Enhanced UV-B radiation alleviates the adverse effects of summer drought in two Mediterranean pines under field conditions. Physiol. Plant 94, 37–44. https://doi.org/10.1023/A:1009706806427.

Porcel, R., Barea, J.M., Ruiz-Lozano, J.M., 2003. Antioxidant activities in mycorrhizal soybean plants under drought stress and their possible relationship to the process of nodule senescence. N. Phytol. 157, 135–143. https://doi.org/10.1046/j.1469-8137.2003.00658.x.

Prudent, M., Salon, C., Souleimanov, A., Emery, R.N., Smith, D.L., 2015. Soybean is less impacted by water stress using *Bradyrhizobium japonicum* and thuricin-17 from *Bacillus thuringiensis*. Agron. Sustain. Dev. 35, 749–757. https://doi.org/10.1007/s13593-014-0256-z.

Prudent, M., Salon, C., Smith, D.L., Emery, R.N., 2016a. Nod factor supply under water stress conditions modulates cytokinin biosynthesis and enhances nodule formation and N nutrition in soybean. Plant Signal. Behav. 11, e1212799 https://doi.org/10.1080/15592324.2016.1212799.

Prudent, M., Vernoud, V., Girodet, S., Salon, C., 2016b. How nitrogen fixation is modulated in response to different water availability levels and during recovery: a structural and functional study at the whole plant level. Plant Soil 399, 1–12. https://doi.org/10.1007/s11104-015-2674-3.

Ramos, M.L.G., Parsons, R., Sprent, J.I., James, E.K., 2003. Effect of water stress on nitrogen fixation and nodule structure of common bean. Pesqui. Agropecu. Bras. 38, 339–347. https://doi.org/10.1590/S0100-204X2003000300002.

Ray, J.D., Heatherly, L.G., Fritschi, F.B., 2006. Influence of large amounts of nitrogen on nonirrigated and irrigated soybean. Crop Sci. 46, 52–60. https://doi.org/10.2135/cropsci2005.0043.

Robson, T.M., Hartikainen, S.M., Aphalo, P.J., 2015. How does solar ultraviolet-B radiation improve drought tolerance of silver birch (*Betula pendula* Roth.) seedlings? Plant Cell Environ. 38, 953–967. https://doi.org/10.1111/pce.12405.

Rogalski, M.A., Merrill, T.S., Gowler, C., Caceres, C., Duffy, M., 2021. Context-dependent host-symbiont interactions: shifts along the parasitism–mutualism continuum. Am. Nat. 198 https://doi.org/10.1086/716635.

Ruiz-Lozano, J.M., Collados, C., Barea, J.M., Azcón, R., 2001. *Arbuscular mycorrhizal* symbiosis can alleviate drought-induced nodule senescence in soybean plants. N. Phytol. 151, 493–502. https://doi.org/10.1046/j.0028-646x.2001.00196.x.

Sadras, V.O., 2007. Evolutionary aspects of the trade-off between seed size and number in crops. Field Crops Res 100, 125–138. https://doi.org/10.1016/j.fcr.2006.07.004.

Sadras, V.O., 2021. Evolutionary and ecological perspectives on the wheat phenotype. Proc. R. Soc. B 288, 20211259. https://doi.org/10.1098/rspb.2021.1259.

Sadras, V.O., Denison, R.F., 2009. Do plant parts compete for resources? An evolutionary viewpoint. N. Phytol. 183, 565–574. https://doi.org/10.1111/j.1469-8137.2009.02848.x.

Sadras, V.O., Lake, L., Li, Y., Farquharson, E.A., Sutton, T., 2016. Phenotypic plasticity and its genetic regulation for yield, nitrogen fixation and δ13C in chickpea crops under varying water regimes. J. Exp. Bot. 67, 4339–4351. https://doi.org/10.1093/jxb/erw221.

Sadras, V.O., Slafer, G.A., 2012. Environmental modulation of yield components in cereals: heritability reveal a hierarchy of phenotypic plasticities. Field Crops Res 127, 215–224. https://doi.org/10.1016/j.fcr.2011.11.014.

Sagan, M., Gresshoff, P.M., 1996. Developmental mapping of nodulation events in pea (*Pisum sativum* L.) using supernodulating plant genotypes and bacterial variability reveals both plant and *Rhizobium* control of nodulation regulation. Plant Sci. 117, 167–179. https://doi.org/10.1016/0168-9452(96)04411-1.

Salvagiotti, F., Specht, J.E., Cassman, K.G., Walters, D.T., et al., 2009. Growth and nitrogen fixation in high-yielding soybean: Impact of nitrogen fertilization. Agron. J. 101, 958–970. https://doi.org/10.2134/agronj2008.0173x.

Sangakkara, U.R., Hartwig, U.A., Nösberger, J., 1996. Soil moisture and potassium affect the performance of symbiotic nitrogen fixation in faba bean and common bean. Plant Soil 184, 123–130. https://doi.org/10.1007/BF00029282.

Sańko-Sawczenko, I., Łotocka, B., Mielecki, J., Rekosz-Burlaga, H., Czarnocka, W., 2019. Transcriptomic changes in *Medicago truncatula* and *Lotus japonicus* root nodules during drought stress. Int. J. Mol. Sci. 20, 1204. https://doi.org/10.3390/ijms20051204.

Sassi, S., Aydi, S., Gonzalez, E.M., Arrese-Igor, C., Abdelly, C., 2010. Understanding osmotic stress tolerance in leaves and nodules of two *Phaseolus vulgaris* cultivars with contrasting drought tolerance. Symbiosis 52, 1–10. https://doi.org/10.1007/s13199-010-0091-1.

Schwinning, S., Ehleringer, J.R., 2001. Water use trade-offs and optimal adaptations to pulse-driven arid ecosystems. J. Ecol. 89, 464–480. https://doi.org/10.1046/j.1365-2745.2001.00576.x.

Serraj, R., Sinclair, T.R., Purcell, L.C., 1999. Symbiotic N2 fixation response to drought. J. Exp. Bot. 50, 143–155. https://doi.org/10.1093/jxb/50.331.143.

Serraj, R., Vadez, V., Sinclair, T., 2001. Feedback regulation of symbiotic N2 fixation under drought stress. Agronomie 21, 621–626. https://doi.org/10.1051/agro:2001153.

Shakoor, A., Shahbaz, M., Farooq, T.H., Sahar, N.E., et al., 2021. A global meta-analysis of greenhouse gases emission and crop yield under no-tillage as compared to conventional tillage. Sci. Total Environ. 750, 142299 https://doi.org/10.1016/j.scitotenv.2020.142299.

Shiozaki, N., Hattori, I., Gojo, R., Tezuka, T., 1999. Activation of growth and nodulation in a symbiotic system between pea plants and leguminous bacteria by near-UV radiation. J. Photochem. Photobiol. B. Biol. 50, 33–37. https://doi.org/10.1016/S1011-1344(99)00065-2.

Shukla, P., Skea, J., Calvo B.E., Masson-Delmotte, V., et al., 2019, IPCC, 2019: Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems. In Press. ⟨https://www.ipcc.ch/srccl/cite-report/⟩.

Silveira, J.A.G., Costa, R.C.L., Oliveira, J.T.A., 2001. Drought-induced effects and recovery of nitrate assimilation and nodule activity in cowpea plants inoculated with *Bradyrhizobium* spp. under moderate nitrate level. Braz. J. Microbiol 32, 187–194. https://doi.org/10.1590/S1517-83822001000300005.

Silvente, S., Sobolev, A.P., Lara, M., 2012. Metabolite adjustments in drought tolerant and sensitive soybean genotypes in response to water stress. PLoS One 7, e38554. https://doi.org/10.1371/journal.pone.0038554.

Sinclair, T.R., Purcell, L.C., King, C.A., Sneller, C.H., Chen, P., Vadez, V., 2007. Drought tolerance and yield increase of soybean resulting from improved symbiotic N2 fixation. Field Crops Res 101, 68–71. https://doi.org/10.1016/j.fcr.2006.09.010.

Smith, C.C., Fretwell, S.D., 1974. The optimal balance between size and number of offspring. Am. Nat. 108, 499–506. https://doi.org/10.1086/282929.

Soba, D., Zhou, B., Arrese-Igor, C., Munné-Bosch, S., Aranjuelo, I., 2019. Physiological, hormonal and metabolic responses of two alfalfa cultivars with contrasting responses to drought. Int. J. Mol. Sci. 20, 5099. https://doi.org/10.3390/ijms20205099.

Solheim, B., Johanson, U., Callaghan, T.V., Lee, J.A., Gwynn-Jones, D., Björn, L.O., 2002. The nitrogen fixation potential of arctic cryptogram species is influenced by enhanced UV-B radiation. Oecologia 133, 90–93. https://doi.org/10.1007/s00442-002-0963-z.

Soper, F.M., Taylor, B.N., Winbourne, J.B., Wong, M.Y., Dynarski, K.A., Reis, C.R., Perakis, S.S., 2021. A roadmap for sampling and scaling biological nitrogen fixation in terrestrial ecosystems. Methods Ecol. Evol. 12, 1122–1137. https://doi.org/10.1111/2041-210X.13586.

Sprent, J.I., Ardley, J., James, E.K., 2017. Biogeography of nodulated legumes and their nitrogen-fixing symbionts. N. Phytol. 215, 40–56. https://doi.org/10.1111/nph.14474.

Sprent, J.I., Platzmann, J., 2001. *Nodulation in legumes*: Royal Botanical Gardens, Kew. S. K. (957); Studies on pollen grains of Cassia species. Bull. Bot. Soc. Bengal 11, 96–103. https://doi.org/10.1093/aob/mcf128.

Stewart, B., Lal, R., 2018. Increasing world average yields of cereal crops: it's all about water. Adv. Agron. 151, 1–44. https://doi.org/10.1016/bs.agron.2018.05.001.

Streeter, J., 2003. Effects of drought on nitrogen fixation in soybean root nodules. Plant Cell Environ. 26, 1199–1204. https://doi.org/10.1046/j.1365-3040.2003.01041.x.

Sulieman, S., Fischinger, S.A., Gresshoff, P.M., Schulze, J., 2010. Asparagine as a major factor in the N-feedback regulation of N2 fixation in *Medicago truncatula*. Physiol. Plant 140, 21–31. https://doi.org/10.1111/j.1399-3054.2010.01380.x.

Sulieman, S., Schulze, J., Mühling, K.H., Tran, L.S.P., 2020. Is N-feedback involved in the regulation of nitrogenase activity in *Medicago truncatula*? J. Plant Nutr. Soil Sci. 183, 42–45. https://doi.org/10.1002/jpln.201900432.

Tajini, F., Trabelsi, M., Drevon, J.J., 2012. Comparison between the reference [i] *Rhizobium tropic* [/i] i CIAT899 and the native [i] *Rhizobium etl* [/i] i 12a3 for some nitrogen fixation parameters in common bean ([i] *Phaseolus vulgaris* [/i] L.) under water stress. Afr. J. Microbiol. Res 6, 4058–4067. https://doi.org/10.5897/AJMR12.566.

Tamagno, S., Sadras, V.O., Haegele, J.W., Armstrong, P.R., Ciampitti, I.A., 2018. Interplay between nitrogen fertilizer and biological nitrogen fixation in soybean: implications on seed yield and biomass allocation. Sci. Rep. 8, 1–11. https://doi.org/10.1038/s41598-018-35672-1.

Tardieu, F., 2012. Any trait or trait-related allele can confer drought tolerance: just design the right drought scenario. J. Exp. Bot. 63, 25–31. https://doi.org/10.1093/jxb/err269.

Tegeder, M., Masclaux-Daubresse, C., 2018. Source and sink mechanisms of nitrogen transport and use. N. Phytol. 217, 35–53. https://doi.org/10.1111/nph.14876.

Ueno, A.C., Gundel, P.E., Ghersa, C.M., Agathokleous, E., Martínez-Ghersa, M.A., 2021. Seed-borne fungal endophytes constrain reproductive success of host plants under ozone pollution. Environ. Res. 202, 111773 https://doi.org/10.1016/j.envres.2021.111773.

Venkateswarlu, B., Saharan, N., Maheswari, M., 1990. Nodulation and N2 (C2H2) fixation in cowpea and groundnut during water stress and recovery. Field Crops Res. 25, 223–232. https://doi.org/10.1016/0378-4290(90)90005-V.

Voisin, A.S., Guéguen, J., Huyghe, C., Jeuffroy, M.H., et al., 2014. Legumes for feed, food, biomaterials and bioenergy in Europe: a review. Agron. Sustain. Dev. 34, 361–380. https://doi.org/10.1007/s13593-013-0189-y.

Wahab, A.A., Abd-Alla, M., 1995. The role of potassium fertilizer in nodulation and nitrogen fixation of faba bean (*Vicia faba* L.) plants under drought stress. Biol. Fertil. Soils 20, 147–150. https://doi.org/10.1007/BF00336594.

West, S., Kiers, E.T., Pen, I., Denison, R., 2002. Sanctions and mutualism stability: when should less beneficial mutualists be tolerated? J. Evol. Biol. 15, 830–837. https://doi.org/10.1046/j.1420-9101.2002.00441.x.

Wojciechowski, M.F., Lavin, M., Sanderson, M.J., 2004. A phylogeny of legumes (Leguminosae) based on analysis of the plastid *matK* gene resolves many well-supported subclades within the family. Am. J. Bot. 91, 1846–1862. https://doi.org/10.3732/ajb.91.11.1846.

Woldeyohannes, W.H., Dasilva, M.C., Gueye, M., 2007. Research note: Nodulation and nitrogen fixation of *Stylosanthes hamata* in response to induced drought stress. Arid. Land Res. Manag. 21, 157–163. https://doi.org/10.1080/15324980701232894.

Yan, Y., 2021. Long-term exposure to solar blue and UV radiation in legumes: pre-acclimation to drought and accession-dependent responses in two successive generations. University of Helsinki,.

Yan, Y., Stoddard, F.L., Neugart, S., Sadras, V.O., Lindfors, A., Morales, L.O., Aphalo, P.J., 2019. Responses of flavonoid profile and associated gene expression to solar blue and UV radiation in two accessions of *Vicia faba* L. from contrasting UV environments. Photochem. Photobiol. Sci. 18, 434–447. https://doi.org/10.1039/c8pp00567b.

Younis, M., 2010. Responses of *Lablab purpureus* (L.) sweet/Rhizobium symbiosis and growth to potassium supply under different water regimes. J. Plant Nutr. 33, 1400–1409. https://doi.org/10.1080/01904167.2010.484287.

Zacarías, J.J.J., Altamirano-Hernández, J., Cabriales, J.J.P., 2004. Nitrogenase activity and trehalose content of nodules of drought-stressed common beans infected with effective (Fix+) and ineffective (Fix−) rhizobia. Soil Biol. Biochem. 36, 1975–1981. https://doi.org/10.1016/j.soilbio.2004.05.016.

Zhang, X., Zou, T., Lassaletta, L., Mueller, N.D., et al., 2021. Quantification of global and national nitrogen budgets for crop production. Nat. Food 2, 529–540. https://doi.org/10.1038/s43016-021-00318-5.

29

CHAPTER 3: Mesorhizobia strain and chickpea variety drive phenotypic plasticity of plant growth and nodulation

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Mesorhizobia strain and chickpea variety drive phenotypic plasticity of plant growth and nodulation

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Abstract

Chickpea establishes symbiotic relationships with several *Mesorhizobium* species and the three-way interaction between chickpea variety, M*esorhizobium* strain, and environment, drives plant growth and nitrogen fixation. Here we quantify the phenotypic plasticity of chickpea varieties and mesorhizobia strains in experiments including five varieties, nine nitrogen sources including seven *Mesorhizobium* strains, and two uninoculated controls (nitrogen fertilised and unfertilised), and three photothermal regimes with photosynthetic photon flux density from 73 to 1021 µmol m^{-2} s^{-1}, temperature from 21 °C to 30 °C, and vapour pressure deficit from 0.76 to 1.29 kPa. We investigated phenotypic plasticity for six traits: shoot dry weight, nodule dry weight, nodules per plant, nodule colour, symbiotic efficiency, and nitrogen cost. We ask two questions. First, is there a hierarchy of phenotypic plasticities between plant growth and nitrogen fixation traits? Second, how does strain-driven plasticity compare with plant-driven plasticity for these traits? Across sources of variation,

shoot dry weight varied 1.9-fold, nodule dry weight 7.7-fold, nodules per plant 5.7-fold, nodule colour 7.0-fold, symbiotic efficiency 2.1-fold, and nitrogen cost 3.4-fold. Phenotypic plasticity was larger for nodules per plant than for shoot dry weight, verifying a hierarchy of plasticities between these traits. Strain-driven plasticity of plant growth and nitrogen fixation traits was larger than variety-driven plasticity for our combination of varieties, strains and photothermal environments. Our study provides insights on the phenotypic plasticity of the legume-rhizobia interaction by considering the plants as part of the rhizobia environment and vice versa.

1. Introduction

Chickpea (*Cicer arietinum*) establishes symbiotic relationships with several *Mesorhizobium* species (López -Bellido et al. 2011; Greenlon et al. 2019) and the three-way interaction between chickpea variety, M*esorhizobium* strain, and environment, drives plant growth, nitrogen fixation, and related traits. *Mesorhizobium* species carry symbiotic genes that can be transferred between strains and potentially alter the symbiont's host range (Mousavi et al. 2016). The horizontal gene transfer drives diversity and shapes the distribution of chickpea symbionts (Greenlon et al. 2019). *Mesorhizobium* strains vary from highly symbiotically effective to entirely ineffective relative to commercial strains (Slattery et al. 2001; Zaw et al., 2021). Variation in symbiotic effectiveness of strains allows for both competition and selection processes; for example, the inoculation of effective strains can compete with native populations of rhizobia and increase nodule number and shoot dry weight of common bean and chickpea (Hungria et al., 2003; Tena et al., 2016).

The ability of *Mesorhizobium* strains to nodulate and fix atmospheric nitrogen varies with chickpea variety (Gunnabo et al. 2020). Similarly, nodulation and symbiotic effectiveness varied among chickpea varieties inoculated with a single strain, and between rhizobia species on the same variety (Biabani et al., 2011). The exchange of chemical signals between host and rhizobia, and genetic compatibility contribute to the observed differences in nodulation and symbiotic effectiveness (Hirsch and Fujishige 2012). Depending on the actual variation of the partners in the symbiosis, variation in nitrogen fixation may be larger among plant varieties (Hafeez et al., 2000) or among strains (Gunnabu et al., 2020; Rigg et al., 2021).

Nutrient availability, radiation, soil pH, and moisture affect nodulation and nitrogen fixation (Martínez-Romero 2009; Divito and Sadras 2014; Iqbal et al., 2022). The availability of nutrients and radiation could limit the nodulation and nitrogen fixation of rhizobia in common bean (Devi et al. 2013), soybean (Collino et al. 2015) and chickpea (Gebremariam and Tesfay, 2021). Plants compensated for reduced photosynthesis by maintaining only half the root nodule mass and nitrogen fixation activity under limited light (Nandanwar et al., 2020). The effect of radiation and temperature on nodulation and nitrogen fixation depends on the legume-rhizobial system. A reduction in radiation from 40 to 8 % of full sunlight delayed nodule development and reduced nitrogen fixation in *Pentaclethra macroloba* (Taylor and Menge, 2018). Temperature above 30 °C delayed nodule initiation and development and reduced nitrogen fixation in temperate legumes, whereas temperature below 10 °C reduced symbiotic activity through a decrease in sugar utilisation in the nodules of tropical legumes (Hungria & Kaschuk, 2014; Hansen, 2017). The nodulation ability of the soybean-*Bradyrhizobium japonicum* system in the tropics was sensitive to temperature below 25 °C (Montanez et al., 1995).

Phenotypic plasticity is the "amount by which the expressions of individual characteristics of a genotype is changed by different environments" (Bradshaw, 1965). Phenotypic plasticity varies with the genotype, trait and the environmental driver, and hierarchies of plasticities have been established for related traits. For example, the high plasticity of seed number is associated with the stability of seed size (Bradshaw, 1965; Sadras, 2007). Likewise, nodule number is plastic in relation to the conserved nodule size in legumes grown under drought at early developmental stages (Iqbal et al. 2022). In some cases, the plasticity of traits is unrelated or positively related, e.g., a positive relation between plasticity of yield and plasticity of phenological and agronomic traits has been reported for grain crops and olive trees (Sadras et al., 2009: Trentacoste et al., 2011).

In this paper, we designed a factorial experiment combining five chickpea varieties, nine sources of rhizobia or nitrogen supply, and three photothermal regimes to address two questions. First, is there a hierarchy of plasticities between plant growth and nitrogen fixation traits? We expect a hierarchy whereby the plasticity of nitrogen fixation is larger than the plasticity of plant growth as found in response to both nutrient and water deficit (Divito and Sadras 2014; Iqbal et al. 2022). Second, how does strain-driven plasticity compare with plant-driven plasticity? We expect that the plasticity of plant growth and nitrogen fixation traits would depend on the genetic variation of each partner but there is insufficient evidence to predict.

2. Materials and Methods

2.1. Plants, strains and environments

We established a factorial experiment combining five chickpea varieties, nine sources of rhizobial inoculant or nitrogen supply, and three photothermal regimes with four replications. Chickpea varieties were Sonali, Howzat, PBA HatTrick, PBA Slasher and PBA Stricker with contrasting nitrogen fixation, and similar seed weight and phenology (Sadras et al., 2016). PBA HatTrick was used as a reference commonly used to screen *Mesorhizobium* strains (Zaw et al. 2021). The seed was obtained from Agriculture Victoria Research, Australia. Nitrogen sources were seven *Mesorhizobium* strains and two uninoculated controls: a positive control, fertilised with 0.5g $KNO_3 L^{-1}$, and a negative control, with no fertilisation. Strains were M036, M065, M067, M075, M078 and M082 selected based on their diversity (Fig. S1) and their symbiotic performance with HatTrick (Zaw et al. 2021). Additionally, we included CC1192, a commercial strain used for inoculant manufacture in Australia (Bullard et al., 2005). The *Mesorhizobium* strains were sourced from a previous study (Zaw et al. 2021) and grown in broth culture (yeast mannitol) in a rotatory shaker at 120 rpm for 48 h before inoculation of seedlings (Vincent, 1970).

Three glasshouse experiments were conducted in early spring (01 September 2020 – 10 October 2020); late spring (09 November 2021 - 21 December 2021), and summer (23 January 2021 - 05 March 2021) to generate variation in photothermal regimes. Radiation and temperature were recorded with Hobo Pendant Temp-Light Data Loggers (OneTemp Pty Ltd., Bourne, MA 02532, USA). The experiments relied on natural sunlight and incident photosynthetic photon flux density (PPFD) ranged from 73 to 1021 μmol m^{-2} s^{-1}. The photothermal ratio was calculated by dividing the PPFD by temperature and varied between 3 to 31 μmol m^{-2} s^{-1} °C^{-1}. Ambient VPD varied from 1.29 to 0.76 kPa.

Seeds of chickpea were surface sterilised with 75 % ethanol for 2 minutes and washed several times with sterile water. Four seeds were sown in non-draining pots (8.5 cm diameter, 12 cm depth) containing sterilised sand. The plants were thinned to two plants per pot 7 days after sowing (DAS) and sterile plastic beads were added to the surface to minimise rhizobial cross-contamination. Plants were inoculated with 1 ml (approximately 10^9 cells ml^{-1}) of rhizobial broth at the base 7 DAS. Sand was initially moistened to approximately 80 % of field capacity using a nitrogen-free nutrient solution, and its moisture level was maintained by providing 20 ml of sterile nitrogen-free nutrient solution once a week (McKnight 1949), and deionized sterile water as needed (Zaw et al. 2021). Positive controls were additionally supplied with 200 ml of KNO_3 (69.2 mg nitrogen per litre) once a week.

2.2. Measurements

Plants were harvested at flowering (40 DAS in the early spring experiment, 42 DAS in late spring, and 43 DAS in the summer experiment) and dissected into shoots, roots and nodules. We measured shoot dry weight and nodule dry weight (70 °C for 48 h); number of nodules per plant; and size and internal colour of individual nodules. The nodules were cut in half, and size and internal colour were scored: 1, white; 2, green; 3 – 3.5 for small (< 0.15 mm) pink nodule; 3.5 – 4 for big (> 0.15 mm) pink nodule; 4 – 4.5 for small (< 0.15 mm) red nodule; 4.5 – 5 for big (> 0.15 mm) red nodule (Judith et al. 2021).

2.3. Symbiotic efficiency, the cost of nitrogen fixation, and phenotypic plasticity

We estimated net plant benefit from symbiosis as symbiotic efficiency, SE, and the cost of nitrogen fixation, N cost (LaRue and Patterson, 1981; Hardarson and Danso, 1993):

$$SE \ (\%) = \frac{DW \ inoculated}{DW_{N-}} \times 100 \qquad \text{(eq. 1)}$$

$$N \ cost \ (\%) = \frac{DW \ inoculated}{DW_{N+}} \times 100 \qquad \text{(eq. 2)}$$

Where DW (g) is shoot dry weight, and subscripts indicate plants inoculated with test strains (inoculated), uninoculated plants with no fertilisation (N-), and uninoculated plants fertilised with KNO_3 (N+).

To quantify phenotypic plasticity of all traits of interest, we assumed that the plant is part of the strain environment, and the strain is part of the plant environment (Sarkar 2004). We thus quantified the plasticity of each strain in 15 environments resulting from the combination of 3 photothermal regimes and 5 plant varieties, and the plasticity of each plant variety in 27 environments resulting from the combination of 3 photothermal regimes and 9 nitrogen sources (7 strains and 2 uninoculated controls). Phenotypic plasticity was calculated as the slope of the reaction norm relating the trait for each plant variety or strain, and the trait environmental mean (Sadras and Richards 2014).

2.4. Statistical analysis

The effects of plant variety, strain, environment and their interactions on shoot dry weight, nodule traits, symbiotic efficiency, and nitrogen cost were analysed with a general linear model using a factorial design with Statistics 8.1 (Tallahassee, FL, USA). Reaction norms relating trait and trait environmental mean were fitted with least squares regression. Following updated statistical recommendations, we avoid the wording "statistically significant", "non-significant", or the variations thereof, thus avoiding dichotomisation based on an arbitrary discrete p-value (Wasserstein et al. 2019). Instead, we report p as a continuous quantity, and Shannon information transform [$s = -\log_2(p)$] as a measure of the information

against the tested hypothesis (Greenland 2019). Although s is a function of p, the additional information provided is not redundant. With base-2 log, the units for measuring this information are bits (binary digits). For example, $p = 0.05$ conveys only $s = - \log_2(0.05) = 4.3$ bits of information, which is hardly more surprising than seeing all heads in 4 "fair tosses (of coins)" (Greenland, 2019).

3. Results

3.1. Environments

Fig. S2 outlines the photothermal regimes during the experiment. The maximum temperature averaged 30 °C in early spring and summer, and 28 °C in late spring. Minimum temperature averaged 22 °C in early spring and late spring, and 21 °C in summer (Fig. S2a). The daily maximum photosynthetic photon flux density averaged 188 µmol m^{-2} s^{-1} in early spring, 574 µmol m^{-2} s^{-1} in late spring, and 484 µmol m^{-2} s^{-1} in summer (Fig. S2b). The photothermal ratio varied between 3 to 13 µmol m^{-2} s^{-1} °C^{-1} in early spring, 5 to 29 µmol m^{-2} s^{-1} °C^{-1} in late spring, and 6 to 31 µmol m^{-2} s^{-1} °C^{-1} in summer (Fig. S2c).

3.2. Variation in shoot dry weight and nitrogen fixing traits with plant variety, nitrogen source, photothermal environment, and their interactions

Shoot dry weight varied with plant variety ($p = 3.70 \times 10^{-14}$, $s = 44.6$), nitrogen source ($p = 1.30 \times 10^{-107}$, $s = 355.0$), photothermal environment ($p = 3.10 \times 10^{-68}$, $s = 224.3$), and with all two-way interactions ($p = 4.20 \times 10^{-20}$, $s = 64.3$), ($p = 4.80 \times 10^{-26}$, $s = 84.1$) and ($p = 1.60 \times 10^{-2}$, $s = 5.9$) (Table 1). Early spring and uninoculated negative control exhibited a significant decrease in shoot dry weight (Fig. S3). Shoot dry weight decreased from 0.84 g plant^{-1} in summer with N fertiliser to 0.31 g plant^{-1} in early spring with negative control (Fig. S3 a-c). It ranged from 0.74 g plant^{-1} for Sonali with N fertiliser to 0.33 g plant^{-1} for PBA HatTrick with negative control.

Table 1 *p* and *s* from ANOVA for shoot dry weight of chickpea in response to plant variety (V), nitrogen source (N), environment (E), and their interactions.

Source of variation	df	*P*	*s*
V	4	3.7×10^{-14}	44.6
N	8	1.3×10^{-107}	355.0
E	2	3.0×10^{-68}	224.3
V × E	8	4.2×10^{-20}	64.3
N × E	16	4.8×10^{-26}	84.1
V × N	32	1.6×10^{-2}	5.9
V × N × E	64	0.5	0.9

Nodule dry weight, nodules per plant, nodule colour, symbiotic efficiency and nitrogen cost varied with plant variety , strain, photothermal environment and their interactions (Table 2). The interaction between plant variety and strain influenced nodule dry weight ($p = 7.00 \times 10^{-6}$, $s = 17.1$), nodules per plant ($p = 6.50 \times 10^{-10}$, $s = 30.5$) and nodule colour ($p = 1.90 \times 10^{-27}$, $s = 88.7$) (Table 2). Nodule dry weight decreased from 33.7 mg plant^{-1} for PBA Slasher with M075 to 5.9 mg plant^{-1} for Howzat with M082 (Fig. S3 d-f). Nodules per plant reduced from 20.0 for PBA Slasher with M075 to 5.5 for Howzat with M082 (Fig S3 g-i). Nodule colour ranged from 5.0 for PBA HatTrick with M067 to 1.2 for Howzat with M065 (Fig. S4 a-c).

The interaction of strain and photothermal environment affected nodule colour ($p = 5.30 \times 10^{-2}$, $s = 4.2$), symbiotic efficiency ($p = 4.0 \times 10^{-6}$, $s = 17.9$) and nitrogen cost ($p = 2.22 \times 10^{-5}$, $s = 15.4$) (Table 2). Nodule colour, symbiotic efficiency and nitrogen cost were lower in summer than in early and late spring (Figure S4). Nodule colour ranged from 4.9 in late spring with M075 to 1.5 in summer with M065 (Fig. S4 a-c). Symbiotic efficiency decreased from 172 % in summer with M075 to 97 % in summer with M065 (Fig. S4 d-f). Nitrogen

cost decreased from 81 % in late spring with M075 to 42 % in summer with M065 (Fig. S4 g-i).

The interaction between plant variety and photothermal environment affected nodule dry weight ($p = 5.61 \times 10^{-3}$, $s = 7.4$), symbiotic efficiency ($p = 1.30 \times 10^{-5}$, $s = 16.1$) and nitrogen cost ($p = 4.20 \times 10^{-7}$, $s = 21.1$) (Table 2). Nodule dry weight decreased from 26 mg plant^{-1} in early spring PBA Slasher to 15 mg plant^{-1} in summer PBA Stricker (Fig. S3 d-f). Symbiotic efficiency decreased from 152 % in early spring PBA Slasher to 117 % in summer PBA Slasher (Fig. S4 d-f). Nitrogen cost decreased from 88 % in late spring PBA Stricker to 48 % in summer PBA HatTrick (Fig. S4 g-i).

The three-way interaction between plant variety, strain and photothermal environment affected the number of nodules per plant ($p = 0.0$, $s = 4.8$) and nodule colour ($p = 3.80 \times 10^{-7}$, $s = 21.3$) (Table 2). Nodules per plant reduced from 22 in early spring PBA Slasher with M075 to 3.5 in summer Howzat with M065 (Fig S3 g-i). Nodule colour reduced from 5 in early spring PBA Slasher with CC1192 to 0.7 in summer PBA HatTrick with M065 (Fig. S4 a-c).

Table 2 p and s from ANOVA for symbiotic efficiency, nitrogen cost, nodule dry weight, nodules per plan, nodule colour of chickpea in response to plant variety (V), strain (S), environment (E), and their interactions.

Source of variation	df	Nodule dry weight	Nodules per plant	Nodule colour	Symbiotic efficiency	Nitrogen cost
			p			
V	4	1.8×10^{-8}	1.1×10^{-7}	2.4×10^{-8}	8.6×10^{-2}	2.0×10^{-18}
S	6	2.6×10^{-56}	1.0×10^{-49}	1.9×10^{-111}	2.5×10^{-18}	1.4×10^{-22}
E	2	1.4×10^{-5}	1.1×10^{-3}	2.7×10^{-20}	4.4×10^{-3}	1.8×10^{-39}
V × E	8	5.6×10^{-3}	0.2	0.1	1.3×10^{-5}	4.2×10^{-7}
S × E	12	0.3	0.2	5.3×10^{-2}	4.0×10^{-6}	2.2×10^{-5}
V × S	24	7.0×10^{-6}	6.5×10^{-10}	1.9×10^{-27}	0.9	0.7

V ×S × E	48	0.4	0.0	3.8×10^{-7}	0.9	0.9
				s		
V	4	25.6	23.0	25.2	3.5	58.7
S	6	184.6	162.7	367.8	58.4	72.5
E	2	16.0	9.7	65.9	7.9	128.7
V × E	8	7.4	2.1	2.8	16.1	21.1
S × E	12	1.5	2.0	4.2	17.9	15.4
V × S	24	17.1	30.5	88.7	0.0	0.4
V × S × E	48	1.0	4.8	21.3	0.0	0.0

3.2.1. Associations between plasticities of plant growth and nitrogen fixation traits

Across sources of variation, phenotypic plasticity of shoot dry weight varied 2.7-fold, nodule dry weight 2.5-fold, nodules per plant 7.3-fold, nodule colour 3.7-fold, symbiotic efficiency 3.0-fold, and nitrogen cost 2.3-fold (Tables 1 and 2).

We used principal component analysis to explore associations between plasticity of shoot dry weight and nitrogen fixation traits (Fig. 1, Table S2). The first component accounted for 51.8 % of the variability and the second component accounted for 22.7 % of the variability. The plasticity of shoot dry weight was positively correlated with the plasticity of nodule dry weight and nodules per plant, and negatively with the plasticity of nitrogen cost and nodule colour. The plasticities of nodules per plant and nodule dry weight were correlated negatively with the plasticity of nitrogen cost and nodule colour (Fig. 1, Table S1).
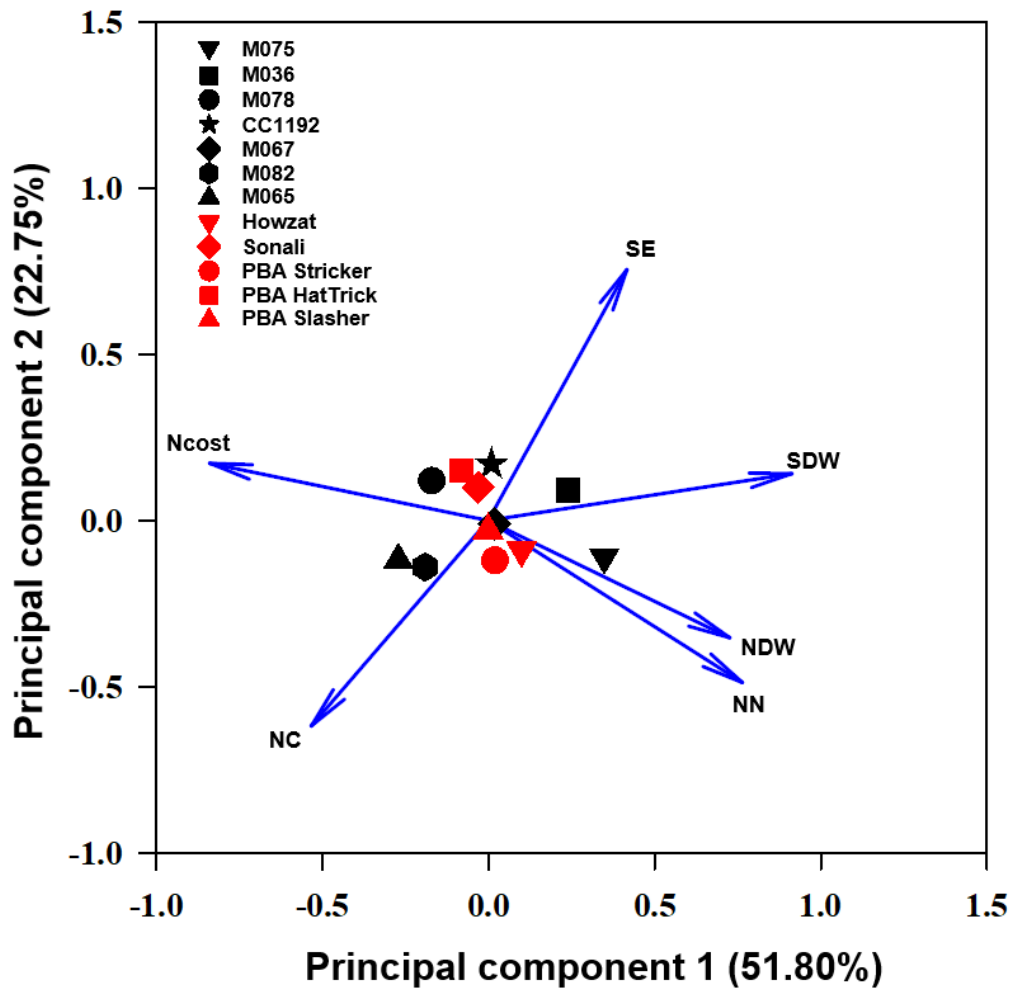
Fig. 1 Principal component analysis for the plasticity of shoot dry weight and nitrogen fixation traits associated with strain and plant variety. Traits are SDW, shoot dry weight; NDW, nodule dry weight; NN, nodules per plant; NC, nodule colour; SE, symbiotic efficiency; Ncost, nitrogen cost.

3.3. Phenotypic plasticity of plant and nitrogen fixation traits for strain and chickpea variety

Strain-driven phenotypic plasticity for shoot dry matter varied 2.7-fold, from 0.5 in M065 to 1.5 in M075, compared with a 1.4-fold variety-driven variation in plasticity, from 0.8 in PBA Slasher to 1.1 in Howzat (Table 3). In the range of environmental mean shoot dry matter

from 0.38 to 0.64, reaction norms of shoot dry matter diverged between strains, highlighting the differential phenotype under more favourable growing conditions (Fig. 2 a)

Table 3 Slope and intercept (± s.e.) of the reaction norms relating shoot dry weight and the environmental mean of shoot dry weight across nitrogen sources and varieties.

Driver	Intercept	Slope
Nitrogen source		
M075	-0.19±0.10	1.53±0.20
M036	-0.18±0.05	1.43±0.11
+ Control	0.01±0.14	1.38±0.28
M078	0.00±0.07	0.93±0.15
CC1192	0.05±0.06	0.90±0.12
M067	0.07±0.04	0.84±0.09
M082	0.06±0.08	0.72±0.17
– Control	0.01±0.14	0.66±0.08
M065	0.12±0.07	0.56±0.15
Variety		
Howzat	-0.04±0.03	1.10±0.06
Sonali	-0.04±0.02	1.06±0.04
PBA Striker	0.03±0.04	1.00±0.09
PBA HatTrick	-0.02±0.04	0.97±0.07
PBA Slasher	0.09±0.03	0.84±0.06

Variation in phenotypic plasticity of nitrogen fixation traits was larger for strain than for plant variety (Table 4). Strain-driven phenotypic plasticity varied 2.5-fold for nodule dry weight, 7.3-fold for nodules per plant, 3.7-fold for nodule colour, 2.9-fold for symbiotic efficiency and 2.3-fold for nitrogen cost (Table 4). In comparison, variety-driven phenotypic plasticity

varied: 1.7-fold for nodule dry weight, 2.1-fold for nodules per plant, 1.7-fold for nodule colour and 1.6-fold for symbiotic efficiency and nitrogen cost (Table 4).

The reaction norms for these traits diverged or converged for strain and plant variety, indicating distinct responses to different environmental conditions (Fig. 2 b-f). The reaction norms for nodule dry weight diverged for both strain and plant variety, highlighting similar nodule dry weight in conditions that impaired nodule development, and differential phenotypes under conditions that favoured nodule development (Fig. 2 b). Similarly, the reaction norms for nodules per plant diverged between strains, highlighting similar nodules per plant in disadvantaged nodulation conditions, and differential phenotypes under conditions that favoured nodulation (Fig. 2 c). The reaction norms for nodules per plant converged between varieties, indicating similar nodules per plant in combinations of strain and photothermal environment that favoured nodulation, and differential response of varieties under conditions that impaired nodulation (Fig. 2 c). The reaction norms for nodule colour converged for both strain and plant variety, highlighting similar nitrogen fixation under favourable conditions, and differential influence of strains and varieties under unfavourable conditions (Fig. 2 d). The reaction norms for symbiotic efficiency diverged for both strain and plant variety, highlighting similar symbiotic efficiency in conditions that compromised symbiotic efficiency, and larger phenotypic differences under conditions that favoured symbiotic efficiency (Fig. 2 e). The reaction norms for nitrogen cost converged, highlighting similar N cost under conditions with high nitrogen cost, and differential phenotypes in environments with lower nitrogen cost (Fig. 2 f).
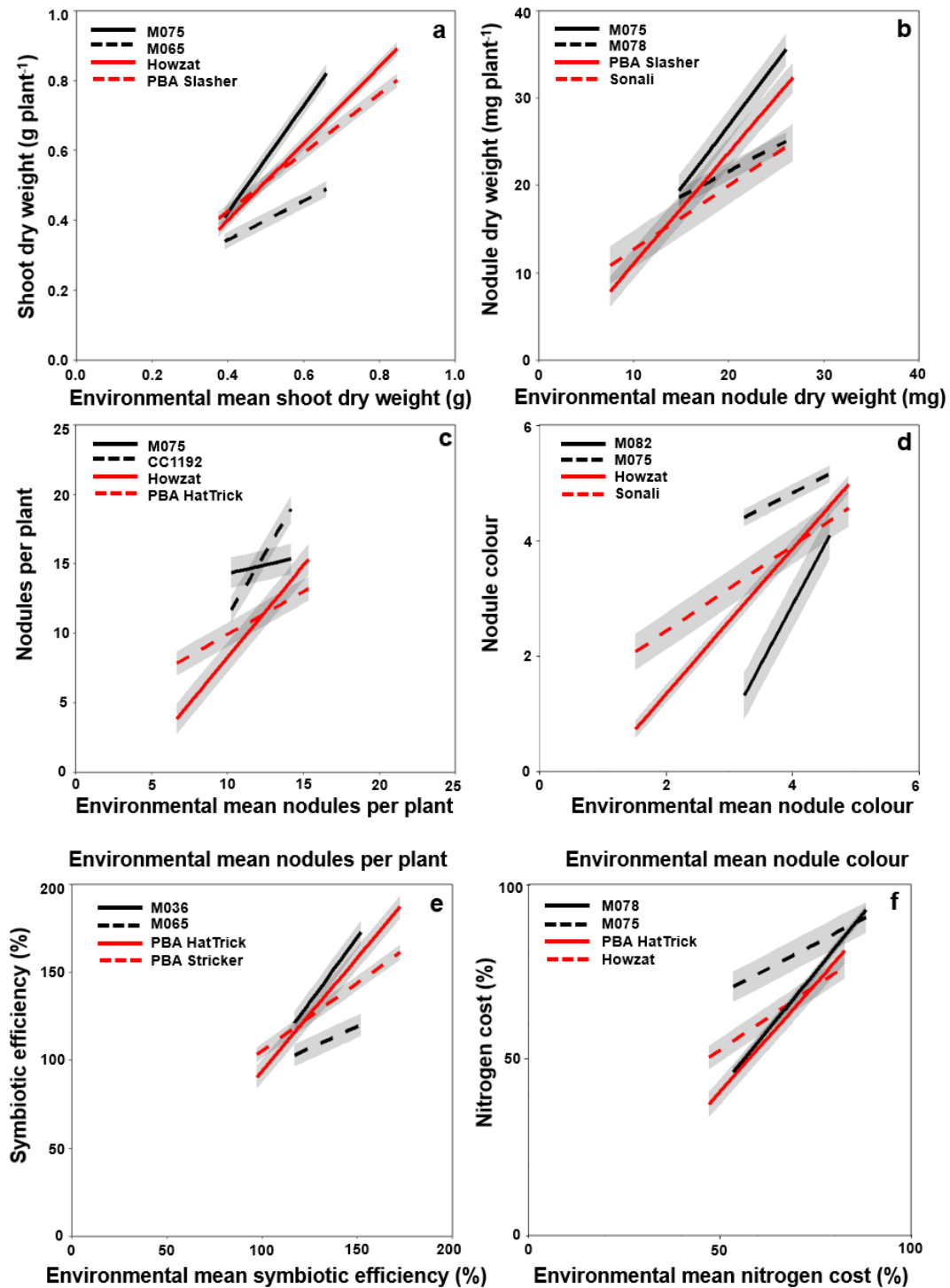
Fig. 2 Reaction norms of (a) shoot dry weight, (b) nodule dry weight, (c) nodules per plant, (d) nodule colour, (e) symbiotic efficiency, and (f) nitrogen cost for the strains and varieties with the most extreme plasticities. For strains, environmental mean is the average across 3 photothermal regimes and 5 plant varieties, returning 15 environments. For varieties, environmental mean is the average across 3 photothermal regimes and 9 nitrogen sources (7

strains and 2 uninoculated controls) for shoot dry weight that provided 27 environments, and 3 photothermal regimes and 7 nitrogen sources for symbiotic efficiency, N cost and nodule traits, that provided 21 environments. Parameters and statistics of fitted lines are in Tables 4 and 5. Shaded area represents standard error of slope.

Table 4 Slope and intercept (± s.e.) of the reaction norms relating nodule dry weight, nodules per plant, nodule colour, symbiotic efficiency and nitrogen cost and the environmental mean of these traits across nitrogen sources and varieties.

	Nodule dry weight		Nodules per plant		Nodule colour		Symbiotic efficiency		Nitrogen cost	
Strain	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope	Intercept	Slope
M036	-0.21±7.06	1.08±0.37	-5.78±5.31	1.48±0.44	1.06±1.40	0.82±0.37	-53.50±46.71	1.49±0.35	22.53±13.97	0.76±0.19
CC1192	2.68±5.89	1.09±0.31	11.79±5.74	0.25±0.47	1.93±1.25	0.67±0.33	-41.07±33.09	1.34±0.25	11.47±9.45	0.86±0.13
M067	1.41±7.16	1.09±0.37	-3.38±5.53	1.40±0.46	1.95±1.56	0.67±0.41	6.05±23.52	0.98±0.17	-5.27±7.97	1.10±0.11
M078	10.20±3.81	0.57±0.20	5.71±5.14	0.61±0.42	1.54±1.71	0.70±0.45	6.75±33.40	0.91±0.25	-24.79±6.60	1.32±0.09
M082	-10.01±7.50	1.11±0.39	-0.55±5.70	0.70±0.47	-5.33±2.18	2.05±0.58	-1.33±44.77	0.88±0.33	-26.48±12.08	1.25±0.17
M075	-1.73±0.35	1.43±0.35	-7.06±5.04	1.83±0.41	2.58±0.79	0.56±0.21	38.81±67.17	0.86±0.50	40.28±18.05	0.57±0.25
M065	-2.11±4.25	0.58±0.22	-0.72±5.25	0.70±0.43	-3.73±1.43	1.50±0.38	44.28±43.80	0.50±0.33	-17.73±13.21	1.10±0.18
Variety										
PBA HatTrick	2.83±2.33	0.81±0.11	3.71±1.59	0.62±0.12	0.31±0.54	0.90±0.13	-33.84±21.74	1.28±0.16	-20.66±11.61	1.23±0.16
Sonali	5.39±3.00	0.73±0.15	4.44±1.64	0.65±0.13	0.94±0.48	0.74±0.12	-1.57±13.98	1.01±0.10	-2.81±6.25	0.96±0.08
Howzat	-4.07±1.99	1.11±0.10	-4.93±2.00	1.32±0.16	-1.19±0.24	1.26±0.06	-2.23±14.99	0.96±0.11	16.26±10.69	0.73±0.15
PBA Slasher	-1.65±2.43	1.27±0.12	-0.36±2.11	1.16±0.17	0.93±0.33	0.84±0.08	9.19±28.72	0.95±0.21	-10.57±5.06	1.17±0.07
PBA Striker	-2.50±1.53	1.07±0.07	-2.85±1.11	1.24±0.09	-0.99±0.36	1.24±0.09	25.45±14.23	0.77±0.10	17.78±9.48	0.89±0.13

4. Discussion

4.1. Variation in shoot dry weight and nitrogen fixing traits with plant variety, nitrogen source, photothermal environment, and their interactions

We assessed the interaction between chickpea variety, *Mesorhizobium* strain and photothermal environment, taking advantage of the documented diversity of chickpea genotypes and *Mesorhizobium* strains (Sadras et al., 2016; Zaw et al., 2021; Zaw et al., 2022).

Nodules per plant and nodule colour were influenced by three-way interaction between plant variety, *Mesorhizobium* strain and photothermal environment. However, there were no significant effects observed on shoot dry weight, symbiotic efficiency and nitrogen cost (Tables 1 and 2). The observed effects on nodules per plant and nodule colour are likely attributed to the impact on signal exchange between plant-strain interaction, along with environmental stress. The specificity of plant-strain interaction relies on the exchange of chemical signals between both partners. This process involves the release of flavonoids by the roots, which vary among plant species and varieties (Hirsch and Fujishige 2012, Cooper 2007). In response, rhizobia produce nod factors (lipochitin oligosaccharides) that induce nodule formation (Oldroyd and Downie 2008). Thus, the interaction between the exchange of signals between plant and strain, along with environmental stresses, can limit the infection process, delay nodulation, and reduce the nodule growth rate (Lira et al. 2015). Symbiotic genes also control the first level of rhizobia specificity in environments that reduce nodulation and nitrogen fixation (Gunnabo et al. 2019). Similar interactions between plant variety, strain and environment affecting nodulation and nitrogen fixation have been reported for soybean and common bean (Argaw and Muleta 2018, Agoyi et al. 2017, Yusuf et al. 2008).

The interaction between plant variety and strain could indicate different infectivity potential of rhizobia with host genotypes as reported in common bean (Neila et al., 2014). In our study, we observed variations in nodule dry weight, nodules per plant, and nodule colour among different chickpea varieties when inoculated with different strains, indicating specificity of the *Mesorhizobium* strains and chickpea variety as reported with common bean - *Rhizobium* specificity (Bouhmouch et al., 2005).

4.2. A hierarchy of plasticities between plant growth and nitrogen fixation traits

Phenotypic plasticity of nodules per plant was higher than the plasticity of shoot dry weight, which was similar to the phenotypic plasticity of nodule dry weight, nodule colour, symbiotic efficiency, and nitrogen cost for both strain and plant variety (Tables 1 and 2). However, there were strain and variety specific departures from this trend. For example, strain CC1192 had larger plasticity for shoot dry weight than for nodule number, and the opposite was true for strain M067. This compares with previous studies showing higher plasticity of nitrogen fixation traits than shoot dry matter in response to drought (Iqbal et al., 2022) and nutrient deficiency (Divito and Sadras 2014). Large variation of nitrogen fixation traits may arise from reduced initiation and development of nodules, increasing nodule senescence or a combination depending on the growth conditions. Temperature above 30 °C and photosynthetic photon flux density of 1320 µmol m^{-2} s^{-1} delayed nodule initiation and reduced nitrogen fixation in soybean, suggesting that nodule initiation and development were more likely sources of variation in our study (Baroniya et al., 2013: Nandanwar et al., 2020).

In comparison to the plasticity of nodules per plant, shoot dry weight was conserved. In soybean inoculated with *B. japonicum* grown in a factorial with two levels of radiation and two levels of soil nitrogen, nodule number per plant was 0.02 at low light and, high nitrogen and 22.6 at high light and low nitrogen whereas average shoot mass varied from 0.3 to 1.8 mg

(Lau et al., 2012). Although both traits were responsive, the 1128-fold variation in number compared with a 5.0-fold variation in shoot dry weight, is consistent with our finding that plasticity of nitrogen fixation traits is higher than the plasticity of shoot biomass. A similar hierarchy of plasticities was found in grain and forage legumes where nodules per plant and nodule mass varied more than shoot mass under deficit of phosphorus, sulphur or potassium (Divito and Sadras, 2014) and for early season drought (Iqbal et al., 2022).

Strain-driven plasticity of nodules per plant associated with plasticity of shoot dry weight (Fig. 2, Table S1). For plant variety, the plasticity of shoot dry weight was negatively associated with plasticity of nodules per plant but positively with nodule colour (Fig. 2, Table S1). Negative correlations between plasticity of seed number and seed size in plants were first interpreted in terms of hierarchies of plasticities (Bradshaw 1965). More recent studies showed associations between plasticities of related traits can be negative or positive in annual and perennial crops, thus expanding the notion of hierarchies (Sadras et al., 2009; Trentacoste et al., 2011).

4.3. The plasticity of plant growth and nitrogen fixation traits was larger for strains than for variety

Under our experimental conditions, the variation in phenotypic plasticity for shoot dry weight and nitrogen fixation traits was larger among strains than among varieties (Fig. 2). Other studies showed genetically diverse strains dominated the variation in plant growth and nitrogen fixation traits, rather than chickpea varieties (Gunnabu et al., 2020; Rigg et al., 2021). Divergent reaction norms for strains indicated shoot and nodule dry weight, symbiotic efficiency and nodules per plant varied more in favourable environments resulting from the combination of chickpea variety and photothermal environment (Fig. 2). Phenotypic plasticity reinforces the notion that large variation in plant growth and nodule traits is linked

with strain potential to benefit from favourable environments. For example, *Bradyrhizobium japonicum* strains improved chickpea growth by producing high phytohormones ratios under favourable growth conditions (Bano et al., 2010). In contrast, convergent reaction norms for nitrogen cost and nodule colour highlighted larger variation in constrained environments (Fig. 2). This reinforces the notion of trait-specific plasticity for the same genotype-environment combinations (Bradshaw, 1965; Sadras and Richards, 2014; Trentacoste et al., 2011).

A quantitative, plasticity perspective assuming plants are part of the rhizobia environment and rhizobia are part of the plant environment provided insights on the three-way interaction between strains, varieties and photothermal environment.

Authors contribution

MDD, VOS, YZ and NI conceived concept and designed experiments. NI carried out experiments, collected and analysed data and wrote original draft. VOS, YZ and MDD supervised the experiments, wrote- review & edit manuscript. All authors read and approved the submitted version.

Data availability statement

The data supporting the findings of this study are available from the corresponding author, Matthew D Denton, and the first author, Nasir Iqbal.

References

Agoyi EE, Odong TL, Tumuhairwe JB, Chigeza G, Diers BW, Tukamuhabwa P (2017) Genotype by environment effects on promiscuous nodulation in soybean (*Glycine max* L. Merrill). Agric Food Secur 6 (1): 1-14

Argaw A, Muleta D (2018) Effect of genotypes-Rhizobium-environment interaction on nodulation and productivity of common bean (*Phaseolus vulgaris* L.) in eastern Ethiopia. Environ Syst Res 6(1): 1-16

Bano A, Batool R, Dazzo F (2010) Adaptation of chickpea to desiccation stress is enhanced by symbiotic rhizobia. Symbiosis 50 (3): 129-133

Baroniya SS, Kataria S, Pandey GP, Guruprasad KN (2014) Growth, photosynthesis and nitrogen metabolism in soybean varieties after exclusion of the UV-B and UV-A/B components of solar radiation. Crop J 2 (6): 388-397

Biabani A, Carpenter-Boggs L, Coyne CJ, Taylor L, Smith JL, Higgins S (2011) Nitrogen fixation potential in global chickpea mini-core collection. Biol Fertil Soils 47 (6): 679-685

Bradshaw AD (1965) Evolutionary significance of phenotypic plasticity in plants. Adv Genet 13: 115–155

Bouhmouch I, Souad-Mouhsine B, Brhada F, Aurag J (2005) Influence of host cultivars and Rhizobium species on the growth and symbiotic performance of *Phaseolus vulgaris* under salt stress. J Plant Physiol 162: 1103–1113

Bullard GK, Roughley RJ, Pulsford DJ (2005) The legume inoculant industry and inoculant quality control in Australia: 1953–2003. Aust J Exp Agric 45(3): 127-140

Collino DJ, Salvagiotti F, Perticari A, Piccinetti C, Ovando G, Urquiaga S, Racca RW (2015) Biological nitrogen fixation in soybean in Argentina: relationships with crop, soil, and meteorological factors. Plant Soil 392: 239–252

Cooper JE (2007) Early interactions between legumes and rhizobia: disclosing complexity in a molecular dialogue. J Appl Microbiol 103 (5): 1355-1365

Devi MJ, Sinclair TR, Beebe SE, Rao IM (2013) Comparison of common bean (*Phaseolus vulgaris* L.) varieties for nitrogen fixation tolerance to soil drying. Plant Soil 364: 29–37

Divito GA, Sadras VO (2014) How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? A meta-analysis. Field Crops Res 156: 161–171

Gebremariam M, Tesfay T (2021) Effect of P Application Rate and Rhizobium Inoculation on Nodulation, Growth, and Yield Performance of Chickpea (*Cicer arietinum* L.). Int J Agron 2021: 1-14

Greenland S (2019) Valid *p*-values behave exactly as they should: Some misleading criticisms of *p*-values and their resolution with *s*-values. Am Stat 73 (suppl 1): 106-114

Greenlon A, Chang PL, Damtew ZM, Muleta A, Carrasquilla-Garcia N, Kim D, Cook DR (2019) Global-level population genomics reveals differential effects of geography and

phylogeny on horizontal gene transfer in soil bacteria. Proceedings of the National Academy of Sciences 116 (30): 15200-15209

Gunnabo AH, Geurts R, Wolde-meskel E, Degefu T, Giller KE, Van Heerwaarden J (2019) Genetic interaction studies reveal superior performance of Rhizobium tropici CIAT899 on a range of diverse East African common bean (*Phaseolus vulgaris* L.) genotypes. Appl Environ Microbiol 85 (24): e01763-19

Gunnabo AH, van Heerwaarden J, Geurts R, Wolde-meskel E, Degefu T, Giller KE (2020) Symbiotic interactions between chickpea (*Cicer arietinum* L.) genotypes and Mesorhizobium strains. Symbiosis 82 (3): 235-248

Hafeez FY, Shah NH, Malik KA (2000) Field evaluation of lentil cultivars inoculated with *Rhizobium leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution. Biol Fertil Soils 31: 65–69

Hansen AP (2017) Legume-Rhizobium symbioses: Significance for sustainable plant production. In: Varma A, Sharma A (eds) Modern tools and techniques to understand microbes: Cham, Springer, pp 159–182

Hardarson G, Danso SKA (1993) Methods for measuring biological nitrogen fixation in grain legumes. Plant soil 152 (1): 19-23

Hirsch AM, Fujishige NA (2012) Molecular signals and receptors: communication between nitrogen-fixing bacteria and their plant hosts. In: Witzany G, Baluška F (eds) Biocommunication of plants: Springer, Berlin, Heidelberg, pp 255-280

Hungria M, Campo RJ, Mendes IC (2003) Benefits of inoculation of the common bean (*Phaseolus vulgaris*) crop with efficient and competitive *Rhizobium tropici* strains. Biol Fertil Soils 39 (2): 88-93

Hungria M, Kaschuk G (2014) Regulation of N_2 fixation and NO_3^-/NH_4^+ assimilation in nodulated and N-fertilized *Phaseolus vulgaris* L. exposed to high temperature stress. Environ Exp Bot 98: 32–39

Iqbal N, Sadras VO, Denison RF, Zhou Y, Denton MD (2022) Clade-dependent effects of drought on nitrogen fixation and its components–Number, size, and activity of nodules in legumes. Field Crops Res *284*: 108586

Judith KW, Fredrick MN, Paul NN, Jeremiah OM, Simon TM, Charles AO (2021) Nodulation and protein content of selected chickpea varieties as impacted by spacing and n-fertiliser rates in Eastern Kenya. Afr J Agric Res 17 (6): 882-895

LaRue TA, Patterson TG (1981) How much nitrogen do legumes fix?. Adv Agron 34: 15-38

Lau JA, Bowling EJ, Gentry LE, Glasser PA, Monarch EA, Olesen WM, Young RT (2012) Direct and interactive effects of light and nutrients on the legume-rhizobia mutualism. Acta Oecol 39: 80–86

Lira Jr MA, Nascimento LR, Fracetto GG (2015) Legume-rhizobia signal exchange: promiscuity and environmental effects. Front Microbiol 6: 945

López-Bellido RJ, López-Bellido L, Benítez-Vega J, Muñoz-Romero V, López-Bellido FJ, Redondo R (2011) Chickpea and faba bean nitrogen fixation in a Mediterranean rainfed Vertisol: Effect of the tillage system. Eur J Agron 34 (4): 222-230

Martínez-Romero E (2009) Coevolution in Rhizobium-legume symbiosis?. DNA Cell Biol 28 (8): 361-370

McKnight T (1949) Efficiency of isolates of Rhizobium in the cowpea group, with proposed additions to this group. Qld J Agric Sci 6: 61–76.

Mukherjee B, Kumar Naskar M, Nath R, Atta K, Visha Kumari V, Banerjee P, ... & Hossain A (2023) Growth, nodulation, yield, nitrogen uptake, and economics of lentil as influenced by sowing time, tillage, and management practices. Front Sustain Food Syst 7:1151111.

Mousavi SA, Li L, Wei G, Räsänen L, Lindström K (2016) Evolution and taxonomy of native mesorhizobia nodulating medicinal *Glycyrrhiza* species in China. Syst Appl Microbiol 39: 260–265

Nandanwar S, Yele Y, Dixit A, Goss-Souza D, Singh R, Shanware A, Kharbikar L (2020) Effects of pesticides, temperature, light, and chemical constituents of soil on nitrogen fixation. In: Everlon CR, Ademar PS (eds) Nitrogen fixation, IntechOpen, pp 1-8.

Neila A, Adnane B, Mustapha F, Manel B, Imen H, Boulbaba L, Cherki G, Bouaziz S (2014) *Phaseolus vulgaris*-rhizobia symbiosis increases the phosphorus uptake and symbiotic N fixation under insoluble phosphorus. J Plant Nutr 37 (5): 643–657

Oldroyd GE, Downie JA (2008) Coordinating nodule morphogenesis with rhizobial infection in legumes. Annu Rev Plant Biol 59: 519-546

Rigg JL, Webster AT, Harvey DM, Orgill SE, Galea F, Dando AG, Hayes RC (2021) Cross-host compatibility of commercial rhizobial strains for new and existing pasture legume cultivars in south-eastern Australia. Crop Pasture Sci 72: 652-665

Sadras VO (2007) Evolutionary aspects of the trade-off between seed size and number in crops. Field Crops Res 100 (2-3): 125-138

Sadras VO, Denison RF (2009) Do plant parts compete for resources? An evolutionary perspective. New Phytol 183: 565–574

Sadras VO, Reynolds M, De la Vega AJ, Petrie PR, Robinson R (2009) Phenotypic plasticity of phenology and yield in wheat, sunflower and grapevine. Field Crops Res 110: 242–250

Sadras VO, Richards RA (2014) Improvement of crop yield in dry environments: benchmarks, levels of organisation and the role of nitrogen. J Exp Bot 65 (8): 1981-1995

Sadras VO, Lake L, Li Y, Farquharson EA, Sutton T (2016) Phenotypic plasticity and its genetic regulation for yield, nitrogen fixation and δ13C in chickpea crops under varying water regimes. J Exp Bot 67: 4339–4351

Sarkar S (2004) From the Reaktionsnorm to the evolution of adaptive plasticity. In: DeWitt TJ, Scheiner SM (eds) Phenotypic Plasticity – Functional and Conceptual Approaches, Oxford University, New York, pp 10–30

Slattery JF, Coventry DR, Slattery WJ (2001) Rhizobial ecology as affected by the soil environment. Aust J Exp Agric 41 (3): 289-298

Taylor BN, Menge DN (2018). Light regulates tropical symbiotic nitrogen fixation more strongly than soil nitrogen. Nat Plants 4(9): 655-661

Tena W, Wolde-Meskel E, Walley F (2016) Response of chickpea (*Cicer arietinum* L.) to inoculation with native and exotic *Mesorhizobium* strains in Southern Ethiopia. Afr J Biotechnol 15 (35): 1920-1929

Trentacoste ER, Sadras VO, Puertas CM (2011) Effects of the source:sink ratio on the phenotypic plasticity of stem water potential in olive (*Olea europaea* L.). J Exp Bot 10 (62): 3535-3543

Vincent JM (1970) A manual for the practical study of the root-nodule bacteria. IBP handbook 15, Blackwell Scientific, Oxford-Edinburgh, pp 164

Wasserstein RL, Schirm AL, Lazar NA (2019) Moving to a world beyond "*p*< 0.05". Am Stat 73 (supp 1): 1-19

Yusuf AA, Abaidoo RC, Iwuafor ENO, Olufajo OO (2008) Genotype effects of cowpea and soybean on nodulation, N_2-fixation and N balance in the Northern Guinea savanna of Nigeria. J Agron 3 (7): 258-264

Zaw M, Rathjen JR, Zhou Y, Ryder MH, Denton MD (2021) Symbiotic effectiveness, ecological adaptation and phylogenetic diversity of chickpea rhizobia isolated from a large-scale Australian soil collection. Plant Soil, 469 (1): 49-71

Zaw M, Rathjen JR, Zhou Y, Ryder MH, Denton MD (2022) Rhizobial diversity is associated with inoculation history at a two-continent scale. FEMS Microbiol Ecol 98 (5): fiac044

Supplementary data

Table S1 Matrix of Pearson's correlation coefficients (r) for the plasticity of shoot dry weight and nitrogen fixation traits associated with strain and variety, and for the pooled data. Shading indicates the strength of correlations, with darker shading denoting stronger positive (blue) or negative (red) correlations.

| Driver		Shoot dry weight	Nodule dry weight	Nodules per plant	Nodule colour	Symbiotic efficiency
Strain	Nodule dry weight	0.62				
	Nodules per plant	0.72	0.58			
	Nodule colour	-0.59	-0.21	-0.35		
	Symbiotic efficiency	0.51	0.39	0.06	-0.43	
	Nitrogen cost	-0.79	-0.70	-0.59	0.50	-0.40
Variety	Nodule dry weight	-0.47				
	Nodules per plant	-0.02	0.87			
	Nodule colour	0.40	0.42	0.75		
	Symbiotic efficiency	-0.05	-0.54	-0.75	-0.50	
	Nitrogen cost	-0.77	-0.13	-0.57	-0.69	0.60
Pooled	Nodule dry weight	0.47				
	Nodules per plant	0.63	0.64			
	Nodule colour	-0.51	-0.10	-0.19		
	Symbiotic efficiency	0.45	0.19	-0.07	-0.44	
	Nitrogen cost	-0.75	-0.55	-0.58	0.28	-0.17

16S-23S rDNA IGS
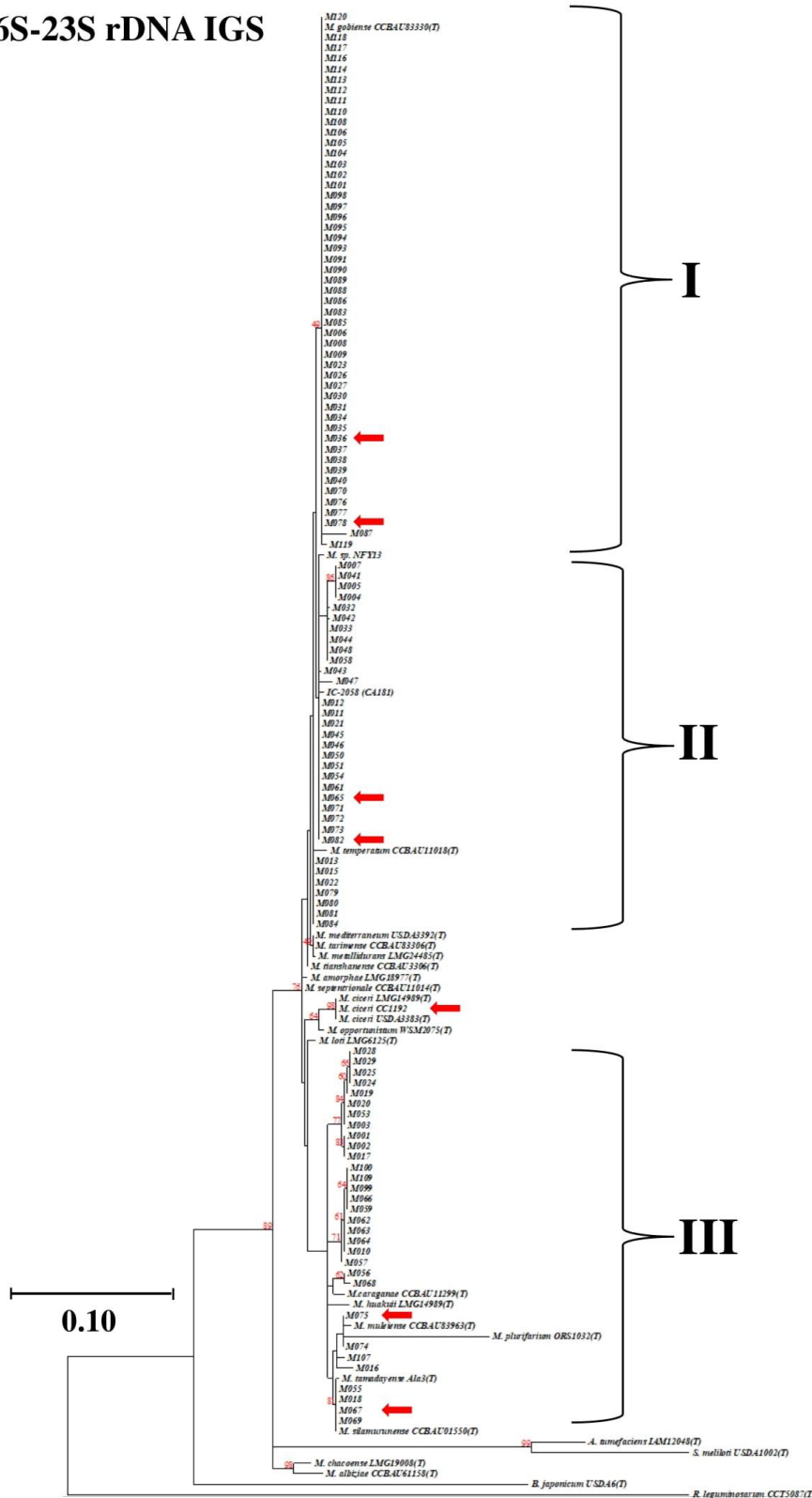
Fig. S1 Maximum likelihood phylogenetic tree based on 16S–23S rDNA IGS sequences of

114 Myanmar rhizobial strains and reference strains. Strains indicated with red arrows were

selected in this study. Bootstrap values were computed based on 1000 replications. The scale

bar (0.10) indicates the percentage of nucleotide substitutions per site. A, *Agrobacterium*, B,

Bradyrhizobium, M, *Mesorhizobium*, S, *Sinorhizobium*, and R, *Rhizobium* (Zaw et al., 2022).
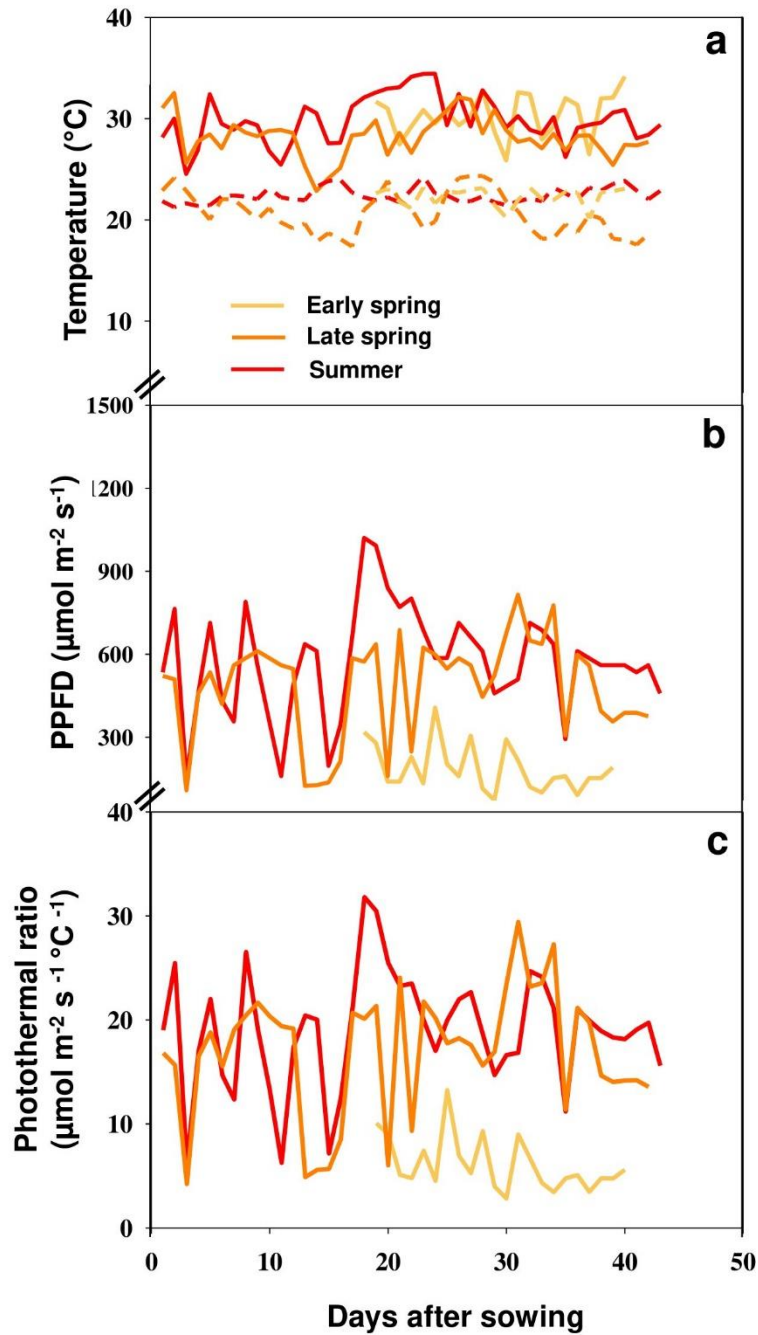
Fig. S2 (a) Daily maximum (solid line) and minimum (dashed line) temperature, (b) photosynthetic photon flux density (PPFD) and (c) photothermal ratio in three experiments during early spring (01 September 2020 – 10 October 2020), late spring (09 November 2021 - 21 December 2021), and summer (23 January 2021 - 05 March 2021). Early spring data were recorded from September 19, 2020 due to delayed availability of sensors.
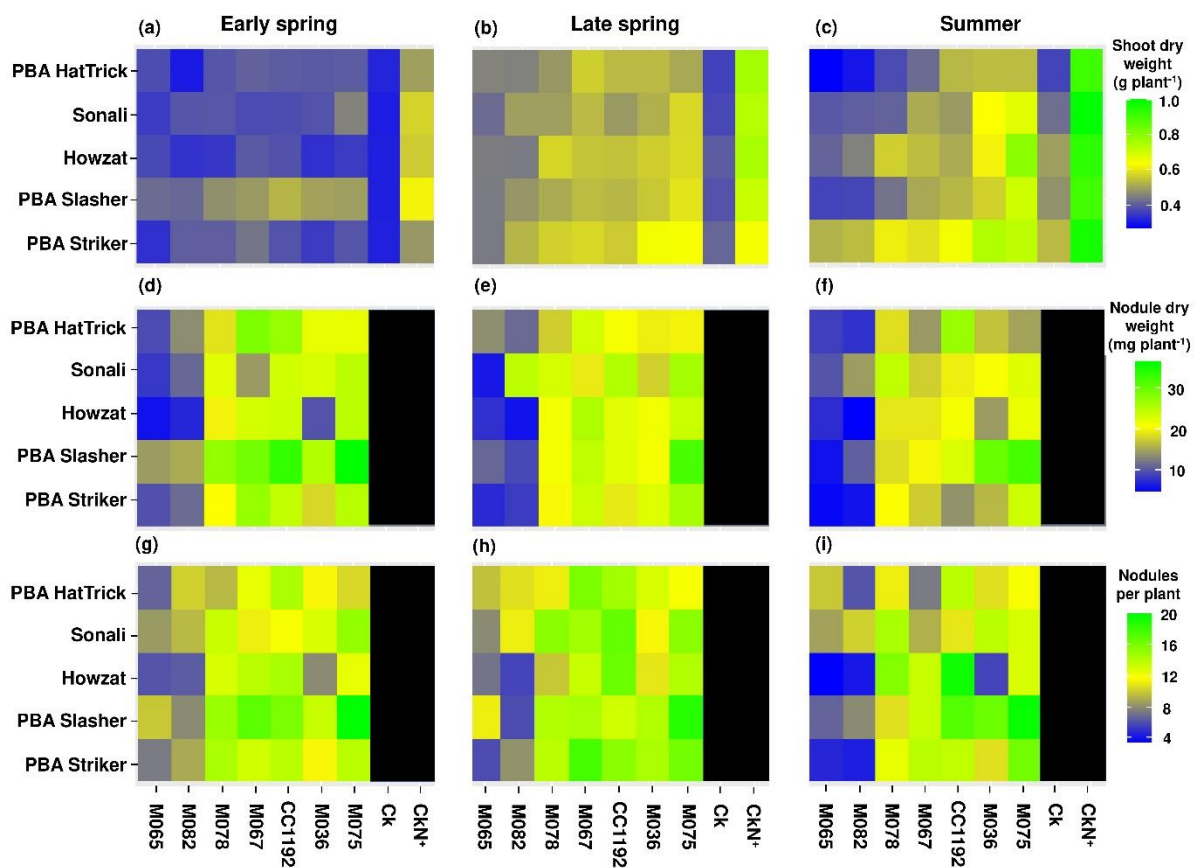
Fig. S3 (a, b, c) Mean shoot dry weight, (d, e, f) nodule dry weight, and (g, h, i) nodules per plant for the combination of five chickpea varieties and nine nitrogen sources including seven *Mesorhizobium* strains, uninoculated negative control (Ck) with no fertilisation and uninoculated positive control (CkN$^+$) fertilised with 0.5g KNO$_3$ L^{-1}. The chickpea varieties were ordered based on average shoot dry weight across strains and photothermal environments. The *Mesorhizobium* strains were ordered based on average shoot dry weight across chickpea genotypes and photothermal environments.
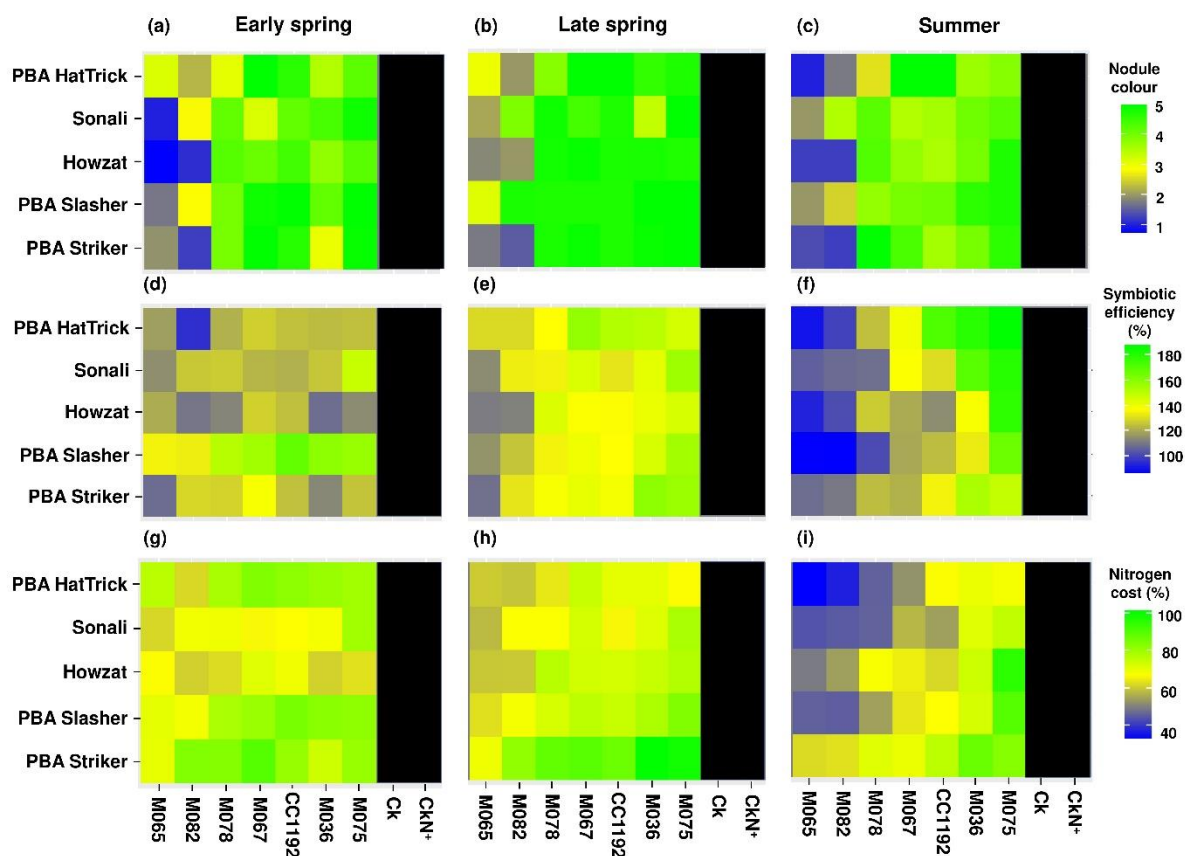
Fig. S4 (a, b, c) Mean nodule colour, (d, e, f) symbiotic efficiency, and (g, h, i) nitrogen cost for the combination of five chickpea varieties and nine nitrogen sources including seven *Mesorhizobium* strains, uninoculated negative control (Ck) with no fertilisation and uninoculated positive control (CkN⁺) fertilised with 0.5g KNO_3 L^{-1}. The chickpea varieties were ordered based on average shoot dry weight across strains and photothermal environments. The *Mesorhizobium* strains were ordered based on average shoot dry weight across chickpea genotypes and photothermal environments.

CHAPTER 4: Variations of Chickpea Growth in Response to *Mesorhizobium* Strains are Temporal Under Well-Watered Conditions and Drought

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Contribution to the Paper	Conceived concept, designed and conducted experiments, data collection, analysis and writing original draft
Overall percentage (%)	80%
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.

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  ii.   permission is granted for the candidate in include the publication in the thesis; and
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Variations of Chickpea Growth in Response to *Mesorhizobium* Strains are Temporal Under Well-Watered Conditions and Drought

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Abstract

Drought can impact the symbiotic relationship between Chickpea (*Cicer arietinum*) and rhizobia, influencing nodulation, nitrogen fixation and plant growth. Here we used a high-throughput shoot phenotyping technology to investigate the effects of *Mesorhizobium* strain on the growth dynamic of chickpea varieties, nodulation and bacteroid morphology under different water regimes in an experiment including four chickpea varieties, four nitrogen sources including two *Mesorhizobium* strains, and two uninoculated controls (nitrogen fertilised and unfertilised) under well-watered and drought conditions. We asked three questions. First, does the impact of rhizobial strains on chickpea growth change over time under well-watered and drought conditions? second, do genetically diverse chickpea varieties

exhibit variations in leaf exudates, biomass and nodule traits in response to different *Mesorhizobium* strains under well-watered and drought conditions? third, are bacteroid size and amount of polyhydroxybutyrate modified by *Mesorhizobium* strain, chickpea variety, water availability and their interactions? Shoot growth rate showed temporal variations in response to both strains CC1192 and M075 under well-watered and drought conditions. Shoot growth rate showed temporal variations in response to both strains CC1192 and M075 under well-watered and drought conditions. Under well-watered conditions, chickpea inoculated with CC1192 showed high shoot growth rates, relative to M075, and continued to accumulate high plant biomass and symbiotic efficiency at harvest. In drought, shoot growth rate was comparable between both CC1192 and M075, indicating no significant difference in plant biomass and symbiotic efficiency at harvest. Across sources of variation, leaf exudates varied 3.4-fold, total plant biomass 3.0-fold, nodules per plant 3.9-fold, nodule dry weight 3.0-fold, symbiotic efficiency 1.5-fold, bacteroid size 1.4-fold and polyhydroxybutyrate 1.4-fold. Plant biomass was negatively correlated with both bacteroid size and amount of polyhydroxybutyrate under well-watered conditions, determining the existence of a trade-off between plant fitness and rhizobia fitness. This research demonstrates the dynamic nature of growth response exhibited by chickpea in relation to *Mesorhizobium* strains, highlighting the critical role that water availability plays in shaping this response.

1. Introduction

Chickpea is one of the most important and valuable 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). For drought adaptation, chickpea possesses traits to improve the efficiency of water use while maintaining growth (Upadhyaya et al., 2012). In particular, plants that exhibit drought-reduced photosynthesis sacrifice leaf expansion to free resources to enhance the production of organic acids to maintain water content under drought (Chtouki et al., 2022). The presence of organic acids in chickpea leaf exudates acts as osmoprotectants, maintaining leaf temperature and cell turgor and reducing water loss (Lauter and Munns, 1986; Singh et al., 2015).

Chickpea (*Cicer arietinum*) establishes symbiotic relationships with rhizobia and fixes atmospheric nitrogen to enhance growth, nutrient acquisition and stress tolerance (López - Bellido et al. 2011, Greenlon et al. 2019). An effective symbiotic rhizobial strain improves plant growth, nodulation, pod number and seed yield relative to other strains under drought (Dimkpa et al., 2009), but growth responses vary with both plant variety and strain (Biabani et al., 2011; Zaw et al., 2021). Effective *Mesorhizobium* and *Sinorhizobium* strains improved nodulation of chickpea and soybean under drought, but did not always translate into increased shoot growth (Kibido et al., 2018; Abdela et al., 2020). Almost all physiological studies of growth responses to symbiotic rhizobia are based on the harvest time point biomass, which may mask temporal responses to rhizobia. To measure shoot growth over time to pinpoint the appearance of growth improvement in order to gain a deeper perspective on rhizobia effects on chickpea growth still needs attention in the literature.

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). These 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). During transformation, swollen bacteroids undergo polyploidisation and compromise their reproductive ability, characterising their differentiation as terminal (Mergaert et al., 2006). 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), while non-swelling bacteroids hoard high-energy lipid poly-3-hydroxybutyrate (PHB) inside their cells for reproduction, 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 bacteroids into swollen and non-swollen is a plant-dependent trait (Van de Velde et al., 2010; Montiel et al., 2017). Therefore, strain, plant variety and water availability could influence the bacteroid morphology to alter plant growth.

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 impact of rhizobial strains on chickpea growth change over time under well-watered and drought conditions? We hypothesise that the effect of rhizobial strains on chickpea growth will vary temporally and be influenced by the water availability as the growth of tomato, barley and *Medicago* change over time with the inoculation of mycorrhizae (Williams et al., 2019).

Second, do genetically diverse chickpea varieties exhibit variations in leaf exudates, biomass and nodule traits in response to different *Mesorhizobium* strains under well-watered and drought conditions? The hypothesis that leaf exudates are an adaptation to drought predicts an increase in leaf exudation with drought. Drought decouples photosynthesis and growth and favours the accumulation of carbon compounds (Granier et al., 2006; Hummel et al., 2010; Muller et al., 2011). In response to short-term drought under controlled conditions, *Thymus vulgaris, T. kotschyanus* and *Aptenia cordifolia* accumulated organic acids including malic, fumaric and citric (Herppich and Peckmann, 1997; Ashrafi et al., 2018).

Third, are bacteroid size and amount of polyhydroxybutyrate modified by *Mesorhizobium* strain, chickpea variety, water availability and their interactions? Although there is no previous evidence that host-imposed bacteroid swelling differs among cultivars of a given plant species, we hypothesised that rhizobia strain, plant variety, or water availability could influence the bacteroid morphology. 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, use of plant C to increase bacteroid size could come at the expense of using that 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).

Materials and Methods

1.1.*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 Australian Plant Phenomics Facility, University of Adelaide, Australia. We selected chickpea varieties that showed contrasting plant biomass under drought: Kyabra, Boundary, Drummond and Pistol (Iqbal et al., 2023a unpublished). 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 0.5g 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 et al., 2023b unpublished) 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).

1.2.*Plant growth and treatments*

Seeds of chickpea were surface sterilised with 75 % ethanol for 2 minutes 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 soil. The soil was free of chickpea rhizobia and sandy (sand: 86 %, silt: 6 % and clay: 8 %) with a pH that ranged from 7.0 to 7.6. 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 after growing them on benches 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) once a week until 28 DAP. 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, Aachen, Germany).

1.3. *Experimental design*

The experiment occupied eight lanes in the southwest smart-house that fitted with conveyor systems and imaging stations (LemnaTec Scanalyzer 3D) for the non-destructive, high-throughput phenotyping of plants. 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 randomized 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 (Brien, 2023a, b), a package for the R statistical computing environment (R Core Team, 2023).

1.4. *Plant growth*

Planting occurred on 19 August 2022, denoted days after planting (DAP) 0. Imaging was carried out daily from DAP 10 to DAP 53 inclusive. The drought treatment (1/3 available soil water represents 33 % pot capacity) for plants in the drought group commenced at DAP 29. 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 trait smooth function from growthPheno, 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 and sPSA RGR on the following points: 10, 15, 20, 29, 32, 41 and 53 DAP.

1.5. *Measurement*

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 efficiency, bacteroid size and polyhydroxybutyrate were also measured (see next section).

1.5.1. *Symbiotic efficiency*

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

$$Symbiotic\ efficiency\ (\%) = \frac{DW\ inoculated}{DW_{N-}} \times 100 \qquad (eq.\ 1)$$

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

1.5.2. *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 3000rpm for 10 minutes 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 10µl of Nile Red stock to cells in one tube (final concentration 100µg/ml) and incubated at room temperature in dark for 1h to estimate polyhydroxybutyrate. Samples were centrifuged at 3000rpm for 10 mins and 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' (585/42nm) channel off blue (488nm) laser.

1.6. *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, 2020) and asremlPlus (Brien, 2023a). The following linear mixed model was fitted to each trait:

$$\mathbf{y} = \mathbf{1}\mu + \mathbf{X}_t\boldsymbol{\tau} + \mathbf{X}_s\boldsymbol{\beta} + \mathbf{Z}\,\mathbf{u} + \mathbf{e},$$

where $\mathbf{y}$ is the response vector of values for the trait being analysed; μ is the overall mean in this experiment for the response; $\boldsymbol{\tau}$ is the vector of fixed effects of interest; $\boldsymbol{\beta}$ is the vector for fixed spatial effects; $\mathbf{u}$ is the vector of random spatial effects allowed for in the design; $\mathbf{1}$ is the vector of ones and the matrices $\mathbf{X}_t$, $\mathbf{X}_s$ and $\mathbf{Z}$ 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 $\boldsymbol{\tau}_t$ of fixed effects of interest is partitioned as $[\boldsymbol{\tau}_V^\top \boldsymbol{\tau}_W^\top \boldsymbol{\tau}_R^\top \boldsymbol{\tau}_{V:W}^\top \boldsymbol{\tau}_{V:R}^\top \boldsymbol{\tau}_{W:R}^\top \boldsymbol{\tau}_{V:W:R}^\top]$, 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 $[\mathbf{u}_{B:L}^\top \ \mathbf{u}_{B:P}^\top]$, where $\mathbf{u}_{B:L}$ and $\mathbf{u}_{B:P}$ 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 population mean equal to zero. Several models assessing individual plant variation were investigated for each trait, namely (i) a single variance, (ii) variances that differed between water regimes and/or nitrogen source control and non-control treatments, and (iii) a smooth spatial surface fitted using tensor-product splines, either natural cubic smoothing splines or P-splines. Firstly, variance models in (i) and (ii) were compared and the model with the smallest Akaike Information Criterion (AIC) was chosen. Secondly, each of the local spatial models in (iii) was fitted and compared to the selected non-spatial model. The model with the smallest AIC was selected as the final model.

Residual-versus-fitted values plots, and normal probability plots of the residuals were inspected to check that the assumptions underlying the analyses are 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 for 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. Least significant differences for $\alpha = 0.05$ [LSD (5 %)] were calculated for determining the significance of pairwise differences between the EMMs.

2. Results

2.1. *Plant growth over time*

Under well-watered conditions, strain CC1192 supported significantly faster plant growth in all four cultivars, relative to M075, but differences were insignificant under drought (Figure 1 1). 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 (Supplementary 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). Well-watered plants inoculated with CC1192 showed projected shoot area of 123 % at 41 DAP and 402 % at 53 DAP compared with water-stressed plants in the negative control (Figure 1). Kyabra inoculated with CC1192 produced projected shoot area of 311 % compared with Drummond as a negative control at 53 DAP (Figure 1).

Relative growth rate of the smoothed projected shoot area was influenced by the main effects of plant variety, water regime and nitrogen source from 41 to 53 DAP (Table S1 and Figure 2).
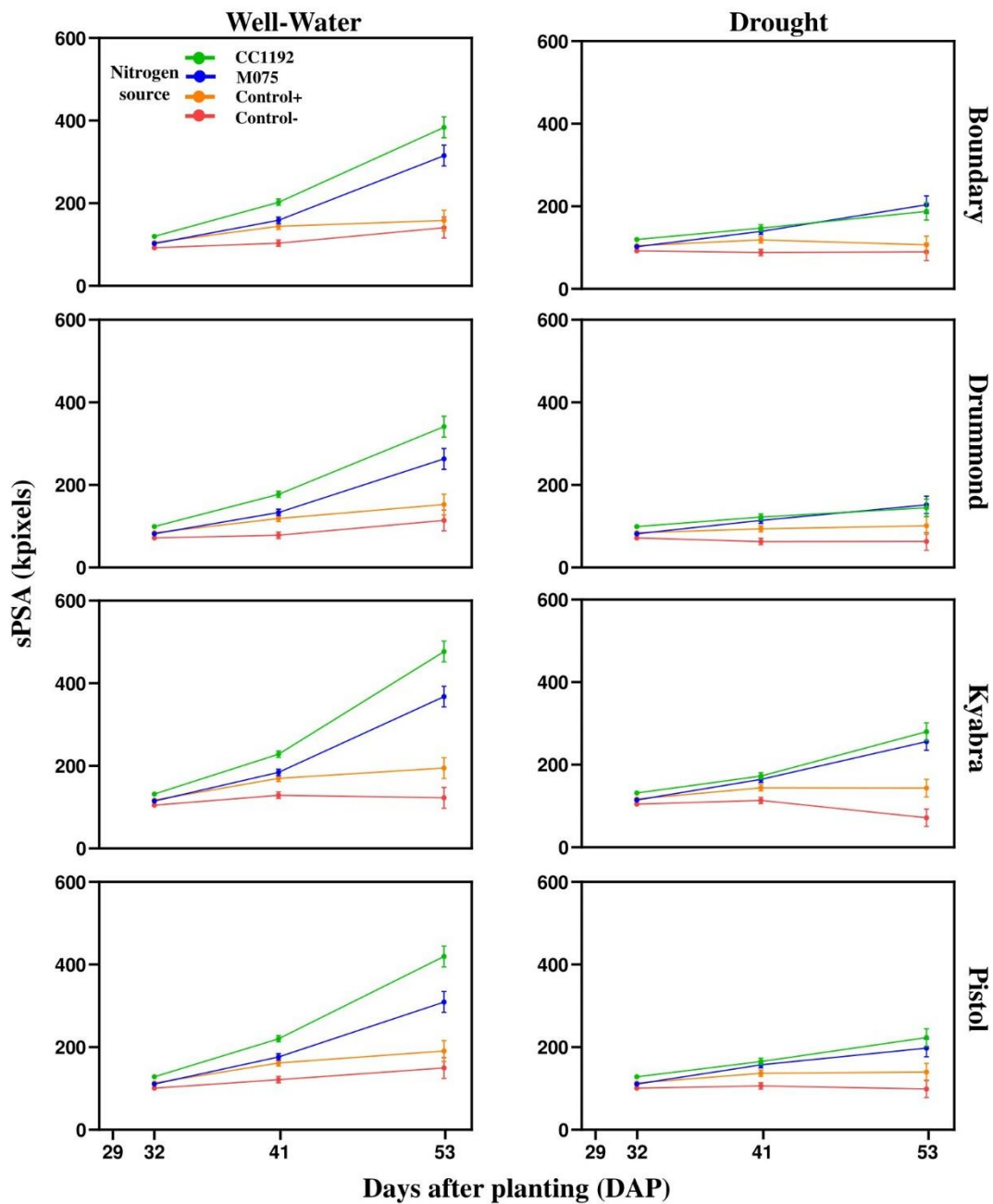
Figure 1. Projected Shoot Area (sPSA) of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192, M075, negative and positive control under well-watered and

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.
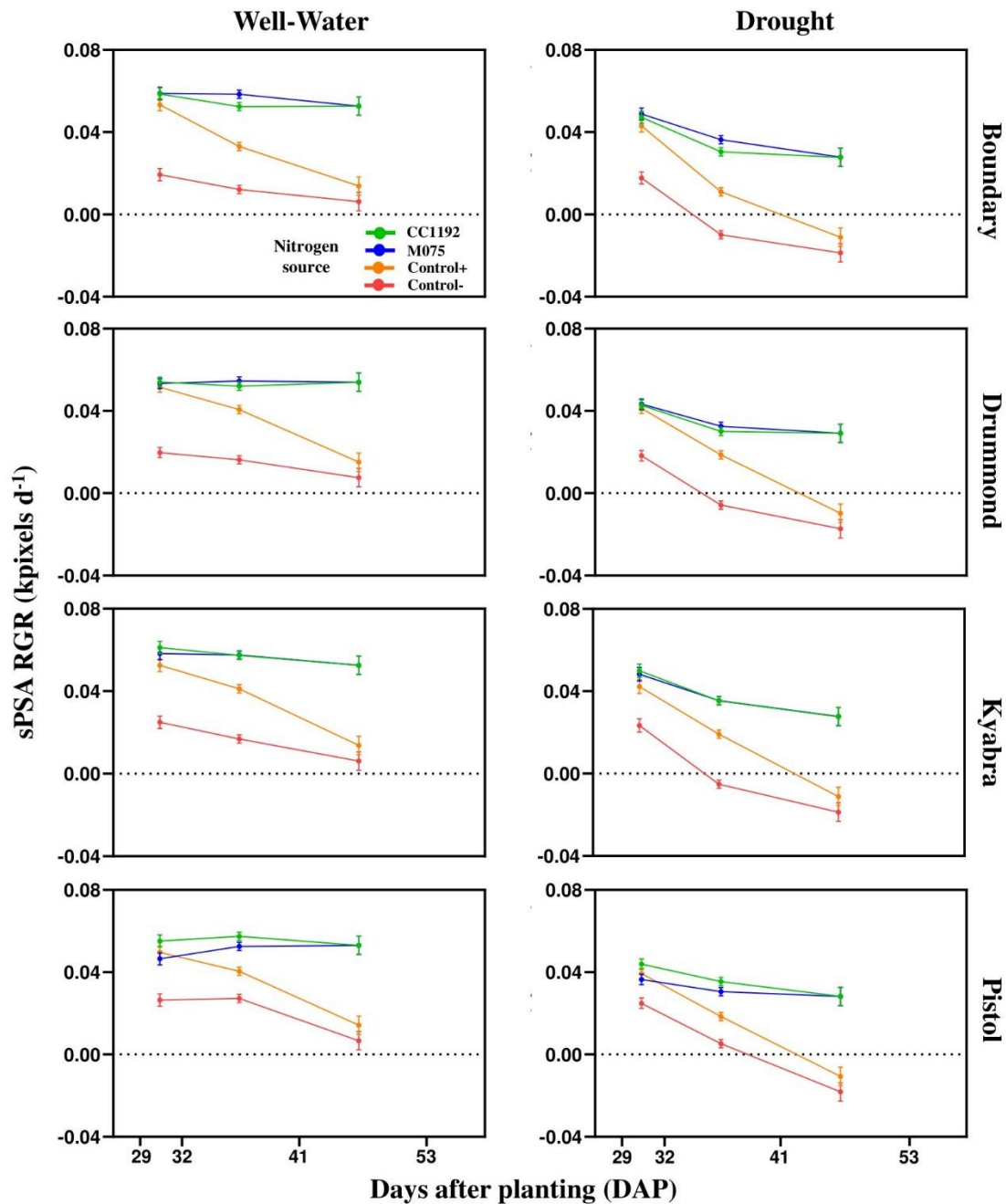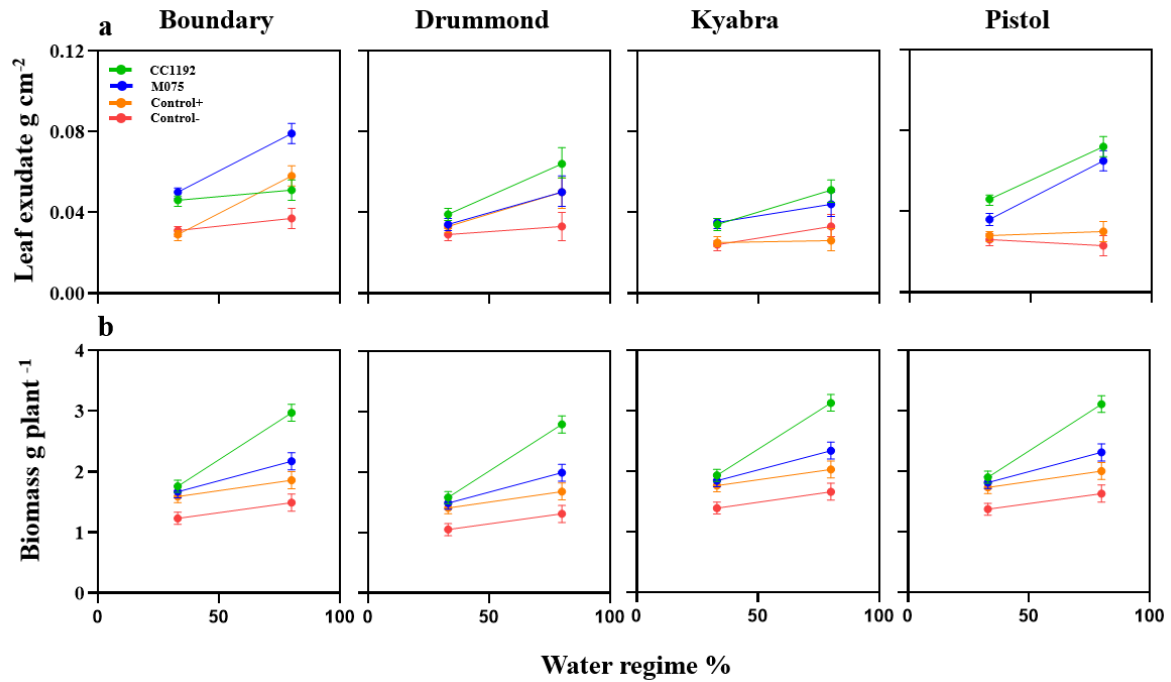

Figure 2. Relative Growth Rates of the smoothed Projected Shoot Area (sPSA RGR) of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192, M075, negative and

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.

2.2. *Variation in leaf exudates and total plant growth at harvest*

In all four cultivars, leaf exudation in the rhizobia-inoculated treatments was reduced under drought compared to well-watered treatment, contradicting the exudate-as-drought-adaptation hypothesis (Figure 3a). In addition, we observed a concurrent decrease in plant biomass under drought stress in comparison to well-watered treatments (Figure 3b). Leaf exudates were reduced from 0.07 g cm^{-2} in well-watered Pistol inoculated with CC1192 to 0.02 g cm^{-2} in water-stressed Kyabra as a negative control (Figure 3a). Plant biomass ranged from 1.2 g plant^{-1} in severe-stressed plants with negative control to 3.0 g plant^{-1} in well-watered plants inoculated with CC1192 (Figure 3b). The three-way interaction between plant variety, water regime and nitrogen source affected the leaf exudates ($p < 0.001$) (Table S1). The interaction of water regime and nitrogen source influenced the total plant biomass ($p < 0.001$) (Table S1).

Figure 3. (a) Leaf exudates and (b) 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.

2.3. *Variation in nodule traits and symbiotic efficiency at harvest*

CC1192's greater benefit to plants (under well-watered conditions, Fig. 1) was not due to greater nodulation, as it made significantly fewer nodules per plant (Fig. 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 (Oono& Denison 2010). 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& Denison 2010, Fig. 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.

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 increased nodules per plant by 54 % in comparison to Kyabra inoculated with CC1192 (Figure 4a). Drummond inoculated with CC1192 increased nodule dry weight by 19 % 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 increased nodules per plant by 143 % in comparison to severe-stressed Drummond (Figure 4a). Nodule dry weight increased in well-watered Drummond by 155 % in comparison to severe-stressed Pistol (Figure 4b).

The interaction of water regime and nitrogen source influenced the symbiotic efficiency ($p = 0.009$) (Table S1). Symbiotic efficiency was reduced from 205 % in well-watered plants inoculated with CC1192 to 141 % in water-stressed plants inoculated with M075 (Figure 4c).
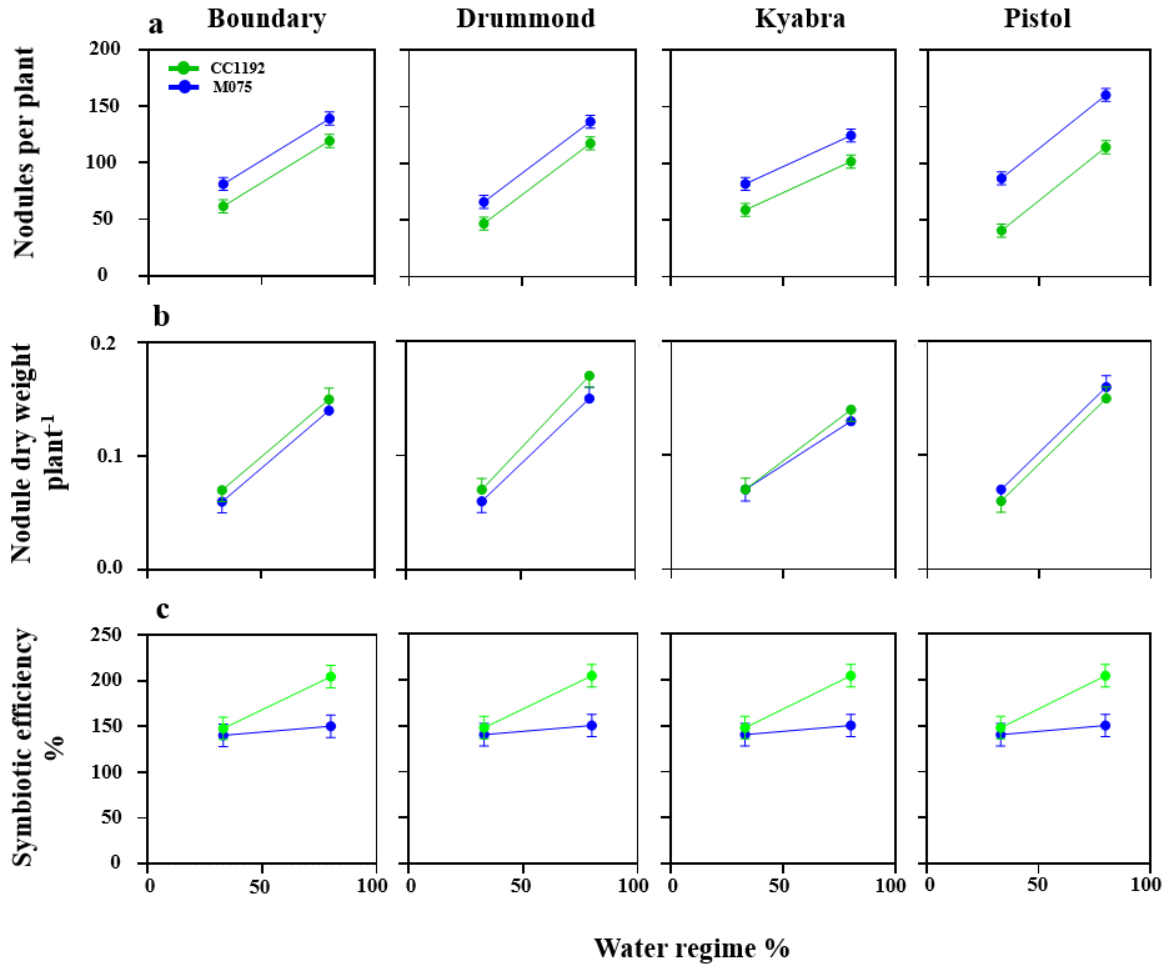
Figure 4. (a) Nodules per plant, (b) nodule dry weight and (c) symbiotic efficiency of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192 and M075 under two water regimes: well-watered control (80 % pot capacity) and drought (1/3 of available soil water represents 33 % pot capacity). Error 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.

2.4. *Variation in bacteroid size and amount of polyhydroxybutyrate*

The bacteroid size was less in all four cultivars under drought in comparison to well-watered treatments (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 increased

bacteroid size by 10 % 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 high 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

Supplementary Table S2).
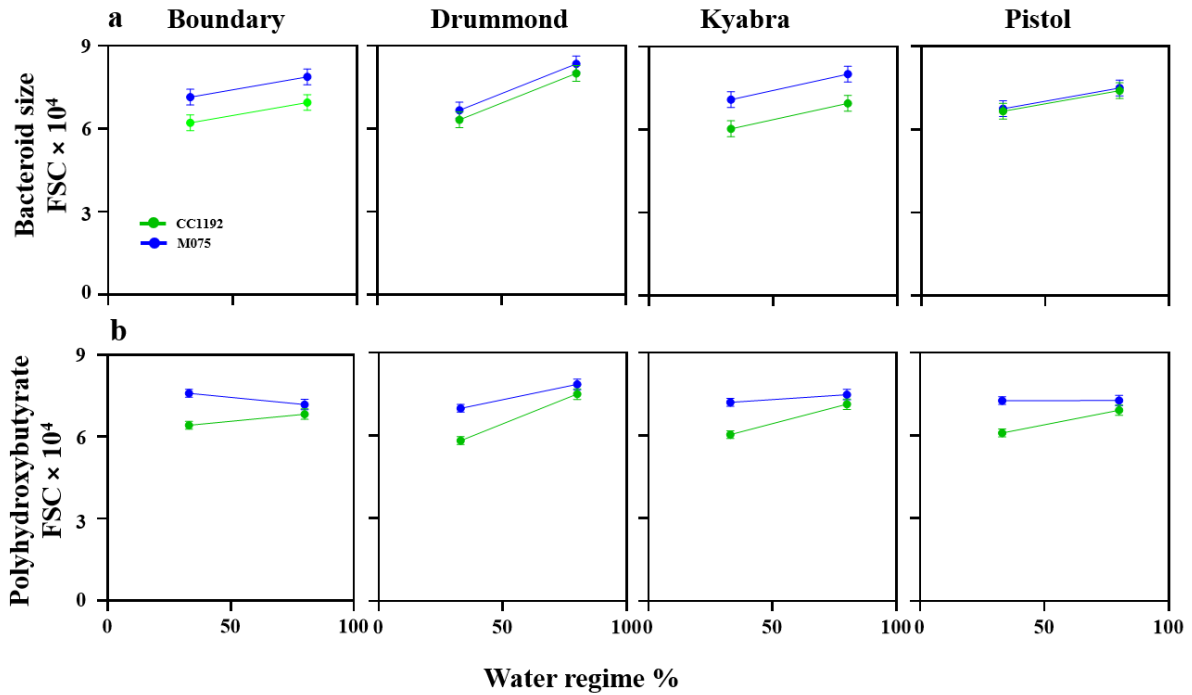
Figure 5. (a) Bacteroid size and (b) the amount of polyhydroxybutyrate of Boundary, Drummond, Kyabra and Pistol inoculated with CC1192 and M075 under two water regimes: well-watered control (80 % pot capacity) and drought (1/3 of available soil water represents 33 % pot capacity). Error bars are an Estimated marginal mean $\pm$ 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.
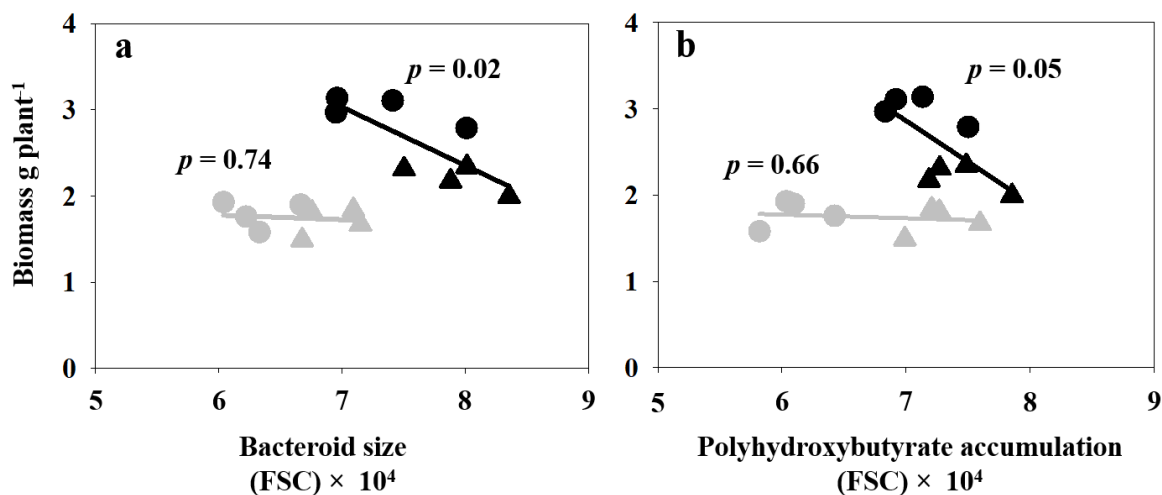
Figure 6. Relationship of plant biomass with (a) bacteroid size and (b) polyhydroxybutyrate accumulation (based on forward and side scatter plots, FSC) of four chickpea varieties inoculated with CC1192 (circle) and M075 (triangle) in response to well-watered (black) and drought (grey).

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3. Discussion

3.1. Chickpea growth responses to M*esorhizobium* strains are temporal under well-watered and drought

The high-throughput phenotyping showed the pattern of chickpea growth in response to *Mesorhizobium* strains, plant variety and water availability. The effect of strain was not significant on the projected shoot area before 41 DAP. At 41 and 53 DAP, projected shoot area patterns diverged between strains depending on water availability (Table S1 and Figure 1). At those points (41 and 53 DAP), chickpea inoculated with CC1192 had a high shoot growth rate compared with other nitrogen sources under well-watered treatments and continued to accumulate high plant biomass and express high symbiotic efficiency at harvest

(Figure 1, 3). This response was particularly in reference to M075 and the negative control. We expected nitrogen-fertilised treatment to produce high shoot growth rate compared with rhizobial and un-inoculated negative control treatments. However, both rhizobial strains showed high shoot growth rate compared to the nitrogen-fertilised treatment presumably due to inadequate nitrogen supplied in the fertilised treatment during the first three weeks of chickpea growth.

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, and accumulated high plant biomass and symbiotic efficiency at harvest (Figure 1, 3). The positive divergence in projected shoot area after 41 and 53 DAP under well-watered conditions can be explained by the relative growth rates of the smoothed projected shoot area between the two strains (Fig 2), indicating higher relative growth rates in the plants inoculated with CC1192 during pre-drought phase (10-29 DAP) (Figure S2). The potential of the commercial strain CC1192 to fix high quantities of nitrogen in the early stages (pre-drought phase) is a possible driver behind the temporal growth response that influences chickpea's capacity to benefit from unfavourable environments later (drought phase). This is consistent with a previous finding that nitrogen fixation significantly affected soybean chlorophyll content and growth during the early stages of development (Kaschuk et al., 2010). However, 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. Thus, the lack of RGR difference between the strains later in growth suggests that early plant growth may depend on some important difference between the strains other than their relative N-fixation contributions. For example, M075's greater number of nodules might result in a greater nodule-construction carbon cost per plant at a time when current C costs have a larger effect than current N benefits.

3.2. **Chickpea varieties vary in leaf exudates, plant biomass and nodule traits in response to different *Mesorhizobium* strains and water regime**

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 (Fig. 4 and 5). CC1192-inoculated plants showed a 1.6-fold increase in plant biomass, while their symbiotic efficiency showed a 1.3-fold improvement compared to plants inoculated with M075 under well-watered conditions (Figure 3b, 4c). These results suggest the potential of commercial strain CC1192 to fix a substantial quantity of nitrogen under well-watered conditions, and are consistent with studies on chickpea-rhizobia and *M. truncatula - S. meliloti* symbiosis under well-watered and drought (Mhadhbi et al. 2009; Esfahani and Mostajeran, 2011).

Robust rhizobial strains persisting 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 indicate 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 that the effectiveness of *Mesorhizobium* strains and chickpea varieties, as reported with common bean-*Rhizobium*, where an isolate of *Rhizobium* spp. induced the highest nodulation with one cultivar, was not consistently observed with the other cultivars (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., 2018; Abdela et al., 2020)..

Leaf exudates play a direct role in plant defence against pests and pathogens (War et al., 2012), and a putative indirect role in mediating interactions with predators and herbivore parasitoids (Dicke et al., 2009). The simplest hypothesis to predict leaf exudates would be that plant growth and leaf exudation depend on the same resources. If resource supply were fixed, this would result in a negative correlation between effects on biomass vs. growth, as seen the contrasting effects of the two rhizobia strains on Boundary under well-watered conditions (Fig. 3). In general, however, Fig. 3 shows a positive correlation between exudates and biomass, consistent with differences in resource availability among plants swamping resource-allocation tradeoffs (Van Noordwijk and de Jong, 1986).

3.3. **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 and

Denison., 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., 2017; Lamouche et al., 2019).

3.4. **Bacteroid size and amount of polyhydroxybutyrate 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 ($R^2 = 0.4$) correlation between cell size and "symbiotic efficiency" 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., 2017). 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).

In conclusion, shoot growth rate responses to *Mesorhizobium* strains undergoes changes 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, water availability but do not determine the symbiotic benefits in the chickpea-*Mesorhizobium* symbiosis. This information advances our understanding of the function of Chickpea-*Mesorhizobium* symbiosis that could lead to development of targeted approaches to promote positive growth responses through rhizobia.

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Declaration of competing interest

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.

Reference

Abdela, A. A., Barka, G. D., & Degefu, T. (2020). Co-inoculation effect of *Mesorhizobium ciceri* and *Pseudomonas fluorescens* on physiological and biochemical responses of *Kabuli* chickpea (*Cicer arietinum* L.) during drought stress. *Plant Physiology Reports*, *25*, 359-369.

Al-Tamimi, N., Brien, C., Oakey, H., Berger, B., Saade, S., Ho, Y. S., ... & Negrão, S. (2016). Salinity tolerance loci revealed in rice using high-throughput non-invasive phenotyping. *Nature communications*, *7*, 13342.

Argaw, A., Muleta, D. (2018). Effect of genotypes-*Rhizobium*-environment interaction on nodulation and productivity of common bean (*Phaseolus vulgaris* L.) in eastern Ethiopia. *Environmental System Research,* 6, 1-16.

Biabani, A., Carpenter-Boggs, L., Coyne, C. J., Taylor, L., Smith, J. L., & Higgins, S. (2011). Nitrogen fixation potential in global chickpea mini-core collection. *Biology and Fertility of Soils*, *47*, 679-685.

Brien, C., Jewell, N., Garnett, T., Watts-Williams, S. J., & Berger, B. (2021). Smoothing and extraction of traits in the growth analysis of noninvasive phenotypic data. *Plant Methods*, 16, 1-21.

Brien, C. J. (2023a) asremlPlus: Augments ASReml-R in fitting mixed models and packages generally in exploring prediction differences. Version 4.3-47.

Brien, C. J. (2023b) dae: Functions useful in the design and ANOVA of experiments. Version 3.2-14.

Brien, C. J. (2023c) growthPheno: Functional analysis of phenotypic growth data to smooth and extract traits. Version 2.1-17.

Butler, D. G., Cullis, B. R., Gilmour, A. R., Gogel, B. J., & Thompson, R. (2021). ASReml-R reference manual, Version 4.

Chen, W. F., Meng, X. F., Jiao, Y. S., Tian, C. F., Sui, X. H., Jiao, J., ... & Ma, S. J. (2023). Bacteroid development, transcriptome, and symbiotic nitrogen-fixing comparison of *Bradyrhizobium arachidis* in nodules of peanut (*Arachis hypogaea*) and medicinal legume *Sophora flavescens*. *Microbiology Spectrum*, 11, e01079-22.

Chtouki, M., Laaziz, F., Naciri, R., Garré, S., Nguyen, F., & Oukarroum, A. (2022). Interactive effect of soil moisture content and phosphorus fertilizer form on chickpea growth, photosynthesis, and nutrient uptake. *Scientific Reports*, *12*, 6671.

Dicke, M., Van Loon, J. J., & Soler, R. (2009). Chemical complexity of volatiles from plants induced by multiple attack. *Nature chemical biology*, 5, 317-324.

Dimkpa, C., Weinand, T., & Asch, F. (2009). Plant–rhizobacteria interactions alleviate abiotic stress conditions. *Plant, cell & environment*, *32*, 1682-1694.

Esfahani, M. N., Mostajeran, A. (2011). Rhizobial strain involvement in symbiosis efficiency of chickpea–rhizobia under drought stress: plant growth, nitrogen fixation and antioxidant enzyme activities. *Acta Physiologiae Plantarum,* 33,1075–1083.

Farkas, A., Maróti, G., Dürgő, H., Györgypál, Z., Lima, R. M., Medzihradszky, K. F., ... & Kondorosi, É. (2014). *Medicago truncatula* symbiotic peptide NCR247 contributes to bacteroid differentiation through multiple mechanisms. *Proceedings of the National Academy of Sciences*, 111, 5183-5188.

Greenlon, A., Chang, P. L., Damtew, Z. M., Muleta, A., Carrasquilla-Garcia, N., Kim, D., Cook, D. R. (2019). Global-level population genomics reveals differential effects of geography and phylogeny on horizontal gene transfer in soil bacteria. *Proceedings of the National Academy of Sciences* 116, 15200-15209.

Hardarson, G., Danso, S. K. A. (1993). Methods for measuring biological nitrogen fixation in grain legumes. *Plant and soil* 152, 19-23.

Iqbal N., Rahman, M. M., Cawthray R. G., Zhou Y., Denton D. M., Sadras O. V. (2023a). Drought and herbivory differentially modulate the leaf exudation of organic acids in chickpea. Unpublished.

Iqbal N., Rahman, M. M., Cawthray R. G., Zhou Y., Denton D. M., Sadras O. V. (2023b). Mesorhizobia strain and chickpea variety drive phenotypic plasticity of plant growth and nodulation. Unpublished.

Kaschuk, G., Hungria, M., Leffelaar, P. A., Giller, K. E., & Kuyper, T. W. (2010). Differences in photosynthetic behaviour and leaf senescence of soybean (*Glycine max* [L.] Merrill) dependent on N2 fixation or nitrate supply. *Plant Biology*, *12*, 60-69.

Kibido, T., Kunert, K., Makgopa, M., Greve, M., & Vorster, J. (2020). Improvement of *rhizobium*-soybean symbiosis and nitrogen fixation under drought. *Food and Energy Security*, *9*, e177.

Kunert, K. J., Vorster, B. J., Fenta, B. A., Kibido, T., Dionisio, G., & Foyer, C. H. (2016). Drought stress responses in soybean roots and nodules. *Frontiers in plant science*, 7, 1015.

Lamouche, F., Bonadé-Bottino, N., Mergaert, P., & Alunni, B. (2019). Symbiotic efficiency of spherical and elongated bacteroids in the *Aeschynomene-Bradyrhizobium* symbiosis. *Frontiers in Plant Science*, 10, 377.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., ... & González, E. M. (2009). Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in *Medicago truncatula* under drought and recovery. *Molecular plant-microbe interactions*, 22, 1565-1576.

LaRue, T. A., Patterson, T. G. (1981). How much nitrogen do legumes fix?. *Advances in Agronomy,* 34, 15-38.

Lauter, D. J., & Munns, D. N. (1986). Water loss via the glandular trichomes of chickpea (*Cicer arietinum* L.). *Journal of experimental botany*, 37, 640-649.

López-Bellido, R. J., López-Bellido, L., Benítez-Vega, J., Muñoz-Romero, V., López-Bellido, F. J., Redondo, R. (2011). Chickpea and faba bean nitrogen fixation in a Mediterranean rainfed Vertisol: Effect of the tillage system. *European Journal of Agronomy* 34, 222-230.

Mandal, D., & Sinharoy, S. (2019). A toolbox for nodule development studies in chickpea: a hairy-root transformation protocol and an efficient laboratory strain of *Mesorhizobium* sp. *Molecular Plant-Microbe Interactions*, *32*, 367-378.

Maqbool, M. A., Aslam, M., & Ali, H. (2017). Breeding for improved drought tolerance in Chickpea (*Cicer arietinum* L.). *Plant Breeding*, *136*, 300-318.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., ... & Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the *Rhizobium–*legume symbiosis. *Proceedings of the National Academy of Sciences*, *103*, 5230-5235.

Mhadhbi, H., Jebara, M., Limam, F., & Aouani, M. E. (2004). Rhizobial strain involvement in plant growth, nodule protein composition and antioxidant enzyme activities of chickpea-rhizobia symbioses: modulation by salt stress. *Plant Physiology and Biochemistry*, 42, 717-722.

Mhadhbi, H., Jebara, M., Zitoun, A., Limam, F., & Aouani, M. E. (2008). Symbiotic effectiveness and response to mannitol-mediated osmotic stress of various chickpea–rhizobia associations. *World Journal of Microbiology and Biotechnology*, 24, 1027-1035.

Mhadhbi, H., Fotopoulos, V., Djebali, N., Polidoros, A. N., & Aouani, M. E. (2009). Behaviours of *Medicago truncatula–Sinorhizobium meliloti* symbioses under osmotic stress

in relation with the symbiotic partner input: effects on nodule functioning and protection. *Journal of Agronomy and Crop Science*, 195, 225-231.

Mnasri, B., Mrabet, M., Laguerre, G., Aouani, M. E., & Mhamdi, R. (2007). Salt-tolerant rhizobia isolated from a Tunisian oasis that are highly effective for symbiotic N 2-fixation with *Phaseolus vulgaris* constitute a novel biovar (bv. mediterranense) of *Sinorhizobium meliloti*. *Archives of Microbiology*, 187, 79-85.

Montiel, J., Szűcs, A., Boboescu, I. Z., Gherman, V. D., Kondorosi, E., & Kereszt, A. (2016). Terminal bacteroid differentiation is associated with variable morphological changes in legume species belonging to the inverted repeat-lacking clade. *Molecular Plant-Microbe Interactions*, *29*, 210-219.

Neila, A., Adnane, B., Mustapha, F., Manel, B., Imen, H., Boulbaba, L., Cherki, G., Bouaziz, S. (2014). *Phaseolus vulgaris*-rhizobia symbiosis increases the phosphorus uptake and symbiotic N fixation under insoluble phosphorus. *Journal of Plant Nutrition*, 37, 643–657.

Rangarajan, A. A., Koropatkin, N. M., & Biteen, J. S. (2020). Nutrient-dependent morphological variability of Bacteroides thetaiotaomicron. *Microbiology*, 166, 624-628.

Searle, S. R., Speed, F. M., & Milliken, G. A. (1980). Population marginal means in the linear model: an alternative to least squares means. *The American Statistician*, 34, 216-221.

Sen, D., & Weaver, R. W. (1984). A basis for different rates of N2-fixation by the same strains of *Rhizobium* in peanut and cowpea root nodules. *Plant Science Letters*, *34*, 239-246.

Singh, M., Kumar, J., Singh, S., Singh, V. P., & Prasad, S. M. (2015). Roles of osmoprotectants in improving salinity and drought tolerance in plants: a review. *Reviews in Environmental Science and Bio/Technology*, *14*, 407-426.

Tejera, N. A., Campos, R., Sanjuan, J., & Lluch, C. (2004). Nitrogenase and antioxidant enzyme activities in *Phaseolus vulgaris* nodules formed by Rhizobium tropici isogenic strains with varying tolerance to salt stress. *Journal of Plant Physiology*, 161, 329-338.

Tena, W., Wolde-Meskel, E., & Walley, F. (2016). Response of chickpea (*Cicer arietinum* L.) to inoculation with native and exotic *Mesorhizobium* strains in Southern Ethiopia. *African Journal of Biotechnology*, *15*, 1920-1929.

R Core Team (2023). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Ratcliff, W. C., Kadam, S. V., & Denison, R. F. (2008). Poly-3-hydroxybutyrate (PHB) supports survival and reproduction in starving rhizobia. *FEMS Microbiology Ecology*, *65*, 391-399.

Romdhane, S. B., Trabelsi, M., Aouani, M. E., De Lajudie, P., & Mhamdi, R. (2009). The diversity of rhizobia nodulating chickpea (*Cicer arietinum*) under water deficiency as a source of more efficient inoculants. *Soil Biology and Biochemistry*, *41*, 2568-2572.

Oono, R., Denison, R. F., & Kiers, E. T. (2009). Controlling the reproductive fate of rhizobia: how universal are legume sanctions?. *New Phytologist*, 183, 967-979.

Oono, R., Schmitt, I., Sprent, J. I., & Denison, R. F. (2010). Multiple evolutionary origins of legume traits leading to extreme rhizobial differentiation. *New Phytologist*, 187, 508-520.

Oono, R., & Denison, R. F. (2010). Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant physiology*, *154*, 1541-1548.

Upadhyaya, H. D., Kashiwagi, J., Varshney, R. K., Gaur, P. M., Saxena, K. B., Krishnamurthy, L., ... & Singh, I. P. (2012). Phenotyping chickpeas and pigeonpeas for adaptation to drought. *Frontiers in physiology*, *3*, 179.

Van de Velde, W., Zehirov, G., Szatmari, A., Debreczeny, M., Ishihara, H., Kevei, Z., ... & Mergaert, P. (2010). Plant peptides govern terminal differentiation of bacteria in symbiosis. *Science*, *327*, 1122-1126.

Van Noordwijk, A. J., & De Jong, G. (1986). Acquisition and allocation of resources: their influence on variation in life history tactics. *The American Naturalist*, 128, 137-142.

Vincent, J. M. (1970). A manual for the practical study of the root-nodule bacteria. A manual for the practical study of the root-nodule bacteria.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., & Sharma, H. C. (2012). Mechanisms of plant defense against insect herbivores. *Plant signaling & behavior*, 7, 1306-1320.

Zaw, M., Rathjen, J. R., Zhou, Y., Ryder, M. H., Denton, M. D. (2021). Symbiotic effectiveness, ecological adaptation and phylogenetic diversity of chickpea rhizobia isolated from a large-scale Australian soil collection. *Plant Soil* 469, 49-71.

Zaw, M. (2022). Symbiotic effectiveness, phylogenetic diversity and ecological adaptation of chickpea rhizobia isolated from Australian and Myanmar soils (Doctoral dissertation).

Supplementary data

Table S1. The *p*-values for the Wald *F*-statistics that test for seven treatment sources, comprising the four interactions of the three treatment factors (Variety, Water regime, Nitrogen source) plus their three main effects. Statistically significant terms ($p \leq 0.05$) are flagged with an asterisk (*). Within any trait, "na" indicates that the test for that term is not applicable because of a significant higher-order term (interaction) involving the factor(s) in the na term.

Trait	V:W:N	V:W	V:N	W:N	V	W	N
sPSA 10	0.705	0.296	0.803	0.164	* <0.001	0.772	* 0.010
sPSA 15	0.738	0.461	0.615	0.452	* <0.001	0.716	* 0.016
sPSA 20	0.583	0.837	0.465	0.436	* <0.001	0.479	* 0.020
sPSA 29	0.516	0.959	0.614	0.16	* <0.001	0.441	* <0.001
sPSA 32	0.483	0.968	0.493	0.193	* <0.001	0.958	* <0.001
sPSA 41	0.716	0.849	0.075	* 0.005	* <0.001	na	na
sPSA 53	0.537	0.879	* 0.006	* <0.001	na	na	na
sPSA RGR 10-15	0.533	0.308	0.105	0.441	* <0.001	0.511	0.098
sPSA RGR 15-20	0.498	0.113	0.446	0.36	* <0.001	0.245	* 0.002
sPSA RGR 20-29	0.84	0.63	0.104	0.623	* <0.001	0.852	* <0.001
sPSA RGR 29-32	0.693	0.893	* <0.001	* 0.033	na	na	na
sPSA RGR 32-41	0.274	0.449	* <0.001	0.203	na	* <0.001	na
sPSA RGR 41-53	0.157	0.061	0.168	0.079	* 0.002	* <0.001	* <0.001

Leaf exudates	* <0.001	na	na	na	na	na	na
Plant biomass	0.865	0.525	0.409	* <0.001	* <0.001	na	na
Symbiotic efficiency	0.997	0.25	0.779	* 0.009	0.207	na	na
Nodule per plant	0.098	* <0.001	* 0.004	0.171	na	na	na
Nodule dry weight	0.056	* <0.001	* 0.005	0.107	na	na	na
Bacteroid size	0.122	* 0.012	* 0.024	0.339	na	na	na
Polyhydroxybutyrate	0.719	* 0.003	0.45	* 0.001	na	na	na

Table S2. Matrix of Pearson's correlation coefficients (r) for plant biomass, symbiotic efficiency and nodule traits of four chickpea varieties inoculated with CC1192 and M075 under well-watered and drought. Shading indicates the strength of correlations, with darker shading denoting stronger positive (blue) or negative (red) correlations.

		Bacteroid size	Polyhydroxybutyrate	Plant biomass	Symbiotic efficiency	Nodules per plant
Well-watered	Polyhydroxybutyrate	0.86				
	Plant biomass	-0.78	-0.71			
	Symbiotic efficiency	-0.63	-0.56	0.94		
	Nodules per plant	0.40	0.31	-0.78	-0.79	
	Nodule dry weight	0.06	0.00	0.06	0.24	0.33
Drought	Polyhydroxybutyrate	0.82				
	Plant biomass	-0.14	-0.18			
	Symbiotic efficiency	-0.81	-0.94	0.31		
	Nodules per plant	0.61	0.91	-0.02	-0.86	
	Nodule dry weight	-0.57	-0.45	0.39	0.42	-0.05
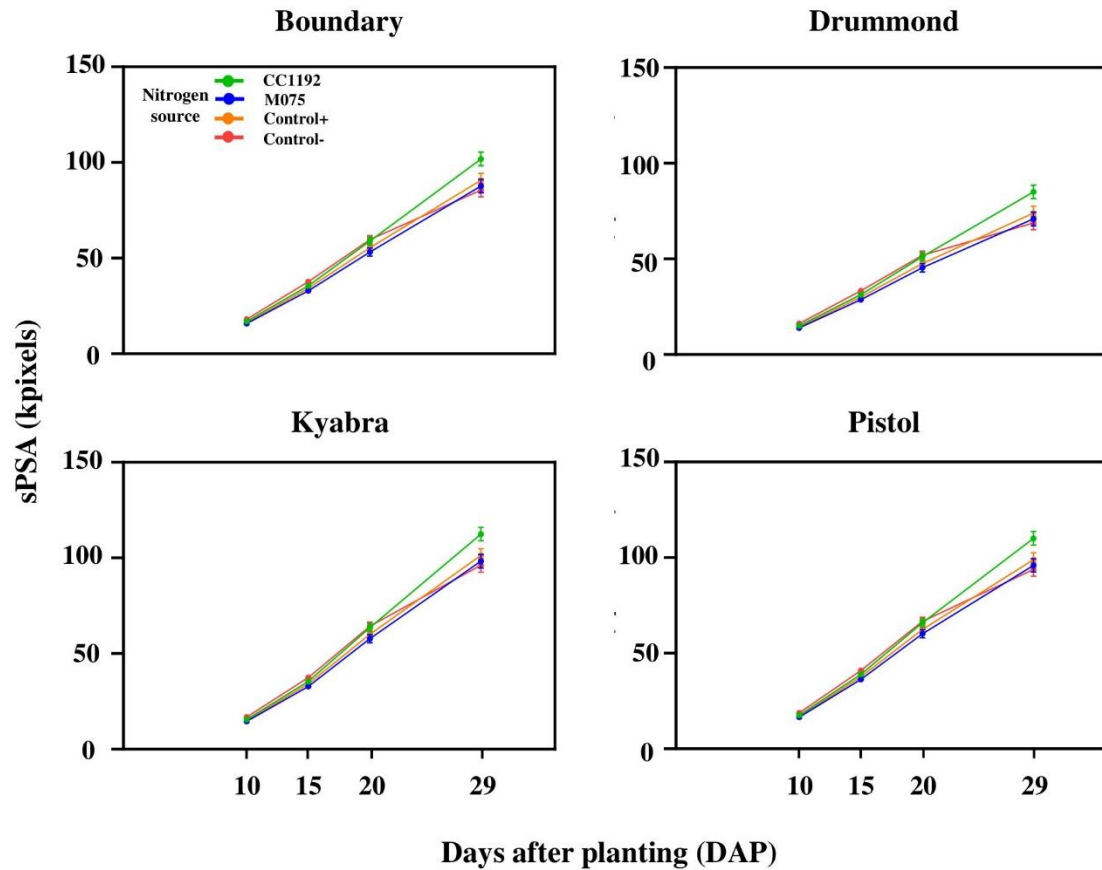
Figure S1. Estimated marginal means (EMMs) for the Smoothed Projected Shoot Area (sPSA) during the pre-drought phase (up to day 29 after planting) for four chickpea varieties and four nitrogen source treatments. Error bars are an EMM $\pm$ half-LSD (5 %). See Table S1 for statistical results. Two EMMs from the same panel and time-point are significantly different if their error bars do not overlap.
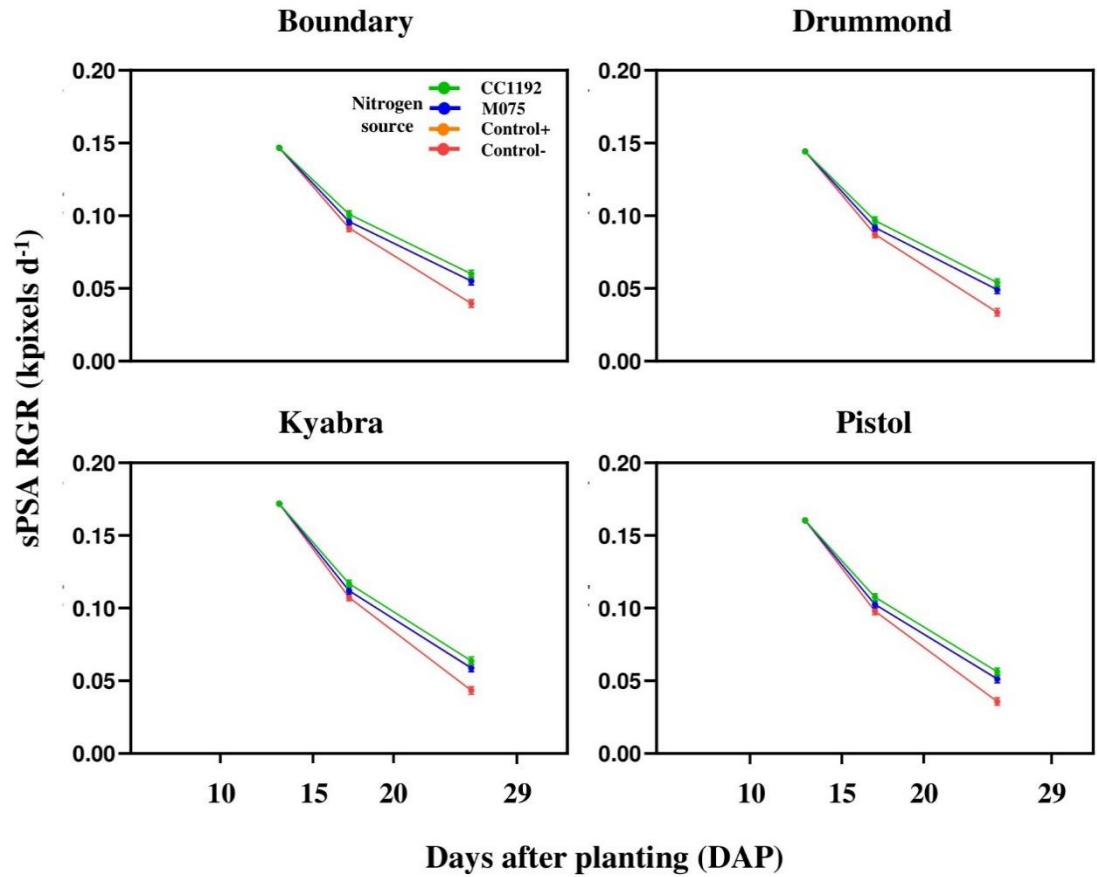
Figure S2. Estimated marginal means (EMMs) for the Relative Growth Rates of the smoothed Projected Shoot Area (sPSA RGR) during the pre-drought phase (up to day 29 after planting) for four chickpea varieties and four nitrogen source treatments. Error bars are an EMM $\pm$ half-LSD (5 %). See Table S1 for statistical results. Two EMMs from the same panel and time-point are significantly different if their error bars do not overlap.

CHAPTER 5: Drought and herbivory differentially modulate the leaf exudation of organic acids in chickpea

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Overall percentage (%)	85%		
Certification:	This paper reports on original research I conducted during the period of my Higher Degree by Research candidature and is not subject to any obligations or contractual agreements with a third party that would constrain its inclusion in this thesis. I am the primary author of this paper.		
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Co-Author Contributions

By signing the Statement of Authorship, each author certifies that:

    i.   the candidate's stated contribution to the publication is accurate (as detailed above);

    ii.   permission is granted for the candidate in include the publication in the thesis; and

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Drought and herbivory differentially modulate the leaf exudation of organic acids in chickpea

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Abstract

Abstract

Purpose: Drought and herbivory challenge chickpea growth and yield. Consequently, we designed two glasshouse experiments to test four hypotheses: (1) drought increases leaf exudation of organic acids; (2) herbivory increases leaf exudation of organic acids; (3) drought modulates leaf exudation of organic acids in response to herbivory; and (4) there is a trade-off between growth and defence associated with leaf exudation of organic acids.

Methods: Experiment 1 combined factorially twelve varieties and three water regimes. Experiment 2 combined factorially six varieties, two water regimes, and two herbivory treatments.

Results: Drought decreased the amount of leaf exudates by 71 % and growth traits by 15 to 48 %. Herbivory increased the amount of leaf exudates by 60 % and reduced shoot biomass by 32 % in well-watered plants, and increased exudates by 49 % and reduced shoot biomass by 38 % under severe water stress. Leaf damage, and survival and size of *H. armigera* larvae were larger in water-stressed plants, and correlated negatively with the leaf exudates in both water regimes. Our study did not support a trade-off between growth and defence: in experiment 1, there was no trade-off between exudates and growth traits in 25 out of 27 cases resulting from the combination of nine traits and three water regimes; weak trade-offs in 2 out of 27 cases were apparent under water stress; in experiment 2, growth and exudates were unrelated.

Conclusions: The complex relationship between drought, herbivory, and plant defence underscores the need for a nuanced understanding of crop management.

Keywords: defence, leaf exudates, oxalic acid, malic acid, photosynthesis, trade-off

1. Introduction

Drought and cotton bollworm (*Helicoverpa armigera*) both reduce chickpea yield globally (Molina et al., 2008; Chrigui et al., 2020). Insecticides are widely used for controlling *H. armigera*, with side effects including development of resistance in pest populations, damage to non-target organisms, and chemical residues in soil and water (Kranthi et al. 2002). In this context, plant resistance to *H. armigera* is advantageous.

Chickpea (*Cicer arietinum*) secretes organic acids from roots (Veneklaas et al. 2003), and green tissues (Toker et al., 2004) in response to pests, pathogens and beneficial microbes. Leaf organic acids play a defence role against *H. armigera* (Chrigui et al., 2020). Intra-specific variation in insect resistance has been reported that is partially correlated with the amount of leaf organic acid exudates (Srivastava et al., 1989; Bhagwat et al., 1995; Yoshida et al., 1995; Yoshida et al., 1997; San et al., 2022). Oxalic acid incorporated into an artificial diet inhibited the growth of *H. armigera* larvae (Yoshida et al., 1995). A large variation in the concentration of oxalic and malic acids was found on chickpea leaves challenged with *H. armigera* (Yoshida et al., 1995; Salimath et al., 2003). Similarly, increased concentration of oxalic acid provide defence against herbivores in cobra lily (*Arisaema species*), Napier grass (*Pennisetum purpurea*) and cacti (*Opuntia* Sp.) (Sidhu et al., 1996; Monje and Baran, 2022).

Drought decouples photosynthesis and growth, leading to an accumulation of carbon compounds (Granier et al., 2006; Hummel et al., 2010; Muller et al., 2011). In response to short-term drought under controlled conditions, *Thymus vulgaris, T. kotschyanus* and *Aptenia cordifolia* accumulated leaf organic acids including malic, fumaric and citric (Herppich and Peckmann, 1997; Ashrafi et al., 2018). The hypothesis that leaf exudates are an adaptation to drought predicts an increase in leaf exudation during drought. Consequently, these organic acids can partially mediate the interaction between drought and herbivory. Previous studies on the interaction between drought and herbivory have tested the two opposing hypotheses: the plant stress hypothesis and the plant vigour hypothesis. The plant stress hypothesis suggests that herbivory benefits from drought-stressed plants due to a trade-off between plant defence and growth as plants divert resources from costly defence mechanisms to enhance survival under drought (Gutbrodt et al., 2011). On the other hand, the plant vigour hypothesis proposes that reduced plant performance resulting from drought has a negative impact on herbivore fitness (Price, 1991). Low water availability decreases photosynthetic rate (Iqbal et

al., 2019), leaf turgor (Gutbrodt et al., 2011), and above-ground biomass (Iqbal et al., 2018), which is predicted to have a negative impact on insects that preferentially feed on fast-growing vigorous plants (Grinnan et al., 2013a).

A trade-off between plant defence and growth might compromise fitness in the absence of herbivores (Baldwin, 1998; Ullmann-Zeunert et al., 2013). It has been argued that elements of the shade-avoidance syndrome, including increased elongation, apical dominance, reduced leaf mass per area, and allocation to roots, are in direct conflict with configurational changes involved in plant defence (Ballare and Austin 2019). In response to intra-specific competition, *Arabidopsis thaliana* upregulated genes associated with photosynthesis and downregulated genes associated with defence under the experimental conditions of Geisler et al. 2012. The growth-defence trade-off involves both carbon and nitrogen; nitrogen allocation in *Nicotiana attenuata* under simulated herbivory was directed to defence-related metabolites, e.g., phenolamides, at the expense of soluble proteins that are critical for carbon assimilation and growth (Ullmann-Zeunert et al., 2013). Induced defences involving organic acids in plants might have a lower cost than constitutive defences (Karban et al., 1997; Hummel et al., 2010). In response to herbivory, chickpea increased defence compounds, including phenols and malic acid, and reduced sugar contents in leaves (Karthik and Vastrad, 2022).

With a focus on chickpea and *H. armigera*, we report results from two experiments designed to test four hypotheses: (1) drought increases leaf exudation of organic acids; (2) herbivory increases leaf exudation of organic acids; (3) drought modulates leaf exudation of organic acids in response to herbivory; and (4) there is trade-off between growth traits and defence associated with leaf exudation of organic acids.

2. Materials and Methods

2.1. *Overview*

Two experiments were established in a glasshouse at The University of Adelaide, Australia, to test the four hypotheses. Plants were grown under natural sunlight and photoperiod conditions. Radiation and temperature were recorded with Hobo Pendant Temp-Light Data Loggers (OneTemp Pty Ltd., Bourne, MA 02532, USA). In both experiments, seeds were sourced from Agriculture Victoria Research, and surface sterilised with 75 % ethanol for 2 minutes, washed several times with distilled water before sowing. Four seeds per pot were sowed and thinned to two plants per pot 7 days after sowing. Pots (17 cm diameter, 10 cm depth) were filled with sandy loam soil, and plants were supplied McKnight's nutrient solution weekly (McKnight, 1949), and tap water as needed to maintain the water regimes outlined below. The experimental design was a completely randomized block design with three (exp. 1) or four (exp. 2) replicates.

2.1.1. Experiment 1: genotype-dependent response to drought

We established a factorial experiment combining twelve varieties and three water regimes. Varieties were HatTrick, Sonali, Seamer, Genesis 838, Drummond, Pistol, Striker, Kyabra, Slasher, Boundary, Captain and Howzat, that represent the pool of commonly used varieties in current cropping systems of Australia. Soil water content was measured gravimetrically; pots were maintained at well-watered conditions until 23 days after sowing (DAS). Afterwards, three water regimes were established: well-watered control (~80 % pot capacity), 2/3 of available soil water (~66 % pot capacity) and 1/3 of available soil water (~33 % pot capacity). The experiment was harvested on 15 June 2021 when plants reached the late flowering stage. Daily maximum photosynthetic photon flux density ranged from 1019 to 203

µmol m^{-2} s^{-1} and photoperiod from 12 h to 14 h. Maximum temperature averaged 32.9 ± 1.9 and minimum temperature averaged 15.3 ± 1.5 °C (Figure S1 a, b).

2.1.2. **Experiment 2: interaction between drought and herbivory**

We selected six varieties that showed contrasting leaf exudates under drought in experiment 1: Drummond, Howzat, Kyabra, Genesis836, Captain and Slasher. Water regimes included a well-watered control (~80 % pot capacity) and 1/3 of available soil water (~33 % pot capacity) applied at 28 DAS. At 36 DAS, we established two herbivore treatments: fifteen *H. armigera* 2nd instar larvae per plant, and untreated controls. The decision to use fifteen larvae per plant was based on a previous study that demonstrated optimal leaf damage in chickpea occurred within a range of ten to fifteen *H. armigera* larvae (Sharma et al., 2005). Plants were placed in transparent nets (base diameter 45 cm, top diameter 45 cm, height 65 cm) (Cunningham et al., 1998). The experiment ended on 5 June 2022 when plants reached the late flowering stage. The larvae were provided by the Commonwealth Scientific and Industrial Research Organisation, Narrabri, Australia. Daily maximum photosynthetic photon flux density ranged from 1452 to 172 µmol m^{-2} s^{-1}. The maximum temperature averaged 34.4 ± 3.0 and minimum temperature averaged 13.1 ± 2.3 °C (Figure S1 c, d).

2.2. **Measurements**

2.2.1. Leaf exudates

In both experiments, we measured leaf exudates from fully matured leaves using the approach of Yoshida et al. (1997) with slight modifications,

$$\text{Leaf exudates (g cm}^{-2}) = (FW_L - WP_L)/LA \qquad \text{(eq. 1)}$$

where FW_L is leaf fresh weight, WP_L is the weight of tissue-wiped leave, and LA is the leaf area. We determined the leaf area using a scanner (Canoscan 9000F Canon Inc.) and Image J software.

In experiment 2 we measured the organic acids from water-washed extracts of mature leaves through high-performance liquid chromatography (Cawthray, 2003; Uloth et al., 2015). Leaves were excised from plant at harvest and placed into test tubes containing 5 ml distilled water and vortexed for 1 minute. The leaves were removed from test tubes, oven-dried at 70 °C for 48 h, and weighed. Leaf-washed extract was freeze-dried and sent to the laboratory for analysis. The freeze-dried samples were redissolved in 2.5 ml of mobile phase, filtered through a 0.2 µm filter and injected into high-performance liquid chromatography (Prevail organic acid column 250×4.6 mm with 5 µm particle size, Grace, USA). The mobile phase consisted of 25 mM potassium phosphate (pH 2.50) and flow rate was 1 ml min^{-1}. We evaluated oxalic and malic acids, as they were consistently detected in all samples. The amounts of oxalic and malic acids were calculated from linear calibration curves based on peak area. Positive identification of malic and oxalic acids was based on retention time, and photodiode array (Waters) spectral properties of peaks compared to standards and expressed in µmol g^{-1} on dry weight basis.

2.2.2. Plant and insect traits

In experiment 1, we used a portable photosynthesis system (CIRAS-3, Amesbury, USA) to measure net photosynthesis (µmol CO_2 m^{-2} s^{-1}), transpiration (mmol H_2O m^{-2} s^{-1}), stomatal conductance (mmol H_2O m^{-2} s^{-1}), and sub-stomatal CO_2 concentration (µmol mol^{-1}) on the day of harvest. The system was set at 1000 µ mol m^{-2} s^{-1} light source, 100 µmol mol^{-1} analyser flow, 50 % stomatal ratio, and 400 µ mol mol^{-1} reference CO_2 concentration.

Photosynthetic traits were measured on the youngest fully expanded leaf between 10:00 AM and 2:00 PM. Three leaves per replicate were measured. Plants were harvested at 40 DAS and dissected into shoots and roots. Roots were washed with water to remove soil, and carefully placed on a plexiglass tray with a 2 to 3 mm water layer. This tray was transferred to root Epson perfection V700 photo scanner. After scanning, we used WinRHIZO (Version 2007d, Regent Instrument Inc., Canada) software to determine total root length, surface area, root volume and diameter (Hussain et al., 2019). Shoot and root were oven-dried at 70 °C for 48 h and weighed.

In experiment 2, plants were harvested at 44 DAS and dissected into shoots and roots. We counted the number of larvae that survived, the number of damaged and undamaged leaves, and estimated the percentage of leaf damaged as the total number of damaged leaves/total number of leaves × 100. We took the image of larvae using a scanner (Canoscan 9000F), and the larvae size was estimated by measuring their length and width using ImageJ® image analysis software (Collins, 2007). Larvae were weighed with a high-resolution balance (readability = 0.1 mg, Ohaus corporation, USA).

2.3.**Statistical analysis**

We used a general linear model with factorial design to assess the effects of plant variety, water regime and their interactions on plant traits (experiment 1), and the effects of plant variety, water regime, herbivory and their interactions on plant traits, and the effect of plant variety, water regime and their interactions on insect traits (experiment 2). Following updated statistical recommendations, we avoid the wording "statistically significant", "non-significant", or the variations thereof, thus avoiding dichotomisation based on an arbitrary discrete p-value (Wasserstein et al. 2019). Instead, we report p as a continuous quantity, and Shannon information transform [$s = -\log_2(p)$] as a measure of the information against the

tested hypothesis (Greenland, 2019). Although s is a function of p, the additional information

provided is not redundant. With base-2 log, the units for measuring this information are bits

(binary digits). For example, $p = 0.05$ conveys only $s = - \log_2(0.05) = 4.3$ bits of information,

"which is hardly more surprising than seeing all heads in 4 fair tosses" (Greenland, 2019).

We used OriginPro, 2022. (OriginLab Corporation, Northampton, MA, USA) and SigmaPlot

11.0 (Systat Software Inc., San Jose, CA, USA) for data analysis and figures.
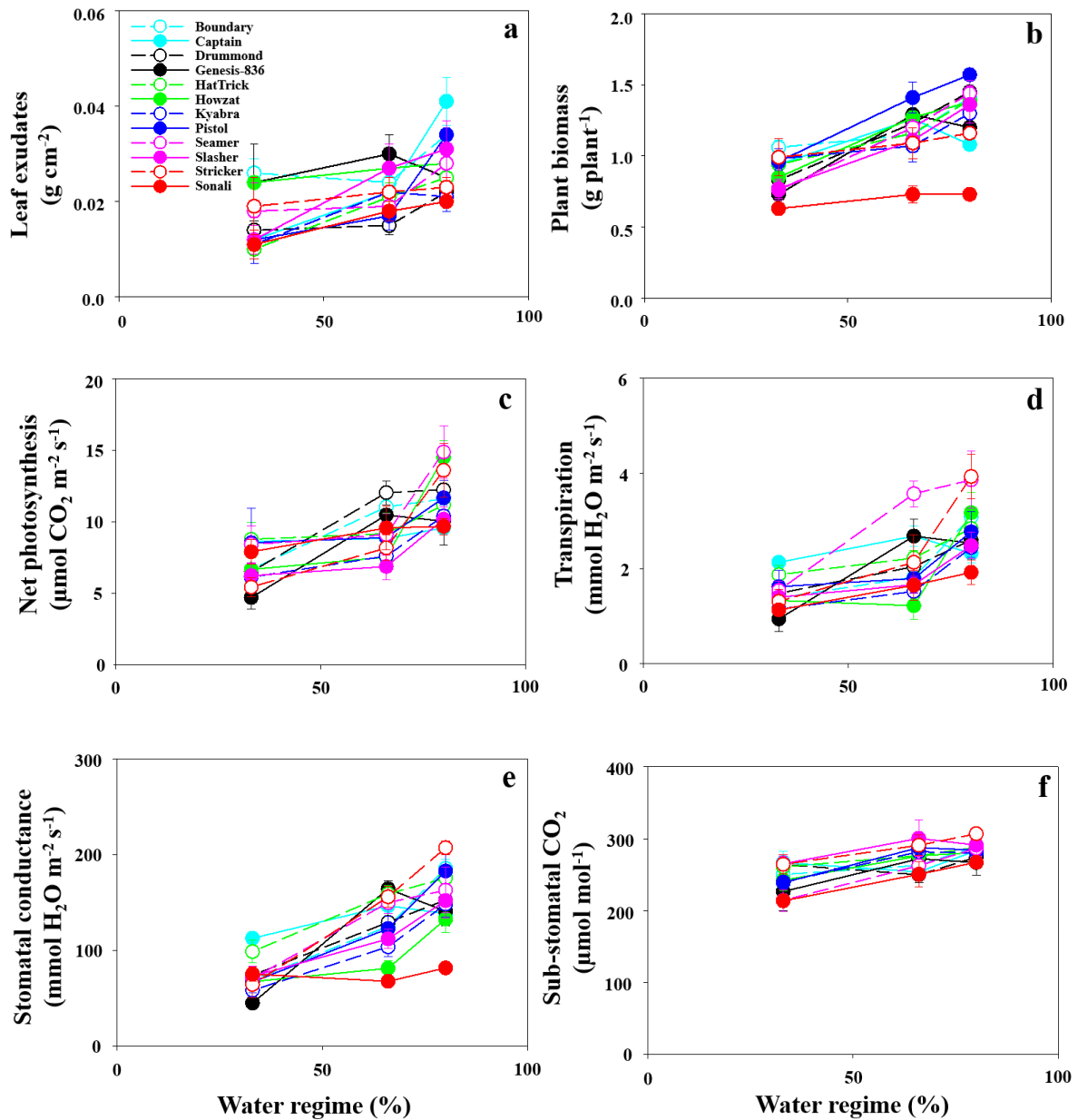
3. Results

3.1.Experiment 1

#### 3.1.1. Variation in plant traits with plant variety, water regime and their interaction

Plant biomass, leaf exudates, transpiration and stomatal conductance varied with plant variety and water regime, except for net photosynthesis and sub-stomatal intercellular CO_2, that only varied with water regime (Table S1). The interaction of plant variety and water regime influenced leaf exudates ($p = 0.02$, $s = 5.6$), plant biomass ($p = 2.49 \times 10^{-4}$, $s = 11.9$), net photosynthesis ($p = 0.02$, $s = 5.6$), transpiration ($p = 2.83 \times 10^{-3}$, $s = 8.4$) and stomatal conductance ($p = 1.55 \times 10^{-4}$, $s = 12.6$) (Table S1). Drought stress at 1/3 of available soil water decreased all plant traits in comparison to well-watered and 2/3 of available soil water (Fig. 1a-f). To illustrate the interactions, the amount of leaf exudates varied 3.4-fold in Captain compared with 1.5-fold in Seamer in response to water stress. Plant biomass varied 2.0-fold in Seamer compared with 1.3-fold in Boundary, net photosynthesis varied 2.5-fold in Striker compared with 1.6-fold in Kyabra, transpiration varied 3.0-fold in Striker compared with a 1.6-fold in Sonali and stomatal conductance varied 3.2-fold in Striker compared with a 1.7-fold in HatTrick under 1/3 of available soil water (Fig. 1a-f).

Fig 1 The effect of drought stress on (a) leaf exudate, (b) plant biomass, (c) net photosynthesis, (d) transpiration, (e) stomatal conductance, and (f) sub-stomatal CO_2 concentration in twelve chickpea varieties exposed to three water regimes: 80, 66 and 33 % pot capacity in experiment 1. Values are means ± standard errors, and errors are not shown when smaller than symbol

3.1.2. *Variation in root traits with plant variety, water regime and their interaction*

Root length, average root diameter, root surface area, and root volume varied with variety and water regime (Table S2). The interaction of variety and water regime modified root surface area and root volume ($p = 0.03$, $s = 5.0$) (Table S2). Root length, average root diameter, root surface area, and root volume decreased under 1/3 of available soil water compared with well-watered and at 2/3 of available soil water (Fig. 2a-d). To illustrate the interactions, root surface area varied 2.3-fold in Slasher compared with a 1.3-fold in Sonali (Fig. 2c) and root volume varied 2.8-fold in HatTrick compared with a 1.5-fold in Captain in response to water stress (Fig. 2d).
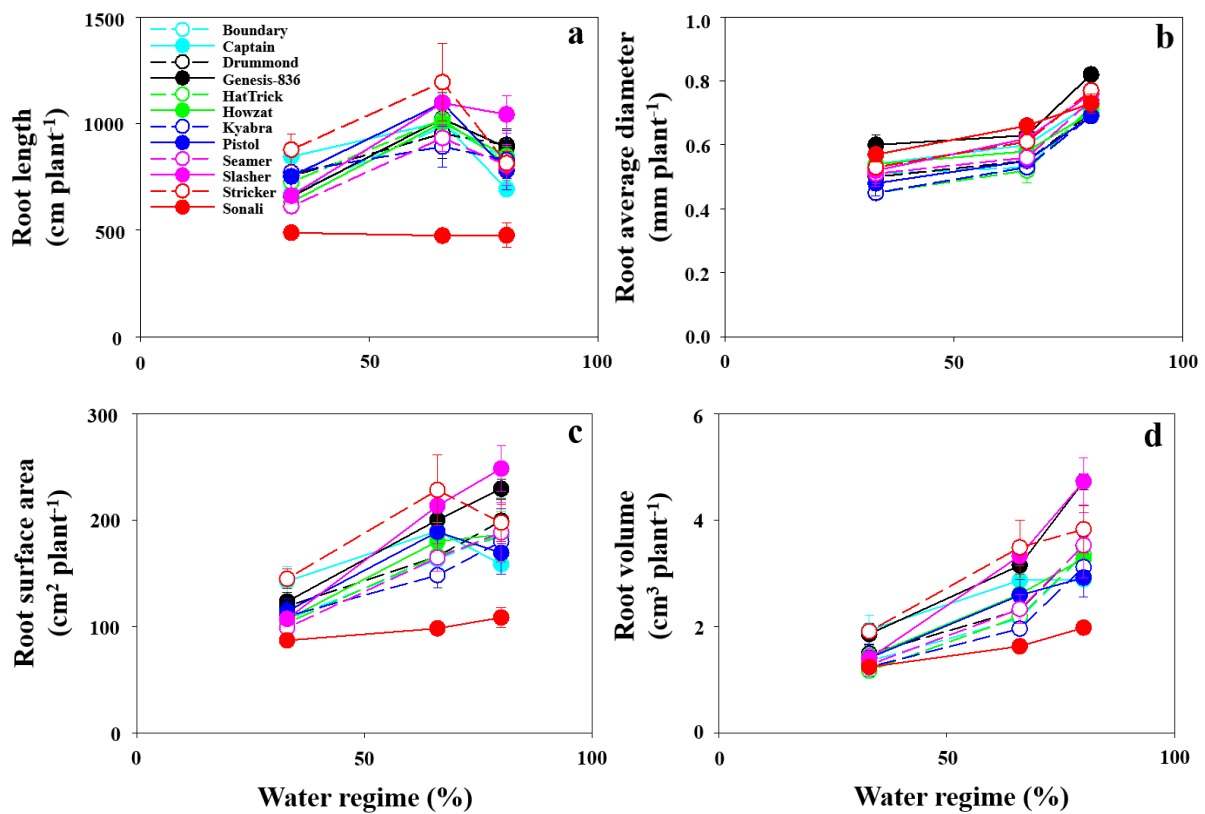
Fig 2 The effect of drought stress on (a) root length, (b) root surface area, (c) root average diameter, and (d) root volume in twelve chickpea varieties exposed to three water regimes: 80, 66 and 33 % pot capacity in experiment 1. Values are means ± standard errors, and errors are not shown when smaller than symbol

3.1.3. Associations between traits

Growth traits and leaf exudates were unrelated in well-watered plants ($p > 0.4$, $s > 1.3$) (Fig. 3a, Supplementary Table S5). There was a weak, negative correlation between leaf exudates and net photosynthesis ($p = 0.08$, $s = 3.6$) and stomatal conductance ($p = 0.09$, $s = 3.4$) under 1/3 of available soil water (Fig. 3c, Table S5). Leaf exudates correlated positively with root volume under 2/3 of available soil water ($p = 0.08$, $s = 3.6$) (Fig. 3b, Table S5).
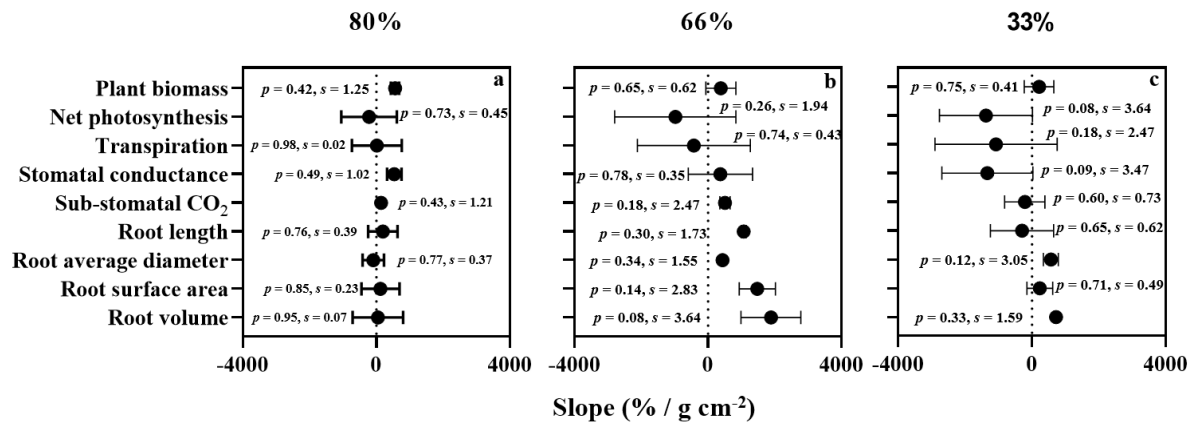
Fig 3 Slopes of linear regressions relating plant and root traits with leaf exudates in chickpea varieties grown under three water regimes; 80, 66 and 33 % pot capacity in experiment 1. Error bars are two standard errors. Traits were normalised relative to maximum value in each treatment for comparison

3.2. Experiment 2:

3.2.1. Variation in plant and larvae traits with plant variety, herbivory, water regime, and their interactions

Plant biomass, amount of leaf exudates, and concentration of malic and oxalic acids varied with variety, water regime, herbivory and their interaction (Table S3). The concentration of both oxalic acid ($p = 2.05 \times 10^{-4}$, $s = 12.2$) and malic acid ($p = 7.74 \times 10^{-4}$, $s = 10.3$) varied with the interaction between variety and herbivory. The concentration of oxalic acid ranged

from 771 µmol g^{-1} in Slasher with no larvae to 262 µmol g^{-1} in Captain challenged with *H. armigera* (Fig. 4e, f). The concentration of malic acid ranged from 138 µmol g^{-1} in Genesis-836 with no larvae to 18 µmol g^{-1} in Kyabra challenged with *H. armigera* (Fig. 4g, h).

The interaction of water regime and herbivory altered leaf exudates ($p = 4.38 \times 10^{-4}$, $s = 11.1$), and the concentration of both oxalic acid ($p = 5.80 \times 10^{-4}$, $s = 10.7$) and malic acid ($p = 6.45 \times 10^{-3}$, $s = 7.2$) (Table S3). Leaf exudates increased from 0.02 g cm^{-2} in water-stressed plants with no larvae to 0.09 g cm^{-2} in well-watered plants challenged with *H. armigera* (Fig. 4c, d). Oxalic acid was reduced from 651 µmol g^{-1} in well-watered plants with no larvae to 287 µmol g^{-1} in water-stressed plants challenged with *H. armigera* (Fig. 4e, f). Malic acid was reduced from 101 µmol g^{-1} in water-stressed plants with no larvae to 27 µmol g^{-1} in well-watered plants challenged with *H. armigera* (Fig. 4g, h).

The three-way interaction between plant variety, water regime and herbivory affected the concentration of oxalic acid ($p = 8.12 \times 10^{-3}$, $s = 6.9$) (Table S3), which reduced from 963 µmol g^{-1} in well-watered Slasher with no larvae to 129 µmol g^{-1} in water-stressed-Captain challenged with *H. armigera* (Fig. 4e, f).

Larval survival varied with plant variety and water regime, and percentage leaf damage only varied with water regime (Table S4). The interaction of plant variety × water regime affected the percentage leaf damage ($p = 0.03$, $s = 5.0$) and larval survival ($p = 5.54 \times 10^{-3}$, $s = 7.4$) (Table S4). Leaf damage ranged from 18 % in water-stressed Captain to 6 % for well-watered Drummond (Fig. 5a). Larval survival ranged from 11 plant^{-1} in water-stressed Captain to 5 plant^{-1} in well-watered Drummond (Fig. 5b).
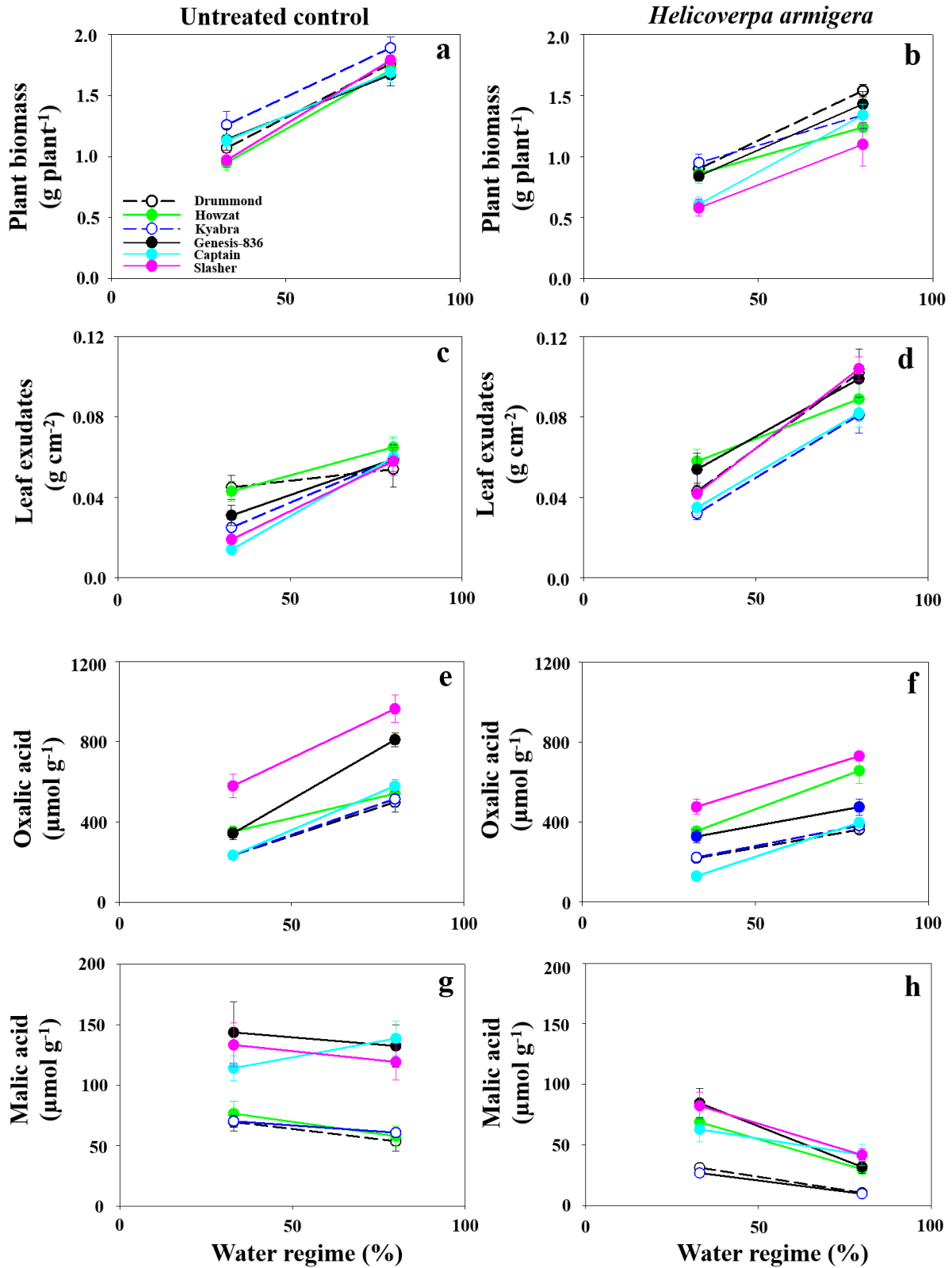
Fig. 4 The effect of drought stress and *Helicoverpa armigera* herbivory on (a) plant biomass,

(b) leaf exudate, (c) oxalic acid and (d) malic acid of six chickpea varieties in experiment 2.

Values are mean ± standard error, and errors are not shown when smaller than symbol
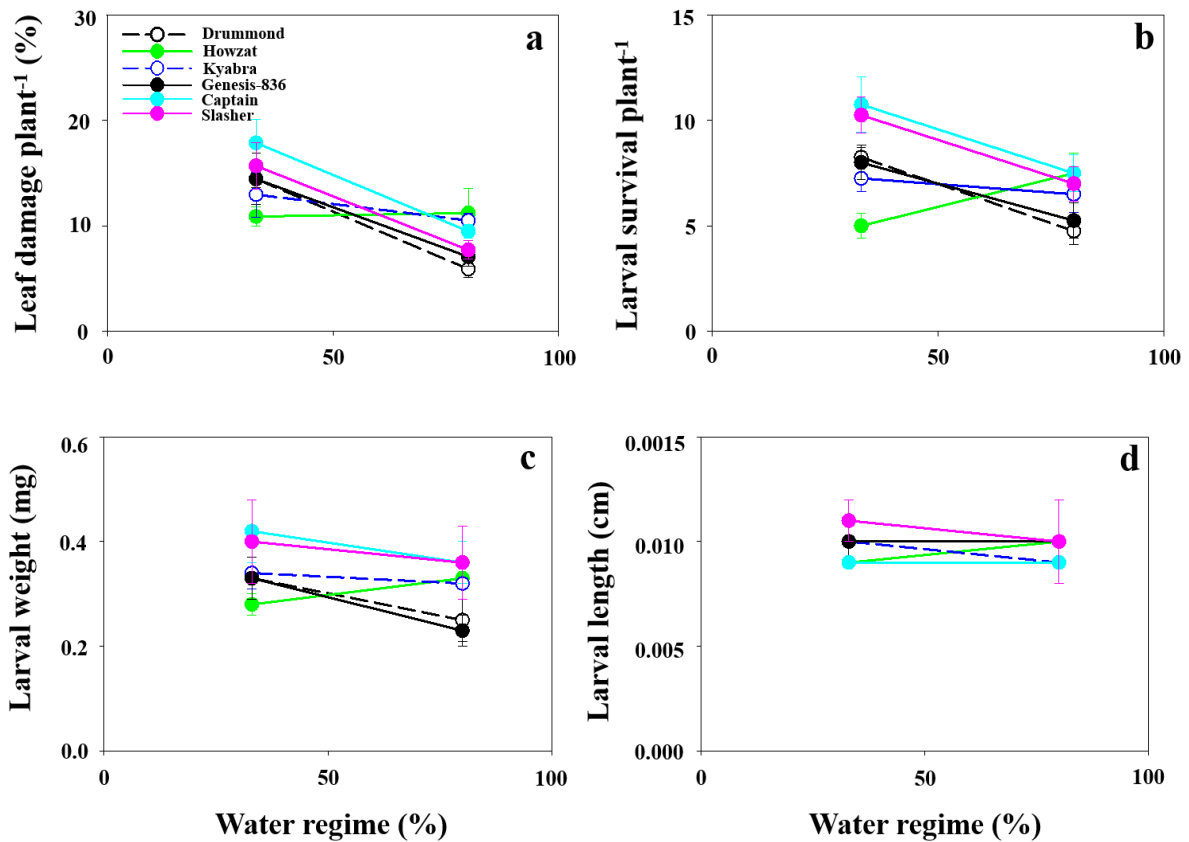
Fig. 5 The effect of drought stress on (a) leave damage per plant, (b) larval survival per plant, (c) larval weight and (d) larval length of six chickpea varieties exposed to two water regimes: 80 and 33 % pot capacity in experiment 2. Values are means ± standard errors, and errors are not shown when smaller than symbol

3.2.2. Association between plant and larvae traits and leaf exudate

Plant biomass was unrelated to the amount of leaf exudates in well-watered ($p = 0.98$, $s = 0.0$) and water-stressed plants ($p = 0.72$, $s = 0.4$) (Fig. 6a, Supplementary Table S6). Leaf damage and larval survival correlated negatively with leaf exudate under both water regimes (Fig. 6b, c, Table S6). Larval weight correlated negatively with leaf exudate, particularly under water stress (Fig. 4d, Table S6).
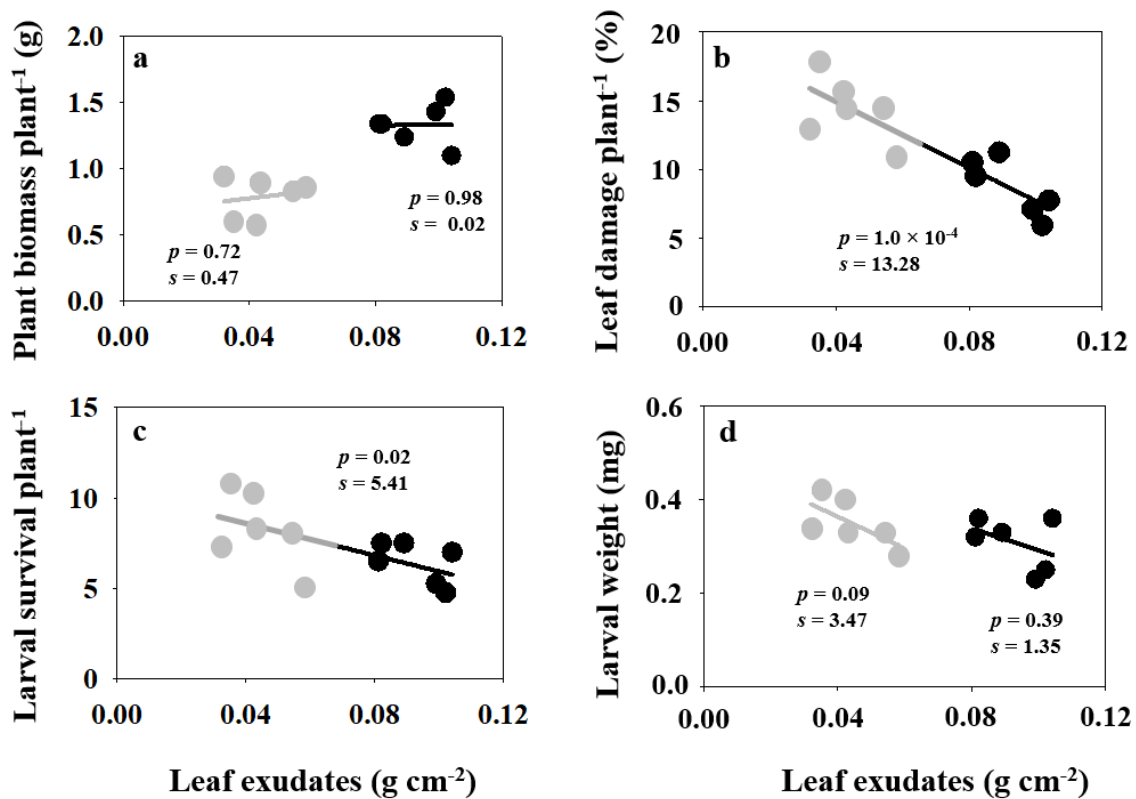
Fig. 6 Relationship of (a) plant biomass, (b) leaf damage, (c) larval survival and (d) larval weight with leaf exudates of six chickpea varieties exposed to two water regimes: 80 % (black) and 33 % (grey) pot capacity under *Helicoverpa armigera* application in experiment 2

4. Discussion

4.1. Hypothesis 1: drought increases the leaf exudation of organic acids

Drought can decouple photosynthesis and growth, leading to an accumulation of reduced carbon (Granier et al., 2006; Hummel et al., 2010; Muller et al., 2011; Körner, 2013). Accordingly, we hypothesised that drought could increase leaf exudates in chickpea. In this study, water stress decreased the amount of leaf exudates by 98 to 72 %, compared with a reduction in shoot biomass of 48 to 64 % (Fig. 1, 2). The hypothesised increase in exudates was not realised in our experiments, where water deficit reduced exudates proportionally more than growth. This could be ascribed to a combination of factors. First, the plant can

produce more exudates under drought, but stomatal closure may limit the release of exudates from the leaves (Flexas et al., 2006). Second, the stressed plant may increase root exudates at the expense of leaf exudates (Yang et al., 2002).

4.2. *Hypothesis 2: herbivory increases the exudation of organic acids*

Herbivory increased the amount of leaf exudates (Fig. 4) consistent with a direct role in plant defence (War et al., 2012), and a putative indirect role mediating interactions with predators and herbivore parasitoids (Dicke et al., 2009). This response indicates the production of inducible leaf exudates in response to *H. armigera,* which implies a complex signalling pathway (Kessler and Baldwin, 2002). Plants challenged with herbivory release hormones such as jasmonic acid and salicylic acid, which in turn activate a number of genes associated with defence compounds, including leaf exudates (War et al., 2011a; War et al., 2012). Under the experimental conditions of War et al. (2011b), salicylic acid was linked to high activities of peroxidase, polyphenol oxidase, total amount of phenols, and hydrogen peroxide that induce defence against biotic stress in chickpea. However, the specific composition of exudation of organic acids varies depending on the plant species, the type of herbivore, and the intensity of the attack (Walters, 2014; Schweiger et al., 2014).

4.3. *Hypothesis 3: drought modulates the effect of herbivory*

Herbivory increased the amount of leaf exudates and water regime modulated this response, which also varied with variety (Fig. 4). Well-watered plants challenged with *H. armigera* increased the amount of leaf exudates by 60 % in comparison to plants with no larvae (Fig. 4c, d). However, water-stressed plants challenged with *H. armigera* increased the amount of leaf exudates by 43 % in comparison to plants with no larvae (Fig. 4c, d). Some plants

allocate more resources to defence when challenged with herbivory (Sampedro et al., 2011; Huot et al., 2014), and defences tend to involve high carbon cost under both herbivory and drought stresses (Cipollini, 2010). Therefore, plants shift resources away from costly defensive compounds and increase nutrient content to maintain growth under drought. Drought-resistant species inherently prioritise resource allocation towards responding to drought rather than investing in defences against herbivore attacks (Montesinos-Navarro et al., 2020; Carvajal Acosta et al., 2023). Tissue turgor, transpiration and stomatal opening also influence the exudation of organic acids (Lu et al., 2020). The production of exudates is constrained due to limitations in gas exchange before resource allocation under drought (Lu et al., 2020).

The percentage of leaf damage and larval survival varied between water regime, plant variety and their interaction, suggesting an antifeeding effect of acid exudates in response to *H. armigera* under well-watered and severe stress (Fig. 5 and Table S4). Antifeedant effects of acid exudates that reduced larval survival have been reported in chickpea (Yoshida et al., 1995; Devi et al., 2013). In our study, Captain recorded higher leaf damage and larval survival than Drummond across water regimes, suggesting that host plant resistance in combination with water availability had a greater effect on *H. armigera*. This is consistent with previous studies showing a variety-dependent effect of drought on resistance to herbivory (Mao et al., 2004; Grinnan et al., 2013).

4.4. *Hypothesis 4: trade-off between exudation of organic acids and growth*

A trade-off between growth and defence has been described in plant-insect and plant-pathogen interactions (Huot et al., 2014). In our study, growth traits (shoot and biomass, and photosynthetic traits) were largely unrelated to leaf exudates, except for a weak correlation

under 1/3 of available soil water (Fig. 3c, Fig. 6a, Table S5). A trade-off, if any, might be contingent on water availability. In milkweed (*Asclepias curassavica*) and pinus pinaster (*pinheiro bravo*), a trade-off between growth and defence was pronounced under low nutrient conditions, but not in well-fertilised plants (Sampedro et al., 2011; López-Goldar et al., 2022).

The negative correlations of leaf damage, larval survival and larval weight with leaf exudates under both water regimes (Fig. 6, Table S6), confirm the defence role of exudates. Oxalic acid in an artificial diet inhibited the growth of H. *armigera* larvae while no such effects were shown by malic acid (Yoshida et al., 1995; 1997). We did not find correlations between herbivory related traits (leaf damage and larval survival) and concentration of oxalic acid and malic acid (Table S6), suggesting the potential involvement of some unknown compounds. A study that characterized the organic acid profiles in the leaf exudates of chickpea showed that some unknown compounds were positively correlated with larval survival, while citric acid, acetic acid and few unknown compounds were negatively correlated with leaf damage, larval survival and larval weight (Narayanamma et al. 2013).

In conclusion, drought decreased, and herbivory increased the amount of leaf exudates. Water regime modulated the response of leaf exudates to herbivory, potentially leading to change in plant-herbivore relationships. A trade-off between defence and growth was not apparent under our experimental conditions.

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Author contribution

Declaration of competing interest

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.

Reference

Ashrafi M, Azimi-Moqadam MR, Moradi P, MohseniFard E, Shekari F, Kompany-Zareh M (2018) Effect of drought stress on metabolite adjustments in drought tolerant and sensitive thyme. Plant Physiol Biochem *132*: 391-399. https://doi.org/10.1016/j.plaphy.2018.09.009

Baldwin IT (1998) Jasmonate-induced responses are costly but benefit plants under attack in native populations. Proceedings of the National Academy of Sciences 95: 8113-8118. https://doi.org/10.1073/pnas.95.14.8113

Ballaré CL, Austin AT (2019) Recalculating growth and defense strategies under competition: key roles of photoreceptors and jasmonates. JXB 70: 3425-3434. https://doi.org/10.1093/jxb/erz237

Bhagwat VR, Aherkar SK, Satpute US, Thakare HS (1995) Screening of chickpea (*Cicer arietinum* L.) genotypes for resistance to gram pod borer, *Helicoverpa armigera* (Hubner) and its relationship with malic acid in leaf exudates. J Entomol Res 19: 249-253.

Carvajal Acosta AN, Agrawal AA, Mooney K (2023) Plant water-use strategies as mediators of herbivore drought response: Ecophysiology, host plant quality and functional traits. J Ecol 111: 687-700. https://doi.org/10.1111/1365-2745.14059

Cawthray GR (2003) An improved reversed-phase liquid chromatographic method for the analysis of low-molecular mass organic acids in plant root exudates. J Chromatogr A 1011: 233-240. https://doi.org/10.1016/S0021-9673(03)01129-4

Cipollini D (2010) Constitutive expression of methyl jasmonate-inducible responses delays reproduction and constrains fitness responses to nutrients in *Arabidopsis thaliana*. Evol Ecol 24: 59-68. https://doi.org/10.1007/s10682-008-9290-0

Collins TJ (2007) ImageJ for microscopy. Biotechniques 43: S25-S30.
https://doi.org/10.2144/000112517

Devi VS, Sharma HC, Rao PA (2013) Influence of oxalic and malic acids in chickpea leaf exudates on the biological activity of CryIAc towards *Helicoverpa armigera*. J Insect Physiol 59: 394-399. https://doi.org/10.1016/j.jinsphys.2013.01.008

Dicke M, Van Loon JJ, Soler R (2009) Chemical complexity of volatiles from plants induced by multiple attack. Nat Chel Biol 5: 317-324. https://doi.org/10.1038/nchembio.169

Flexas J, Bota J, Galmes J, Medrano H, Ribas-Carbó M (2006) Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. Physiol Plant *127*: 343-352. https://doi.org/10.1111/j.1399-3054.2006.00621.x

Geisler M, Gibson DJ, Lindsey KJ, Millar K, Wood AJ (2012) Upregulation of photosynthesis genes, and downregulation of stress defense genes, is the response of *Arabidopsis thaliana* shoots to intraspecific competition. Bot Stud 53(1).

Granier C, Aguirrezabal L, Chenu K, Cookson SJ, Dauzat M, Hamard P, Tardieu F (2006) PHENOPSIS, an automated platform for reproducible phenotyping of plant responses to soil water deficit in *Arabidopsis thaliana* permitted the identification of an accession with low sensitivity to soil water deficit. New Phytol 169: 623-635. https://doi.org/10.1111/j.1469-8137.2005.01609.x

Greenland S (2019) Valid p-values behave exactly as they should: Some misleading criticisms of p-values and their resolution with s-values. American Stat 73: 106-114.
https://doi.org/10.1080/00031305.2018.1529625

Grinnan R, Carter Jr TE, Johnson MT (2013a) The effects of drought and herbivory on plant–herbivore interactions across 16 soybean genotypes in a field experiment. Ecol Entomol 38: 290-302. https://doi.org/10.1111/een.12017

Grinnan R, Carter TE, Johnson MT (2013b) Effects of drought, temperature, herbivory, and genotype on plant–insect interactions in soybean (*Glycine max*). Arthropod Plant Interact 7: 201-215. https://doi.org/10.1007/s11829-012-9234-z

Gutbrodt B, Mody K, Dorn S (2011) Drought changes plant chemistry and causes contrasting responses in lepidopteran herbivores. Oikos 120: 1732-1740. https://doi.org/10.1111/j.1600-0706.2011.19558.x

Herppich WB, Peckmann K (1997) Responses of gas exchange, photosynthesis, nocturnal acid accumulation and water relations of *Aptenia cordifolia* to short-term drought and rewatering. J Plant Physiol 150: 467-474. https://doi.org/10.1016/S0176-1617(97)80100-9

Hummel I, Pantin F, Sulpice R, Piques M, Rolland G, Dauzat M, Muller B (2010) Arabidopsis plants acclimate to water deficit at low cost through changes of carbon usage: an integrated perspective using growth, metabolite, enzyme, and gene expression analysis. Plant Physiol 154: 357-372. https://doi.org/10.1104/pp.110.157008

Huot B, Yao J, Montgomery BL, He SY (2014) Growth – defense trade-offs in plants: a balancing act to optimize fitness. *Mol. Plant* 7: 1267–1287. doi: 10.1093/mp/ssu049.

Hussain S, Iqbal N, Brestic M, Raza MA, Pang T, Langham DR, Yang W (2019) Changes in morphology, chlorophyll fluorescence performance and Rubisco activity of soybean in response to foliar application of ionic titanium under normal light and shade environment. Sci Total Environ 658: 626-637. https://doi.org/10.1016/j.scitotenv.2018.12.182

Iqbal N, Hussain S, Zhang XW, Yang CQ, Raza MA, Deng JC, Liu J (2018) Imbalance water deficit improves the seed yield and quality of soybean. Agron 8: 168. https://doi.org/10.3390/agronomy8090168

Iqbal N, Hussain S, Raza MA, Yang CQ, Safdar ME, Brestic M, Liu J (2019) Drought tolerance of soybean (*Glycine max* L. Merr.) by improved photosynthetic characteristics and an efficient antioxidant enzyme activities under a split-root system. Front Physiol 10: 786. https://doi.org/10.3389/fphys.2019.00786

Karban R, Agrawal AA, Mangel M (1997) The benefits of induced defenses against herbivores. Ecol 78: 1351-1355. https://doi.org/10.1890/0012-9658(1997)078[1351:TBOIDA]2.0.CO;2

Karthik GS, Vastrad AS (2022) Biochemical Response of Chickpea Genotypes as Influenced by Pod Borer *Helicoverpa armigera* (Hubner). Indian J Entomol 84: 627-630. https://doi.org/10.55446/IJE.2021.61

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annu Rev Plant Biol 53: 299-328. https://doi.org/10.1146/annurev.arplant.53.100301.135207

Körner C (2013) Growth controls photosynthesis–mostly. Nova Acta Leopoldina NF 114: 273-283.

Kranthi KR, Jadhav DR, Kranthi S, Wanjari RR, Ali SS, Russel DA (2002) Insecticide resistance in five major pests of cotton in India. Crop Prot 21: 449–460. https://doi.org/10.1016/S0261-2194(01)00131-4

López-Goldar X, Hastings A, Züst T, Agrawal A (2022) Evidence for tissue-specific defence-offence interactions between milkweed and its community of specialized herbivores. Mol Ecol 31: 3254-3265. https://doi.org/10.1111/mec.16450

Lu Y, Duursma RA, Farrior CE, Medlyn BE, Feng X (2020) Optimal stomatal drought response shaped by competition for water and hydraulic risk can explain plant trait covariation. New Phytol 225: 1206-1217. https://doi.org/10.1111/nph.16207

Mao L, Jett LE, Story RN, Hammond AM, Peterson JK, Labonte DR (2004) Influence of drought stress on sweetpotato resistance to sweetpotato weevil, *Cylas formicarius* (Coleoptera: Apoinidae), and storage root chemistry. Fla Entomol 87: 261-267. https://doi.org/10.1653/0015-4040(2004)087[0261:IODSOS]2.0.CO;2

McKnight T (1949) Efficiency of isolates of Rhizobium in the cowpea group, with proposed additions to this group. Qd J Agr Sci 6: 61–76.

Molina C, Rotter B, Horres R et al. (2008). Super SAGE: the drought stress-responsive transcriptome of chickpea roots. BMC Genom 9: 553–581. https://doi.org/10.1186/1471-2164-9-553

Monje PV, Baran EJ (2002) Characterization of calcium oxalates generated as biominerals in cacti. Plant physiol 128: 707-713. https://doi.org/10.1104/pp.010630

Montesinos-Navarro A, Pérez-Clemente RM, Sánchez-Martín R, Gomez-Cadenas A, Verdú M (2020) Phylogenetic analysis of secondary metabolites in a plant community provides evidence for trade-offs between biotic and abiotic stress tolerance. Evol Ecol 34: 439-451. https://doi.org/10.1007/s10682-020-10044-2

Muller B, Pantin F, Génard, M, Turc O, Freixes S, Piques M, Gibon Y (2011) Water deficits uncouple growth from photosynthesis, increase C content, and modify the relationships between C and growth in sink organs. JXB 62: 1715-1729. https://doi.org/10.1093/jxb/erq438

Narayanamma VL, Sharma HC, Vijay PM, Gowda CLL, Sriramulu M (2013) Expression of resistance to the pod borer *Helicoverpa armigera* (Lepidoptera: Noctuidae), in relation to high-performance liquid chromatography fingerprints of leaf exudates of chickpea. Int J Trop Insect Sci 33: 276-282. https://doi.org/10.1017/S1742758413000234

Price PW (1991) The plant vigor hypothesis and herbivore attack. Oikos 62: 244-251. https://doi.org/10.2307/3545270

Riaz S, Johnson JB, Ahmad M, Fitt GP, Naiker M (2021) A review on biological interactions and management of the cotton bollworm, *Helicoverpa armigera* (Lepidoptera: Noctuidae). J Appl Entomol 145: 467-498. https://doi.org/10.1111/jen.12880

Salimath PM, Shahapur SC, Nijagun HG, Khajjidoni ST, Kumar RR, Patil BS (2003) Genetic analysis of pod borer tolerance and malic acid content in chickpea (*Cicer arietinum* L). In Chickpea research for the millennium. Proceedings of the International chickpea conference. Indira Gandhi Agricultural University, Raipur, Chattisgarh 81-85.

Sampedro L, Moreira X, Zas R (2011) Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. J Ecol 99: 818-827. https://doi.org/10.1111/j.1365-2745.2011.01814.x

San SH, Sagar D, Kalia VK, Krishnan V (2022) Effect of different chickpea genotypes and its biochemical constituents on biological attributes of *Helicoverpa armigera* (hubner). Legum Res 45: 514-520. https://doi.org/10.18805/LR-4474

Schweiger R, Heise AM, Persicke M, Müller C (2014) Interactions between the jasmonic and salicylic acid pathway modulate the plant metabolome and affect herbivores of different feeding types. Plant Cell Environ 37: 1574-1585. https://doi.org/10.1111/pce.12257

Sharma HC, Pampapathy G, Dhillon MK, Ridsdill-Smith JT (2005) Detached leaf assay to screen for host plant resistance to *Helicoverpa armigera*. J Econ Entomol 98: 568-576. https://doi.org/10.1093/jee/98.2.568

Sidhu PK, Joshi DV, Srivastava AK (1996) Oxalate toxicity in ruminants fed overgrown Napier grass (*Pennisetum purpureum*). IJAN 13: 181-183.

Srivastava CP, Srivastava RP (1989) Screening for resistance to gram pod borer, *Heliothis armigera* (Hubner), in chickpea (*Cicer arietinum* L.) genotypes and observations on its mechanism of resistance in India. Int J Trop Insect Sci 10: 255-258. https://doi.org/10.1017/S1742758400003477

Toker C, Karhan M, Ulger S (2004) Endogenous organic acid variations in different chickpea (*Cicer arietinum* L.) genotypes. Acta Agric Scand, B Soil Plant Sci 54: 42-44. https://doi.org/10.1080/09064710310019720

Ullmann-Zeunert L, Stanton MA, Wielsch N, Bartram S, Hummert C, Svatoš A, Groten K (2013) Quantification of growth–defense trade-offs in a common currency: nitrogen required for phenolamide biosynthesis is not derived from ribulose-1, 5-bisphosphate carboxylase/oxygenase turnover. Plant J 75: 417-429. https://doi.org/10.1111/tpj.12210

Uloth MB, You MP, Cawthray G, Barbetti MJ (2015) Temperature adaptation in isolates of *Sclerotinia sclerotiorum* affects their ability to infect *Brassica carinata*. Plant Pathol 64: 1140-1148. https://doi.org/10.1111/ppa.12338

Veneklaas EJ, Stevens J, Cawthray GR, Turner S, Grigg AM, Lambers H (2003) Chickpea and white lupin rhizosphere carboxylates vary with soil properties and enhance phosphorus uptake. Plant Soil 248: 187-197. https://doi.org/10.1023/A:1022367312851

Walters D (2011) Plant defense: warding off attack by pathogens, herbivores and parasitic plants. Hoboken, NJ: Blackwell Publishing. https://doi.org/10.1002/9781444328547

War AR, Paulraj MG, War MY, Ignacimuthu S (2011a) Jasmonic acid-mediated-induced resistance in groundnut (*Arachis hypogaea* L.) against *Helicoverpa armigera* (Hubner)(Lepidoptera: Noctuidae). J Plant Growth Regul 30: 512-523. https://doi.org/10.1007/s00344-011-9213-0

War AR, Paulraj MG, War MY, Ignacimuthu S (2011b) Role of salicylic acid in induction of plant defense system in chickpea (*Cicer arietinum* L.). Plant Signal Behav 6: 1787-1792. https://doi.org/10.4161/psb.6.11.17685

War AR, Paulraj MG, Ahmad T, Buhroo AA, Hussain B, Ignacimuthu S, Sharma HC (2012) Mechanisms of plant defense against insect herbivores. Plant Signal Behav 7: 1306-1320. https://doi.org/10.4161/psb.21663

Wasserstein RL, Schirm AL, Lazar NA (2019) Moving to a world beyond "p< 0.05". Am Stat 73(sup1): 1-19. https://doi.org/10.1080/00031305.2019.1583913

Yang J, Zhang J, Wang Z, Zhu Q, Liu L (2002) Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. Planta 215: 645-652. https://doi.org/10.1007/s00425-002-0789-2

Yoshida M, Cowgill SE, Wightman JA (1995) Mechanism of resistance to *Helicoverpa armigera* (Lepidoptera: Noctuidae) in chickpea: role of oxalic acid m leaf exudate as an antibiotic factor. J Econ Entomol 88: 1783-1786. https://doi.org/10.1093/jee/88.6.1783

Yoshida M, Cowgill SE, Wightman JA (1997) Roles of oxalic and malic acids in chickpea trichome exudate in host-plant resistance to *Helicoverpa armigera*. J Chem Ecol 23: 1195-1210. https://doi.org/10.1023/B:JOEC.0000006395.45516.e8.
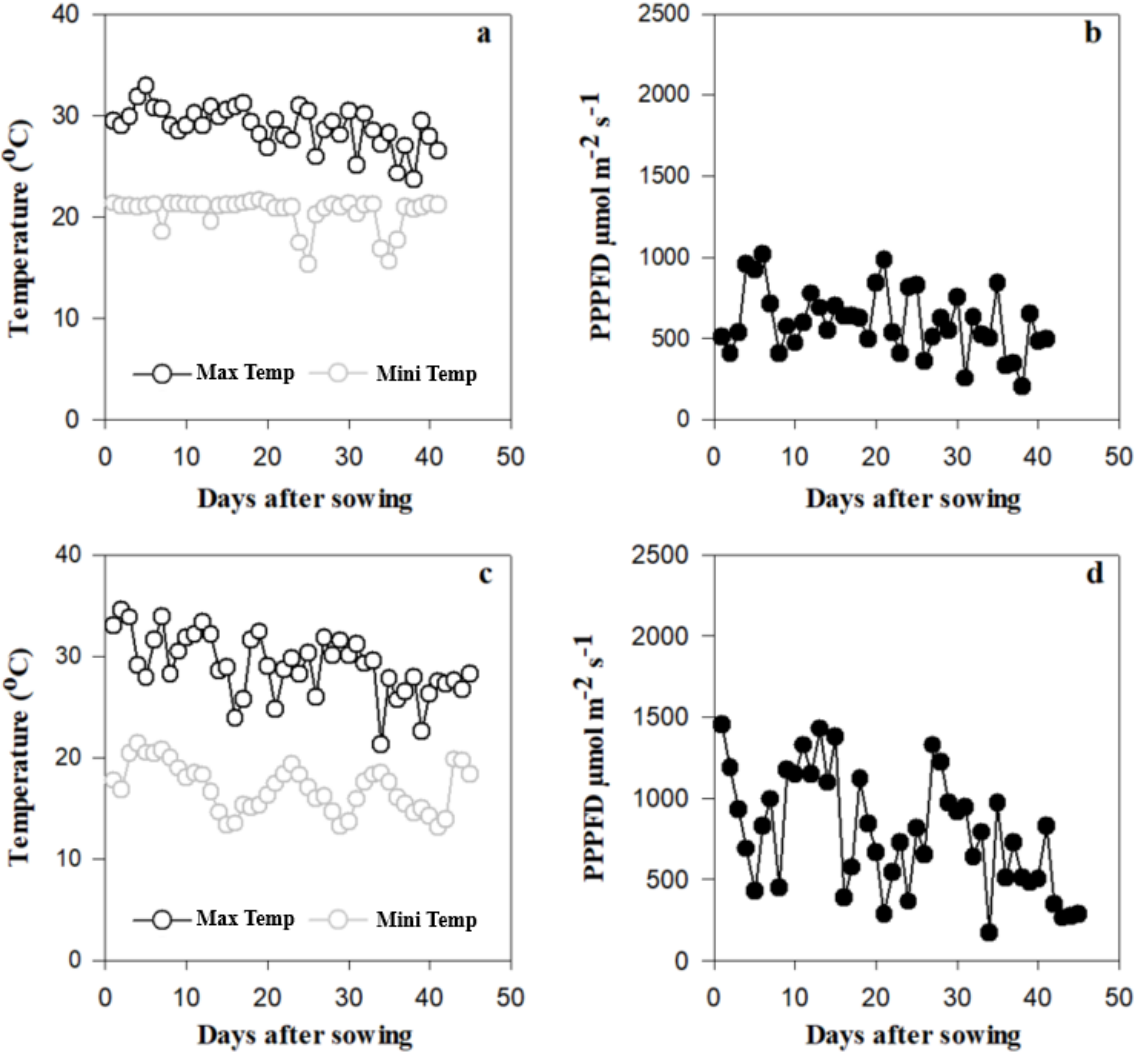
Fig S1 Daily maximum and minimum temperature and photosynthetic photon flux density (PPFD) during experiment 1 (a-b) chickpea varieties x water regimes and experiment 2 (c-d) chickpea varieties x water regimes x herbivory treatments

Table S1 *p* and *s* from ANOVA for leaf exudates, plant biomass, net photosynthesis, transpiration, stomatal conductance, and sub-stomatal CO_2 of chickpea in response to plant variety (V), water regime (W) and their interactions in experiment 1

Sources	df	Leaf exudates	Plant biomass	Net photosynthesis	Transpiration	Stomatal conductance	Sub-stomatal CO2
				p			
V	11	6.30×10^{-4}	4.12×10^{-13}	0.08	2.28×10^{-5}	1.05×10^{-7}	0.15
W	2	2.21×10^{-10}	8.11×10^{-23}	1.61×10^{-13}	1.88×10^{-16}	1.93×10^{-23}	4.93×10^{-6}
V × W	22	0.02	2.49×10^{-4}	0.02	2.83×10^{-3}	1.55×10^{-4}	0.96
				s			
V		10.63	41.14	3.64	15.42	23.18	2.73
W		32.07	73.38	42.50	52.24	75.46	17.63
V × W		5.64	11.97	5.64	8.46	12.65	0.05

Table S2 *p* and *s* from ANOVA for root length, root surface area, root average diameter and root volume of chickpea in response to plant variety (V), water regime (W) and their interactions in experiment 1

Sources	df	Root length	Root surface area	Root average diameter	Root volume
			p		
V	11	3.82×10^{-9}	5.11×10^{-9}	7.84×10^{-8}	1.74×10^{-8}
W	2	8.42×10^{-13}	2.15×10^{-19}	5.10×10^{-32}	7.88×10^{-25}
V × W	22	0.05	0.03	0.74	0.03
			s		
V		27.96	27.54	23.60	25.77
W		40.11	62.01	103.95	80.07
V × W		4.32	5.05	0.43	5.05

Table S3 *p* and *s* from ANOVA for plant biomass, leaf exudates, oxalic and malic acids of chickpea in response to plant variety (V), water regime (W), herbivory (H) and their interactions in experiment 2

Sources	df	Plant biomass	Leaf exudates	Oxalic acid	Malic Acid
			p		
V	5	1.97×10^{-3}	2.21×10^{-3}	1.37×10^{-23}	1.88×10^{-14}
W	1	3.51×10^{-26}	7.99×10^{-24}	1.14×10^{-27}	2.27×10^{-5}
H	1	6.74×10^{-15}	8.78×10^{-14}	1.63×10^{-8}	1.14×10^{-19}
V × W	5	0.76	0.11	0.12	0.23
V × H	5	0.08	0.25	2.05×10^{-4}	7.74×10^{-4}
W × H	1	0.10	4.38×10^{-4}	5.80×10^{-4}	6.45×10^{-3}
V × W × H	5	0.22	0.11	8.12×10^{-3}	0.67
			s		
V	5	8.98	8.82	75.95	45.6
W	1	84.56	76.73	89.50	15.42
H	1	47.08	43.37	25.87	62.93
V × W	5	0.39	3.18	3.05	2.12
V × H	5	3.64	2.00	12.25	10.33
W × H	1	3.32	11.15	10.75	7.27
V × W × H	5	2.18	3.18	6.94	0.57

Table S4 *p* and *s* from ANOVA for percentage leaf damage, larval survival, larval weight, and larval length of chickpea in response to plant variety (V), water regime (W) and their interactions under *Helicoverpa armigera* application in experiment 2

Sources	df	% Leaf damage	Larval survival	Larval weight	Larval length
			p		
V	5	0.34	3.52×10^{-3}	0.07	0.85
W	1	3.43×10^{-7}	5.07×10^{-4}	0.10	0.56
V × W	5	0.03	5.54×10^{-3}	0.61	0.97
			s		
V	5	1.55	8.15	3.83	0.23
W	1	21.47	10.94	3.32	0.83
V × W	5	5.05	7.49	0.71	0.04

Table S5 Matrix of Pearson's correlation coefficients (r) for plant biomass, leaf exudate, photosynthetic and root traits under well-watered, moderate, and severe drought in experiment 1. Shading indicates the strength of correlations, with darker shading denoting stronger positive (blue) or negative (red) correlations

		Plant biomass	Leaf exudates	Net photosynthesis	Transpiration	Stomatal conductance	Sub-stomatal CO_2	Root length	Root surface area	Root average diameter
Well-watered	Leaf exudates	0.25								
	Net photosynthesis	0.47	-0.11							
	Transpiration	0.43	0.01	0.87						
	Stomatal conductance	0.65	0.22	0.41	0.70					
	Sub-stomatal CO_2	0.30	0.25	0.42	0.69	0.79				
	Root length	0.71	0.10	0.21	0.29	0.50	0.32			
	Root surface area	0.54	0.06	0.13	0.29	0.45	0.29	0.96		
	Root average diameter	-0.34	-0.09	-0.16	0.10	-0.01	0.00	0.18	0.46	
	Root volume	0.38	0.02	0.07	0.27	0.38	0.23	0.86	0.97	0.65
Moderate drought	Leaf exudates	0.14								
	Net photosynthesis	0.08	-0.35							

Transpiration	0.24	-0.10	0.28					
Stomatal conductance	0.49	0.09	0.27	0.73				
Sub-stomatal CO_2	0.21	0.41	-0.70	-0.31	0.08			
Root length	0.74	0.32	-0.22	0.12	0.61	0.62		
Root surface area	0.61	0.45	-0.29	0.16	0.58	0.63	0.93	
Root average diameter	-0.42	0.30	-0.16	0.02	-0.21	-0.03	-0.30	0.06
Root volume	0.43	0.52	-0.32	0.17	0.48	0.57	0.77	0.95	0.37

Severe drought

Leaf exudates	0.10							
Net photosynthesis	0.04	-0.52						
Transpiration	0.38	-0.41	0.76					
Stomatal conductance	0.17	-0.50	0.73	0.90				
Sub-stomatal CO_2	0.56	-0.17	-0.09	0.51	0.46			
Root length	0.73	-0.14	-0.10	0.44	0.24	0.73		
Root surface area	0.44	0.12	-0.30	0.27	0.13	0.59	0.86	
Root average diameter	-0.59	0.47	-0.38	-0.40	-0.24	-0.32	-0.37	0.16
Root volume	0.10	0.31	-0.40	0.06	0.00	0.34	0.55	0.90	0.57

Table S6. Matrix of Pearson's correlation coefficients (r) for plant biomass, exudation of organic acids and larval traits in response to well-watered and severe stress under *Helicoverpa armigera* application in experiment 2. Shading indicates the strength of correlations, with darker shading denoting stronger positive (blue) or negative (red) correlations.

		Plant biomass	Leaf exudate	Oxalic acid	Malic acid	Leave damage	Larval survival	Larval weight
Well-watered	Leaf exudate	0.01						
	Oxalic acid	-0.85	0.38					
	Malic acid	-0.61	0.10	0.58				
	Leave damage	-0.46	-0.81	0.19	0.08			
	Larval survival	-0.77	-0.55	0.49	0.57	0.80		
	Larval weight	-0.79	-0.43	0.43	0.49	0.61	0.92	
	Larval length	-0.54	0.48	0.84	0.52	0.01	0.16	-0.03
Severe stress	Leaf exudate	0.19						
	Oxalic acid	-0.23	0.51					
	Malic acid	-0.61	0.57	0.62				
	Leave damage	-0.74	-0.54	-0.31	0.19			
	Larval survival	-0.78	-0.58	-0.14	0.19	0.97		
	Larval weight	-0.82	-0.67	-0.16	0.17	0.93	0.96	
	Larval length	-0.17	-0.18	0.60	0.11	0.15	0.35	0.26

CHAPTER 6: General discussion and conclusion

Genetic factors related to rhizobia and host plant, along with environmental conditions, impact plant growth and defence, nitrogen fixation and its related traits. This thesis investigated the effects of genetics and environmental factors and their interactions on plant traits. This research provides insights into the phenotypic plasticity of the legume-rhizobia interaction, advancing our understanding of the function of the chickpea-*Mesorhizobium* symbiosis and the consequences of genetic and environmental factors for growth and defence of chickpea.

Drought constrains crop yield, and is projected to increase in frequency and severity with climate change (Stewart and Lal, 2018). The effects of drought on growth, nodulation and nitrogen fixation of legumes vary depending on host, rhizobial strain and their interaction (Brockwell and Hely, 1966; Israel et al., 1986; Hafeez et al., 2000; Esfahani and Mostajeran, 2011; Duc et al., 2015). We reviewed the relative variation of shoot growth, nitrogen fixation and its related traits in the light of a hierarchy of phenotypic plasticities (Chapter 2). Drought reduced total nitrogen fixation and average nodule mass more severely than plant shoot mass, and elicited a hierarchy of plasticities whereby number of nodules per plant varied substantially, and average nodule mass and nitrogen fixation per unit nodule mass were relatively conserved (Chapter 2). It suggests that the plant prioritizes the maintenance of shoot growth over nodule development and maintains the function of existing nodules in preference to forming new nodules. This information can be used to develop management strategies that balance the need for plant growth and nitrogen fixation under drought. To achieve this, it is essential to consider implementing precise irrigation techniques, intercropping systems, or the use of soil amendment strategies, which could enhance water availability and improve nitrogen fixation efficiency in legume crops. In addition, this study adds to the existing list of number-size hierarchies in various biological systems. For

example, annual plants adjust their seed production to accommodate environmental variations while conserving seed size (Sadras, 2007; Gambín and Borrás, 2010; Sadras and Slafer, 2012). Similarly, in cell biology, the number of organelles scales with cell size, while the average size of organelles remains relatively conserved (Okie et al. 2016).

Other environmental factors such as moisture, radiation and temperature also modulate the responses of plant growth, nodulation and nitrogen fixation (Hungria & Kaschuk, 2014; Hansen, 2017; Jemo et al., 2017). Considering, the variation in nitrogen fixation among chickpea varieties and efficiency among rhizobial strains that have implications for plastic responses to environmental conditions, we investigated the phenotypic plasticity among chickpea varieties and *Mesorhizobium* strains under varying environments (Chapter 3). Nodulation showed greater phenotypic plasticity than shoot dry weight under varying environments, which aligned with previous studies showing higher plasticity of nitrogen fixation traits than shoot dry matter in response to drought (Iqbal et al., 2022) and nutrient deficit (Divito and Sadras 2014). This also suggests developing the management strategies that balance the need for plant growth and nitrogen fixation not only under drought (Chapter 2) but in different photothermal environments. Further, the plasticity of plant growth and nitrogen fixation traits was influenced by strain-related factors to a greater extent (Chapter 3), indicating that the dominance of strains benefitted from favourable environments, resulting from the combination of chickpea variety and photothermal environment (Bano et al., 2010). These findings suggest the implementation of strain-specific management strategies to create favourable environments for strains that can mitigate the negative impact of environmental factors on nitrogen fixation and productivity. Further, this study provides insights on the phenotypic plasticity of the chickpea-rhizobia symbiosis that led us to further investigate the dynamics of chickpea growth, nodule traits and bacteroid morphology with different rhizobial strains under well-watered and drought conditions (Chapter 4). In this study, chickpea

inoculated with CC1192 showed temporal response in growth rate, depending on the water availability, and continued to accumulate high plant biomass and express high symbiotic efficiency at harvest than other sources of nitrogen (Chapter 4). This suggests the potential of commercial strain CC1192 to fix high nitrogen in early stages (pre-drought phase) and influence chickpea's capacity to benefit from unfavourable environments later (drought phase). In this study, we expected nitrogen-fertilised treatment to produce high shoot growth rate compared with rhizobial and un-inoculated negative control treatments. However, both rhizobial strains showed high shoot growth rate compared to the nitrogen-fertilised treatment presumably due to inadequate nitrogen supplied in the fertilised treatment during the first three weeks of chickpea growth.

Symbiotic rhizobia differentiate into nitrogen fixing bacteroid in nodules and genetic and environmental factors influence the morphology of bacteroid (Mergaert et al., 2006; Oono and Denison., 2010; Oono et al., 2009; Rangarajan et al., 2020). In the present research, bacteroid size and the amount of polyhydroxybutyrate varied between rhizobial strains and varieties (Chapter 4), suggesting genetic variations inherent within each variety and strain that interact with environmental conditions influencing bacteroid morphology (Larrainzar et al., 2009). In addition, the negative correlation of bacteroid size and amount of polyhydroxybutyrate with total plant biomass under well-watered conditions suggests a potential compromise in plant growth due to bacteroid differentiation (Chapter 4). This may be due to the allocation of resources within the plant, where increased investment in bacteroid size and polyhydroxybutyrate synthesis could reduce overall plant biomass (Kiers et al., 2003; Lodwig and Poole, 2003). These findings indicate that there might be a regulatory feedback mechanism, where the plant modulates bacteroid size and amount of polyhydroxybutyrate in response to environmental or physiological cues. It is worth mentioning that this correlation does not indicate a cause-and-effect relationship, as other

factors such as environmental conditions and host-strain interaction may also contribute to the observed compromise in plant growth (Chapter 3).

The interaction between chickpea variety, water regime and nitrogen source influenced the amount of leaf exudates (Chapter 4). Leaf exudates directly contribute to plant defence and affect interactions with herbivores (Dicke et al., 2009; War et al., 2012). The influence of the *Mesorhizobium* strain on leaf exudates and subsequent plant defence provide valuable insights into development of inoculant to enhance plant resilience against herbivory. These findings prompted us to further investigate the effects of drought and herbivory on chickpea growth and defence (Chapter 5). Drought decreased the amount of leaf exudates likely due to stomatal closure limiting the release of exudates and prioritization of root exudates in stressed plant (Yang et al., 2002; Flexas et al., 2006). However, herbivory increased the amount of leaf exudates and water availability modulated this response (Chapter 5). These findings suggest that plants allocate resources to defence (Sampedro et al., 2011; Huot et al., 2014), but defences tend to involve high carbon cost under both herbivory and drought (Cipollini, 2010). Further, the negative correlations of leaf damage, larval survival and larval weight with leaf exudates under well-watered conditions and drought confirm the defence role of exudates (Chapter 5). However, growth traits were largely unrelated to the amount of leaf exudates, except for a weak correlation under severe stress (Chapter 5), suggesting a trade-off between growth and defence contingent on water availability. Studies in milkweed (*Asclepias curassavica*) and pinus pinaster (*Pinheiro bravo*) showed a trade-off between growth and defence under low nutrient conditions, but not in well-fertilised plants (Sampedro et al., 2011; López-Goldar et al., 2022). This study contributes to a better understanding of the effects of herbivory and drought on the defence and growth of chickpea.

6.1. **Conclusion**

This thesis aimed to investigate the effects of genetic factors related to both rhizobia and host plant, along with environmental conditions, on various aspects of growth, defence, nitrogen fixation, and related traits, to advance the understanding to maximise growth and productivity and develop the resilience of chickpea. The hierarchy of phenotypic plasticities observed between plant growth and nitrogen fixation traits indicates that different traits exhibit varying degrees of adaptability to changing environments. The substantial variation in phenotypic plasticity among rhizobial strains compared to varieties highlights the influence of strain-specific genetic factors on the chickpea-*Mesorhizobium* symbiosis. Furthermore, shoot growth rate responses to *Mesorhizobium* strains undergoes changes over chickpea ontogeny, and the data collected at the harvest time point reflects the plant's growth patterns throughout its lifespan. Additionally, the importance of considering the environmental conditions to develop strategies for enhanced chickpea productivity and resilience was revealed. The effects of drought and herbivory on chickpea growth and defence show the modulating role of water availability in the response to herbivory and the potential trade-off between growth and defence under water stress. In summary, this thesis provides insights on the phenotypic plasticity of the legume-rhizobia interaction and advances our understanding of the function of the chickpea-*Mesorhizobium* symbiosis and the consequences of genetic and environmental factors for maximising the growth and defence of chickpea. This study provides groundwork for future studies, which are important for improving chickpea productivity.

6.2. **Limitation of the study and directions for future research**

One limitation of this study is that the experiments were conducted in pots, which restricts our ability to extrapolate the findings to the field environment. We can significantly enhance

the practical usefulness of data acquired in controlled environments by repeating these experiments under field conditions. Therefore, it is crucial to understand the role of genetic and environmental factors that modulate the chickpea – *Mesorhizobium* symbiosis through field studies to develop chickpea resilience in diverse environments for sustainable agriculture. Some areas that future studies should consider are identified below.

The potential of rhizobial strains to improve chickpea resistance against herbivory

Rhizobia can increase leaf exudates and improve plant defence, likely through the provision of additional nitrogen to the host plant (Chapter 4). Lima bean (*Phaseolus lunatus*) and *Rhizobium* Sp. symbiosis increased nitrogen content in plant tissue, which led to the production of nitrogen-containing cyanogenic defence compounds (Thamer et al., 2011). It would be valuable to further investigate the mechanism and potential of *Mesorhizobium* strains to improve chickpea resistance against herbivory. The mechanism of how rhizobia produce high leaf exudate and how nitrogen that rhizobia provide allocate towards the production of specific defensive nitrogen-containing compounds. Future studies should explore the diversity of rhizobial strains and their differential effects on plant defence to identify effective strains that can be used to reduce crop damage.

Current study showed that plants inoculated with rhizobial strains produced high leaf exudate compared with fertilised and un-fertilised treatments under well-watered conditions (Chapter 4). Future studies should investigate that how rhizobia influence stomata opening to release leaf exudates as a defence against herbivores. Future studies should investigate the effects of rhizobia-mediated leaf exudation on herbivore behaviour and performance to highlight the ecological consequences. In our study, nitrogen fertilised treatment had inadequate supply of nitrogen, which poses implications for the interpretation of results (Chapter 4). Further research is needed to compare the effects of *Mesorhizobium* strains and optimal nitrogen

fertilisation to investigate whether this effect is due to fixed nitrogen *per se* or other underlying mechanisms.

The role of plant associated microorganisms in plant defence

There are many microorganisms present in the rhizosphere that could influence the plant defence mechanisms (Pozo and Azcón-Aguilar 2007; Thamer et al., 2011). Future studies should investigate how these different microorganisms interact with each other and with the host to affect herbivore resistance. Additionally, the root zone harbours a substantial number of beneficial microorganisms due to the high organic derivatives originating from root exudates (Raaijmakers et al., 2009). Similarly, chickpea resistance against insect infestation can be attributed to the presence of leaf exudates rich in organic acids and other organic compounds, which were not part of this study. Besides this, the high amount of leaf exudates on the leaf surface could create favourable conditions for microbial growth, some of which may possess beneficial properties, including suppression of pests. Therefore, the antagonistic effects observed in the current study may be due to the organic compounds present in the leaf exudate (Toker et al., 2004) or the presence of phyllosphere microbes on the leaf surface (Sivakumar et al., 2020). Hence, further investigation is needed to examine the potential benefits of phyllosphere microbiomes on the leaf surface. Additionally, further studies should investigate whether the phyllosphere microbiomes are associated with the beneficial microbiomes in the root zone.

References

Bano, A., Batool, R., Dazzo, F. (2010). Adaptation of chickpea to desiccation stress is enhanced by symbiotic rhizobia. *Symbiosis*, *50*, 129-133.

Brockwell, J., Hely, F., (1966). Symbiotic characteristics of *Rhizobium meliloti*: and appraisal of the systematic treatment of nodulation and nitrogen fixation interactions between hosts and rhizobia of diverse origins. *Australian Journal of Agricultural Research,* 17, 885-899.

Cipollini, D. (2010). Constitutive expression of methyl jasmonate-inducible responses delays reproduction and constrains fitness responses to nutrients in *Arabidopsis thaliana*. *Evolutionary Ecology*, *24*, 59-68.

Dicke, M., Van Loon, J. J., & Soler, R. (2009). Chemical complexity of volatiles from plants induced by multiple attack. *Nature chemical biology*, 5, 317-324.

Divito, G.A., Sadras, V.O. (2014). How do phosphorus, potassium and sulphur affect plant growth and biological nitrogen fixation in crop and pasture legumes? A meta-analysis. *Field Crops Research*, 156, 161–171.

Duc, G., Agrama, H., Bao, S., Berger, J., Bourion, V., De-Ron, A. M., Gowda, C. L., Mikic, A., Millot, D., Singh, K. B. (2015). Breeding annual grain legumes for sustainable agriculture: new methods to approach complex traits and target new cultivar ideotypes. *Critical reviews in plant sciences*, 34, 381-411.

Esfahani, M. N., Mostajeran, A. (2011). Rhizobial strain involvement in symbiosis efficiency of chickpea–rhizobia under drought stress: plant growth, nitrogen fixation and antioxidant enzyme activities. *Journal of Plant Physiology*, 33, 1075-1083.

Flexas, J., Bota, J., Galmes, J., Medrano, H., & Ribas-Carbó, M. (2006). Keeping a positive carbon balance under adverse conditions: responses of photosynthesis and respiration to water stress. *Physiologia Plantarum*, *127*, 343-352.

Gambín, B., Borrás, L. (2010). Resource distribution and the trade-off between seed number and seed weight: a comparison across crop species. *Annals of Applied Biology*, 156, 91-102.

Hafeez, F., Shah, N., Malik, K., (2000). Field evaluation of lentil cultivars inoculated with *Rhizobium leguminosarum* bv. *viciae* strains for nitrogen fixation using nitrogen-15 isotope dilution. *Biology and Fertility of Soils*, 31, 65-69.

Hansen, A.P. (2017). Legume-Rhizobium symbioses: Significance for sustainable plant production. In: Varma A, Sharma A (eds) *Modern tools and techniques to understand microbes*. Cham, Springer, pp. 159–182.

Hungria, M., Kaschuk, G. (2014). Regulation of N2 fixation and NO3−/NH4+ assimilation in nodulated and N-fertilized *Phaseolus vulgaris* L. exposed to high temperature stress. *Environmental and Experimental Botany*, 98, 32–39.

Huot, B., Yao, J., Montgomery, B. L., and He, S. Y. (2014). Growth – defence trade-offs in plants: a balancing act to optimize fitness. *Molecular plant*, 7, 1267–1287.

Israel, D. W., Mathis, J. N., Barbour, W. M., Elkan, G. H. (1986). Symbiotic effectiveness and host-strain interactions of *Rhizobium fredii* USDA 191 on different soybean cultivars. *Applied and environmental microbiology*, 51, 898-903.

Iqbal, N., Sadras, V.O., Denison, R.F., Zhou, Y., Denton, M.D. (2022). Clade-dependent effects of drought on nitrogen fixation and its components–Number, size, and activity of nodules in legumes. *Field Crops Research, 284*, 108586.

Jemo, M., Sulieman, S., Bekkaoui, F., Olomide, O. A., et al. (2017). Comparative analysis of the combined effects of different water and phosphate levels on growth and biological nitrogen fixation of nine cowpea varieties. *Frontiers in Plant Science*, 8, 2111.

Kiers, E. T., Rousseau, R. A., West, S. A., & Denison, R. F. (2003). Host sanctions and the legume–rhizobium mutualism. *Nature*, 425, 78-81.

Larrainzar, E., Wienkoop, S., Scherling, C., Kempa, S., Ladrera, R., Arrese-Igor, C., ... & González, E. M. (2009). Carbon metabolism and bacteroid functioning are involved in the regulation of nitrogen fixation in Medicago truncatula under drought and recovery. *Molecular plant-microbe interactions*, 22, 1565-1576.

Lodwig, E., & Poole, P. (2003). Metabolism of Rhizobium bacteroids. *Critical reviews in plant sciences*, 22, 37-78.

López-Goldar, X., Hastings, A., Züst, T., & Agrawal, A. (2022). Evidence for tissue-specific defence-offence interactions between milkweed and its community of specialized herbivores. *Molecular Ecology*, *31*, 3254-3265.

Mergaert, P., Uchiumi, T., Alunni, B., Evanno, G., Cheron, A., Catrice, O., ... & Kondorosi, E. (2006). Eukaryotic control on bacterial cell cycle and differentiation in the Rhizobium–legume symbiosis. *Proceedings of the National Academy of Sciences*, *103*, 5230-5235.

Okie, J. G., Smith, V. H., Martin-Cereceda, M. (2016). Major evolutionary transitions of life, metabolic scaling and the number and size of mitochondria and chloroplasts. *Proceedings of the Royal Society B: Biological Sciences*, 283, 20160611.

Oono, R., & Denison, R. F. (2010). Comparing symbiotic efficiency between swollen versus nonswollen rhizobial bacteroids. *Plant physiology*, *154*, 1541-1548.

Pozo, M. J., & Azcón-Aguilar, C. (2007). Unraveling *mycorrhiza*-induced resistance. Current opinion in plant biology, 10, 393-398.

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., & Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant Soil*, 321, 341–361.

Rangarajan, A. A., Koropatkin, N. M., & Biteen, J. S. (2020). Nutrient-dependent morphological variability of Bacteroides *thetaiotaomicron*. *Microbiology*, 166, 624-628.

Sadras, V. O. (2007). Evolutionary aspects of the trade-off between seed size and number in crops. *Field Crops Research*, 100, 125-138.

Sadras, V. O., Slafer, G. A. (2012). Environmental modulation of yield components in cereals: heritabilities reveal a hierarchy of phenotypic plasticities. *Field Crops Research*, 127, 215-224.

Sampedro, L., Moreira, X., & Zas, R. (2011). Costs of constitutive and herbivore-induced chemical defences in pine trees emerge only under low nutrient availability. *Journal of Ecology*, *99*, 818-827.

Sivakumar, N., Sathishkumar, R., Selvakumar, G., Shyamkumar, R., & Arjunekumar, K. (2020). Phyllospheric microbiomes: diversity, ecological significance, and biotechnological applications. *Plant microbiomes for sustainable agriculture*, 113-172.

Stewart, B., Lal, R. (2018). Increasing world average yields of cereal crops: it's all about water. *Advances in agronomy*, 151, 1-44.

Thamer, S., Schädler, M., Bonte, D., & Ballhorn, D. J. (2011). Dual benefit from a belowground symbiosis: nitrogen fixing rhizobia promote growth and defense against a specialist herbivore in a cyanogenic plant. *Plant and Soil*, 341, 209-219.

Toker, C., Karhan, M., & Ulger, S. (2004). Endogenous organic acid variations in different chickpea (Cicer arietinum L.) genotypes. *Acta Agriculturae Scandinavica, Section B-Soil & Plant Science*, 54, 42-44.

War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., & Sharma, H. C. (2012). Mechanisms of plant defense against insect herbivores. *Plant signaling & behavior*, 7, 1306-1320.

Yang, J., Zhang, J., Wang, Z., Zhu, Q., & Liu, L. (2002). Abscisic acid and cytokinins in the root exudates and leaves and their relationship to senescence and remobilization of carbon reserves in rice subjected to water stress during grain filling. *Planta*, 215, 645-652.