Symbiotic effectiveness, phylogenetic diversity and ecological adaptation of chickpea rhizobia isolated from Australian and Myanmar soils

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Acronyms and abbreviations

ABARES	Australian Bureau of Agricultural and Resource Economics
ACIAR	Australian Centre for International Agricultural Research
AGRF	Australian Genomic Research Facility
$AlCl_3$	Aluminium chloride
ANI	Average nucleotide identity
ANOVA	Analysis of variance
APAL	Australian Precision Agricultural Laboratory
BLAST	Basic local alignment search tool
BNF	Biological nitrogen fixation
BOM	Bureau of Meteorology
CDZ	Central Dry Zone
CFU	Colony forming unit
cm	Centimetre
cv.	Cultivar
$CoSo_4$	Cobalt sulphate
$CuCl_2$	Copper chloride
^{o} C	Degree Celsius
CEC	Cation exchange capacity
DNA	Deoxyribonucleic acid
EC	Electrical conductivity
FAOSTAT	Food and Agriculture Organisation Statistics
g	Gram
GRDC	Grain Research and Development Corporation
h	hour
ha	hectare
HCl	Hydrochloric acid

HGT	Horizontal gene transfer
H_2O	Water
ICEs	Integrated and Conjugative Elements
ICP	Inductively coupled plasma spectrometry
IGS	Intergenic spacer
kg	Kilogram
KNO_3	Potassium nitrate
KOH	Potassium hydroxide
L	Litre
LPO	Lipo-Chito Oligosaccharide
LSD	Least standard deviation
M	Molar
MEGA	Molecular Evolutionary Genetics Analysis
mg	Milligram
mL	Millilitre
µg	Microgram
µmol	Micromole
µL	Microlitre
min	Minute
MLSA	Multilocus sequence analysis
mm	Millimetre
mM	Millimolar
mmol	Millimole
$MnSo_4$	Manganese sulphate
MOALI	Ministry of Agriculture, livestock and Irrigation
N	Nitrogen
N_2	Dinitrogen

NCBI	National Centre for Biotechnology Information
NaOCl	Sodium hypochloride
NaCl	Sodium chloride
Ndfa	Nitrogen derived from atmosphere
NDW	Nodule dry weight
NF	Nodulation (Nod) factor
NH_3	Ammonia
NH_4	Ammonium
$NiSo_4$	Nickel sulphate
NN	Nodule number
NSW	New South Whales
NVT	National Variety Trial
p	Probability
PCR	Polymerase chain reaction
pH	Potential hydrogen
Qld	Queensland
RCB	Randomised completed block
rDNA	Ribosomal deoxyribonucleic acid
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
r.p.m	Revolution per minute
s	Second
SA	South Australia
SDW	Shoot dry weight
SNP	Single nucleotide polymorphism
SE	Symbiotic effectiveness
SPAD	Soil Plant Analysis Development

t	Tonne
Tg	Teragram
v	Volume
VIC	Victoria
w	Weight
WA	Western Australia
YAU	Yezin Agricultural University
YMA	Yeast mannitol agar
YMB	Yeast mannitol broth
$ZnCl_2$	Zinc chloride

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Abstract

The presence of rhizobia has significant impact on the nodulation, nitrogen fixation and productivity of chickpea. However, inoculation practices for chickpea differ globally, which may impact the genetics, physiology and utility of rhizobia. Nodulation and symbiotic effectiveness (SE) of 80 rhizobial strains isolated from across Australian cropping regions were evaluated in a glasshouse experiment. Tolerance of these rhizobial strains to pH, temperature, antibiotics, heavy metals and NaCl were examined on supplemented YMA plates *in vitro*. In addition, the phylogenetic diversity among Australian chickpea rhizobia were investigated by sequencing of 16S-23S rRNA, *atpD*, *recA*, *nodC* and *nifH* genes. Phylogenetic analysis of 16-23S rRNA IGS, *atpD* and *recA* genes revealed that most Australian strains belonged to *Mesorhizobium ciceri* (68%), with most of the remaining strains closely associated with *M. temperatum, M. huakuii* and *M. tianshanense.* Although the strains were diverse in 16S-23S rRNA IGS-based phylogeny, they shared similar symbiosis genes with common chickpea symbionts. Inoculation of chickpea with 80 strains collected from Australia revealed that variation in SE% among isolated strains was correlated with phylogenetic relatedness to the commercial inoculant strain *Mesorhizobium ciceri* CC1192. Strain A47 collected from Queensland gave the highest shoot biomass and two strains (A78 and A79) from Western Australia grew under acidic conditions (pH 4.4), indicating the potential adaptation of these strains to different environmental conditions. Some isolated strains had equal or superior SE relative to inoculant strain CC1192. The incongruence between core and symbiosis gene phylogenies of rhizobial strains in this study suggests the potential occurrence of HGT of symbiosis genes in Australian soils.

A total of 120 *Mesorhizobium* strains were isolated from 103 soils sampled from fields in the central dry zone (CDZ) of Myanmar and evaluated their infectivity and effectiveness in a pot experiment. Nodulation and SE varied considerably among these strains and the majority

(about 90%) provided high shoot dry weight, which was comparable with CC1192. Strain M082 had the highest SE, followed by M009 in a pot experiment and were considered as potential strains to be evaluated under field conditions. Some strains showed potential tolerance of low pH (e.g., M094 and M113), high temperatures (M.021, M075) and 3% NaCl (w/v) such as M062 and M107. The 16-23S rDNA IGS phylogeny confirmed that all Myanmar strains were members of the genus *Mesorhizobium* and most Myanmar strains were most closely related to *M. gobiense*, *M. muleiense*, *M. silamurunense*, *M. tamadayense* and *M. temperatum.* Three of the four main species groups *Mesorhizobium*; *M. gobiense*, *M. huakuii* and *M. muleiense*, were distributed throughout Myanmar but *M. temperatum* was not found in the Southwestern area of Magway. Nearly 70% of Myanmar strains were most closely related to Indian strain IC-2058 (CA-181), which is also most closely related to *M. gobiense*, while none of Myanmar strains were closely related to the cognate chickpea rhizobial species *M. ciceri* and *M. mediterraneum*. However, Myanmar strains shared shared similar *nodC* and *nifH g*ene sequences with chickpea symbionts. Detailed sequence analysis of the *nodC* and *nifH* found that the strains in Myanmar were slightly divergent from the group of cognate chickpea rhizobia and were more closely related to symbiotic genes of *M. muleiense* and the Indian strain IC-2058-CA181. Mutation (substitution) in the *nodC* protein had no significant effect on nodulation and SE of the test strains.

Comparative analysis of 16S-23S rDNA sequences of strains from Australia and Myanmar revealed that there was little overlap in species found in the two countries. The only species found in both Myanmar and Australia were *M. tamadayense* and *M. silumurunense*. The isolated Australian and Myanmar strains showed similar adaptive traits, and the adaptation traits were phylogenetically related within each country. The genetic discrepancy between Australian and Myanmar strains was not only due to inoculation history but to adaptation to soil conditions and crop management over time in each country, and there has been virtually

no loss of symbiotic efficiency of Myanmar strains relative to Australian commercial inoculant CC1192, in pot experiments.

Twelve strains were selected based on their improved SE for chickpea in pot experiments, and acid tolerance in laboratory experiments, and were then tested in five separate field experiments. In low pH soils, the strains collected in Australia were vastly superior compared with those from Myanmar, however in most cases they did not perform better than CC1192. Myanmar strains had lower SE in Australian field conditions compared with performance under glasshouse conditions. As an exception, two Myanmar strains (M022 and M065) provided significantly higher SDW than CC1192 in the low rainfall and high pH sandy soil environment in Loxton. A strong positive association was found between survival of rhizobia on seed and nodulation. Two Australian strains (A21 and A47) and CC1192 showed higher survival on seed at 3 to 24 hours after inoculation and better nodulation across experimental sites. Strain A47 improved symbiotic N_2 fixation and strain A78 showed some tolerance to strongly acidic soil (pH 4.18). The inconsistent performance of Myanmar strains in this study may be due to genetic divergence from common inoculant species, or they simply are not able to survive as an inoculant (on the seed) or in Australian soils. This may not be the case in Myanmar soils, and strains isolated from Myanmar should be further tested in Myanmar soil conditions. Future research in this area should consider the testing of these strains across multiple variable field environments to further evaluate their potential as inoculants to improve chickpea performance.

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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Myint Zaw 29/01/2022

Chapter 1. General introduction

1.1. Introduction

Chickpea (*Cicer arietinum*) is the second most important food legume globally after common bean (*Phaseolus vulgaris*). Chickpea was domesticated from the wild species (*C. reticulatum*) and its sister species (*C. echinospermum*) in fertile crescent between 10,000 and 12,000 years ago (Redden and Berger 2007; Von Wettberg *et al.* 2018). After domestication chickpea was then distributed from the Middle East and Mediterranean basin to Indian subcontinent during the Bronze Age (Redden and Berger 2007; Plekhanova *et al.* 2017) and to Ethiopia between 2,000 and 3,000 years ago (Redden and Berger 2007). Chickpea can contribute N_2 in shoot biomass up to 141 kg N ha^{-1} when in symbiotic association with specific *Mesorhizobium* species (Unkovich *et al.* 2010; López-Bellido *et al.* 2011). However, the rhizobial inoculation practice varies among countries, with rare or absent inoculation in countries like India and Ethiopia to frequent application of effective commercial inoculants in developed countries like Canada, U.S.A and Australia (Greenlon *et al.* 2019).

Australia is the largest exporter of desi chickpea in the world and chickpea is most common pulse crop in Australia (ABARES 2019). Chickpea was introduced to Australia in 1970s and is mainly grown in northern eastern and parts of the southern cropping regions of Australia, with only minor production in the western parts of the grains belt. Since initial cultivation, the commercial inoculant strain *M. ciceri* CC1192 has been frequently applied to Australian chickpea crops (Corbin *et al.* 1977). Despite the use of a single inoculant strain over time, the symbiotic effectiveness of rhizobia residing in soil has been observed to decline from that of the inoculant strain (Sullivan *et al.* 1995; Batista *et al.* 2007; Elias and Herridge 2015). This scenario has been studied in chickpea soils in a relatively small sampling region in northern NSW, where strains re-isolated from soils showed low to similar symbiotic effectiveness (SE)

to the commercial strain, with no strains exceeding the SE of the commercial inoculant strain CC1192. However, large-scale assessment of genetic and phenotypic diversity in chickpea rhizobia across Australia has not been conducted.

In Myanmar, chickpea is an important cash crop that is sown on stored moisture as a second crop in the post-monsoon season. Chickpea is mainly cultivated in Sagaing, Mandalay and Magway regions, that are collectively known as the central dry zone area (CDZ) of Myanmar. The demand for both domestic consumption and export of Myanmar chickpea is increasing, with about 108,000 MT production per annum, worth approximately 100 million USD in 2018 (https://www.selinawamucii.com/insights/market/myanmar/chickpeas/); the harvested areas increased from 120k ha to 380k ha between 1994 and 2019 (FOASTAT 2021). Despite the importance of chickpea to Myanmar, this crop is mostly cultivated by small-holder farmers with very limited inputs, including inoculation. There is no current established supply chain for rhizobial inoculants in Myanmar (Denton *et al.* 2017), although some attempts have been made to produce inoculants on a small scale using indigenous strains selected for chickpea; these covered < 25% of chickpea growing areas (Herridge *et al.* 2008; ACIAR report 2019). Despite very low levels of inoculation, chickpea plants in Myanmar can be well-nodulated, and soils have high populations of resident rhizobial strains. The resident symbiotic rhizobia probably evolved with chickpea plants over a long period of cultivation and may have improved adaptation and symbiotic efficiency.

In *Mesorhizobium*, symbiosis genes (*nod, nif, fix*) that are required for nodulation and nitrogen fixation are in the integrative and conjugative element (ICE) region, which is mobile genetic material (Hill *et al.* 2021). The nodulation (*nod*) genes in *Mesorhizobium* are considered highly specific, and all chickpea-nodulating rhizobia share highly similar *nodC* sequences (Greenlon *et al.* 2019; Laranjo *et al.* 2008). Although symbiosis genes of *Mesorhizobium* are encoded in

chromosomes, they may be easily transferred within or between species because they are located on ICEs. As an example, the horizontal transfer of the symbiosis islands (HGT) of Australian commercial inoculant *M. ciceri* CC1192 *in vitro* to an ICE-cured derivative of *M. japonicum* (Hill *et al*. 2021) HGT may have contributed to variation and diversity in chickpea-nodulating rhizobia in Australian and Myanmar soils through the transfer of nodulation genes from symbiotically capable *Mesorhizobium* inoculants or co-introduced symbionts with chickpea.

The success of growing chickpea depends on the presence of well-adapted rhizobia. Success of inoculation under field conditions is limited by several factors, in particular the soil environment and the survival of rhizobia on the seed (Brockwell *et al.* 1995). Unpredictability of rainfall events in Australia, and dry conditions during the growing season in Myanmar have significant negative impacts on legume production. It is imperative that rhizobial inoculants applied on seed survive and colonise the rhizosphere for effective nodulation (Drew *et al.* 2012a). Soil acidification is also of growing concern throughout Australian cropping soils the extent of this problem is potentially increasing due to inadequate treatment and intensification of land management (McKenzie *et al.* 2017), while in Myanmar soils, rhizobial strains must survive in limited moisture conditions and higher temperatures compared with Australia. These abiotic stresses together are particularly challenging for rhizobial survival and nodulation on such difficult soils (Eberbach and Douglas 1989; Brockwell *et al.* 1991; Howieson 1995; Howieson and Ballard 2004; Evans 2005; Denton *et al.* 2007; Rathjen *et al.* 2020; Yates *et al.* 2021). It is possible that soils in Australia and Myanmar may contain diverse isolates, which may provide the basis of new chickpea inoculants.

1.2. Objectives

This thesis aims to investigate the phenotypic and genetic diversity of chickpea rhizobia from Australia and Myanmar soils. Research objectives in chapters 2 to 5 focus on specific aspects of achieving this aim (Fig. 1.1):

(1) Chapter 2 reviews the process of biological nitrogen fixation (BNF), abiotic factors affecting BNF, useful techniques in studying diversity of legume rhizobia and biogeographic distribution of chickpea rhizobia worldwide.

(2) Chapter 3 investigates phenotypic and phylogenetic diversity of resident chickpea-nodulating rhizobia from a large-scale Australian soil collection.

(3) Chapter 4 analyses the phylogenetic and phenotypic diversity of rhizobia in Myanmar to provide a more detailed picture of the diversity of chickpea nodulating rhizobia, particularly in comparison with Australia.

(4) Chapter 5 assesses highly adapted strains for their use as inoculants to fix nitrogen and improve chickpea productivity in acid and dry soils.

(5) Chapter 6 provides a general synthesis of the results.

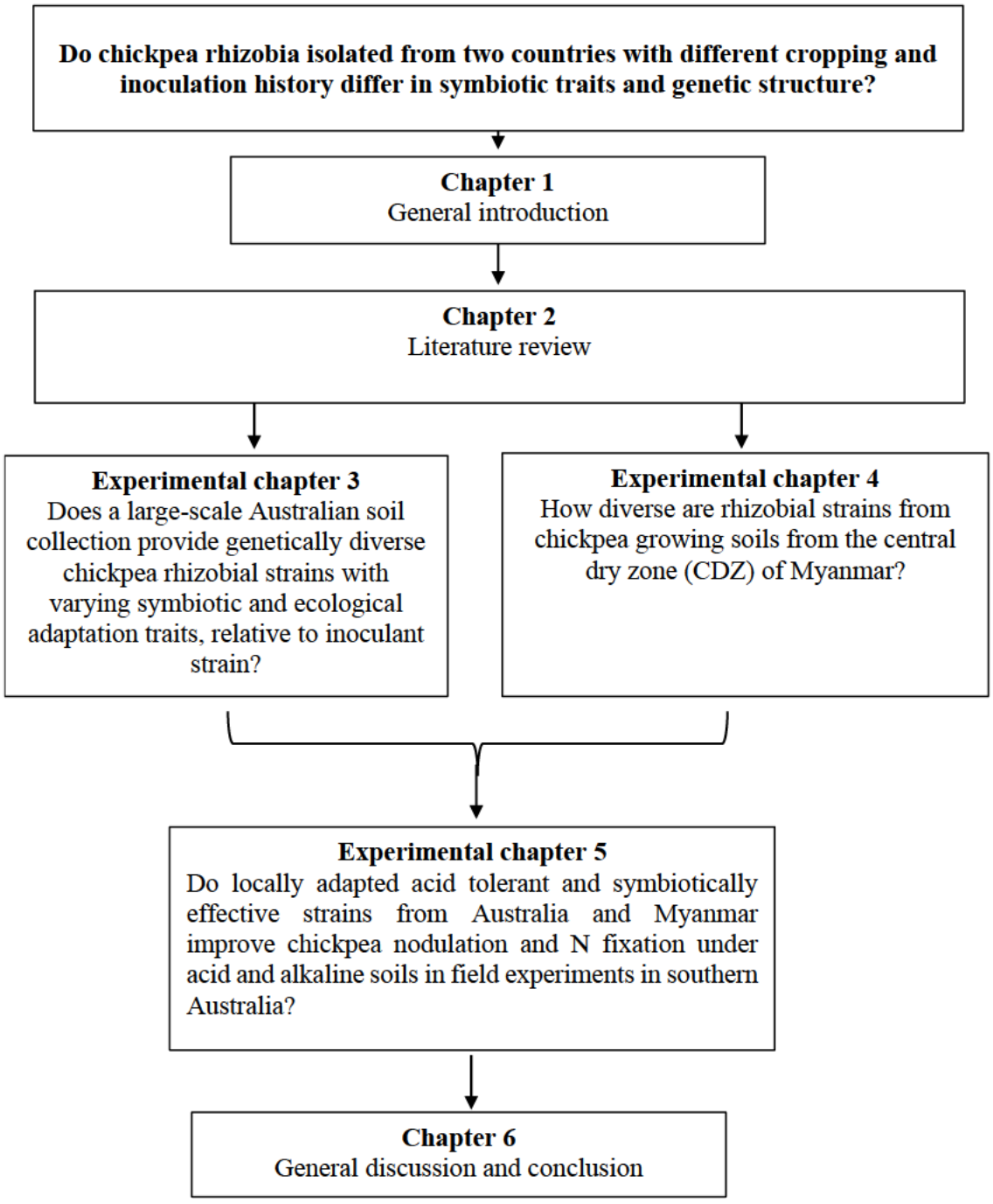

Figure 1.1. Thesis structure

1.3. References

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Chapter 2. Literature review

2.1. Chickpeas in Australia and Myanmar

Chickpea (*Cicer arietinum*) is the world's second most cultivated pulse legume after common bean. The global chickpea production has increased to 14.7 million MT, with 85.5% of total production was accounted by Asian countries (FAOSTAT 2021). Globally, about 2.4 million tons of chickpea each year enter the world market, as it is a high demand food in many countries. India is the largest producer and importer of chickpea as well as the second leading exporter after Australia. Australia is the major leading exporter of chickpea accounting for nearly half of world chickpea exports, with an estimate of over 926,802 tons annually (FAOSTAT 2021). Australian chickpea export earned about $906 million (ABARES 2019). The harvested areas of chickpea in Myanmar have also increased from 120k ha to 380k ha during 1994-2019 (FAOSTAT 2021). There is a high demand for Myanmar-produced chickpea in India, Singapore, and Pakistan.

Chickpea was domesticated from the wild species (*C. reticulatum*) and its sister species (*C. echinospermum*) in fertile crescent between 10,000 and 12,000 years ago (Redden and Berger 2007; Von Wettberg *et al.* 2018). Globally, chickpea is currently grown in more than 50 countries from the Indian subcontinent, the Middle East, the southern Europe, North Africa, America, and Australia (Jukanti *et al.* 2012). There are two distinct types of domesticated chickpea: Desi and Kabuli. Desi chickpeas account for more than 80% of the world total chickpea area and are mainly grown in Asia and Africa (Pande *et al.* 2005), while Kabuli types are mostly grown in West Asia, North Africa, North America and Europe (Jukanti *et al.* 2012). The main importers of Desi chickpea are India and Pakistan, while Kabuli types are mainly imported by Middle East and Europe.

Chickpea is an important protein source for human nutrition. Due to its nutritional values for energy and health benefits, the demand for chickpea is increasing worldwide (Wood and Grusak 2007; Jukanti *et al.* 2012). Chickpea provides vegetable protein for human diets in developing countries where animal proteins are not affordable for many people, or for people who are vegetarians. Both Desi and Kabuli chickpeas can be eaten raw as a fresh vegetable or after boiling, roasting, processing into flour that can be used in making different meals such as Myanmar tofu. The split Desi chickpea is mostly used in making a soup to serve with rice in most South Asian countries (Merga and Haji 2019).

Due to its ability to grow on infertile soils under low rainfall conditions, chickpea provides the benefits in agricultural productivity and sustainability through reducing N fertiliser input, which improves the productivity of chickpea itself and benefits following crops (Herridge *et al.* 1995; Dalal *et al.* 1998; Felton *et al.* 1998). In the northern and south-eastern regions of Australia, chickpea is an valuable rotation crop with wheat, barley, and sorghum, as it minimizes the incidence of cereal diseases (Felton *et al.* 1998; Kirkegaard *et al.* 2004), provides more effective weed control than continuous cereals through greater herbicide options (Lucy *et al.* 2004), in addition to providing biological nitrogen fixation ((Herridge *et al.* 1995; Dalal *et al.* 1998; Felton *et al.* 1998).

In Myanmar, chickpea is an important cash crop with increasing demand for both domestic consumption and export. Chickpea cultivation in Myanmar is concentrated in central dry zone (CDZ), where chickpea is usually sown as a second crop on residual soil moisture after rice on lowlands and in rotation with other oil-seeded and pulse crops on uplands. In CDZ, lowlands are mostly Vertisols, while course-textured sandy soils with low organic matter are common in uplands. With increasing demand for Myanmar chickpea in the Indian market, chickpea cultivation has expended from120k ha to 380k ha between 1994-2019 (FAOSTAT 2021).

Myanmar earned approximately 100 million USD from chickpea export in 2018 (https://www.selinawamucii.com/insights/market/myanmar/chickpeas/).

2.2. Nodulation and nitrogen fixation in chickpea

Biological nitrogen fixation (BNF) is one of the most important processes for plants after photosynthesis. Although the atmosphere contains about 79% nitrogen (N_2), its reduction into plant available form ($NH4^+$) is an energy-consuming process. Several soil beneficial microbes such free living free-living diazotrophs, symbiotic and endophytic diazotrophic bacteria can perform BNF in association with legumes (Sylvia *et al.* 2005). BNF by various legume-nodulating bacteria is an important supply of N for both natural system and agricultural crops (Vance 2002), and globally it contributes about 50-70 Tg fixed N $year^{-1}$ in agriculture (Herridge *et al.* 2008). The rhizobial symbiotic association with crop legumes (pulses and oil-seeded legumes) and pasture legumes contributes annual N supplies of 21.5 Tg N and 12-25 Tg N annually, respectively (Herridge *et al.* 2008).

Nodule formation is a result of chemical signalling between legume hosts and rhizobia. Nodulation is initiated by secretion of plant-derived flavonoids to synthesise *nodD* protein for the transcription of other nodulation genes (*nodABC*) that synthesize nod factor (NF) or lipochitooligosaccharides (LCOs) (Nelson and Sadowsky 2015). The chemical modification of NFs/LCOs vary depending on the specific rhizobial species that determine host specificity (Via *et al.* 2016). The NFs are recognized by LysM receptors and even a single mutation in these receptors may change the specificity at the species level of rhizobia (Radutoiu *et al.* 2007). The NF/LCOs comprise two parts: N-acetylglucosamine units and a long-chain fatty acid. Failure in nodulation will occur when either of these two parts of LCOs are disrupted (Wang *et al.* 2019).

Nodule initiation occurs when the root hair deforms and curls and forms infection thread by enzymatic dissolution of root-hair cell wall (Djordjevic and Weinman 1991) (Fig. 2.1). Rhizobial cells release cellulase to make an entry hole on root hair through which they enter a plant-derived infection thread, then colonise and proliferate in the extracellular spaces (Brewin 1991; Brewin 2004; Poole *et al*. 2018; Sprent *et al*. 2017). After infection and formation of nodules, the cells of rhizobia differentiate into bacteroids inside the nodules. These bacteroids are enclosed by a symbiosome, which provides ideal conditions for the nitrogenase enzyme to transform N_2 to ammonia. The conversion of one mole of N_2 to ammonia requires about 8 moles of electrons and 16 moles of ATP hydrolysis (Seefeldt *et al.* 2009) (Fig. 2.1). This association can benefit both legumes and rhizobia as the host plants provide carbon and an energy source to rhizobia, and the rhizobia fix atmospheric nitrogen that is used by the plants (Poole and Allaway 2000).

Chickpea is considered a highly specific host that can only be nodulated by a single genus, *Mesorhizobium*. In nodulation, there are different levels of specificity between the groups of host plants and compatible rhizobial species or strains for symbiotic association (Simms and Taylor 2002; Oldroyd 2013; Andrews and Andrews 2017). Firstly, specific flavonoids secreted by some legumes are only recognised by specific species or strains of rhizobia (Wang *et al.* 2012). In this case, the rhizobial transcription regulator (*nodD*) recognises the specific flavonoids that trigger the production of NF. Only NF recognised by the plant can induce deformation and curling of root hair formation of infection thread, and nodule organogenesis since the primary structure of NFs and their decorations or functional groups differ. Secondly, the plant controls the specificity by regulating the movement of the rhizobia via the infection thread and their release into the cortical cells of the roots (Simms and Taylor 2002; Wang *et al.* 2012). The plants release receptor lectins that only recognise the surface polysaccharides of specific rhizobia, thereby promoting multiplication and release of the correct rhizobia into

cortical cells before differentiation (Simms and Taylor 2002). Generally, the *nod* gene inducers (flavonoids) are flavones and flavonones for fast growing rhizobia, while these are the isoflavones in slow growing rhizobia. The two flavonoids: naringenin and daidzein have been identified in *M. ciceri* (Srivastava *et al.* 1999).

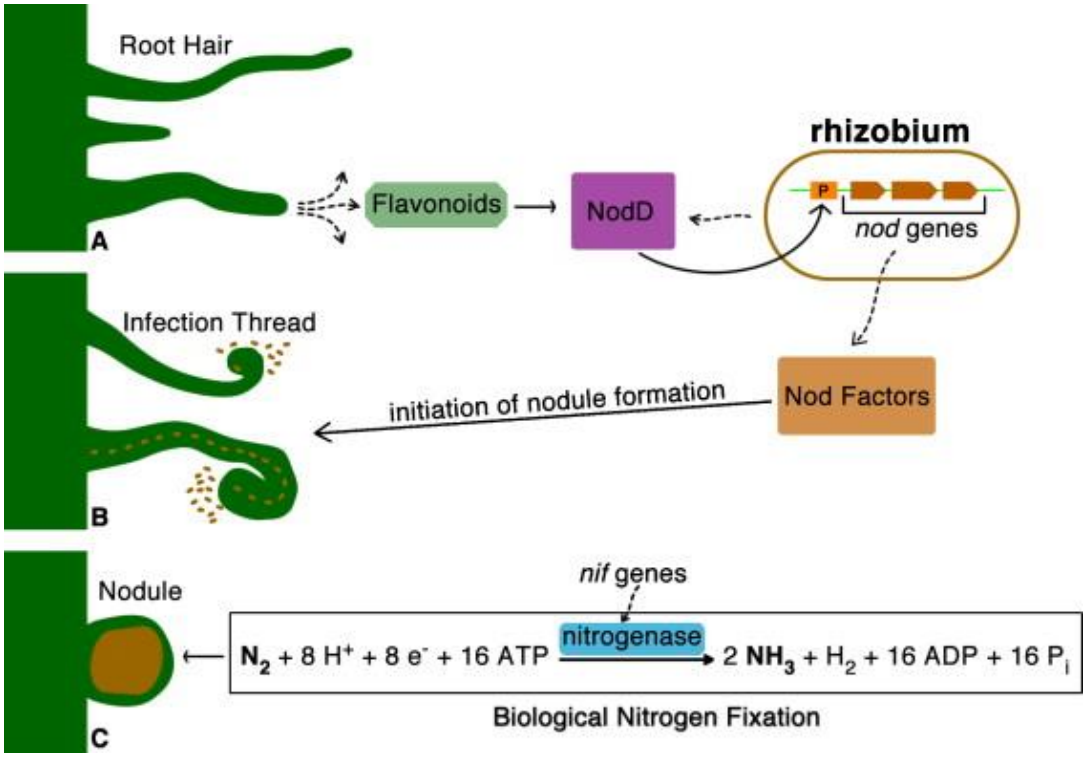

Fig. 2.1 A schematic diagram of nodulation and N_2 fixation process in symbiotic association between legume and rhizobia (Laranjo *et al.* 2014).

2.3. Symbiotic genes and host specificity

All symbiotic rhizobia contain common nodulation genes (*nodABC, nodD, nodIJ*) except for some *Bradyrhizobium* strains (Giraud *et al.* 2007). The *nodABC* genes encode for the proteins involved in the synthesis of the LCOs. The LCOs are then modified by species-specific enzymes that offer specific nucleotide substitutions for specific legumes (Long 1996). These nucleotide substitutions are responsible for symbiotic specificity (Lewin *et al.* 1990; Long

1996). The specific NF receptors (NFRs) must recognise specific LCOs to initiate nodulation (Nelson and Sadowsky 2015), although some promiscuous legumes such as *Sophora flavescens* may have one or more different NFRs and could be nodulated by various *nodC*-specific rhizobia (Jiao *et al.* 2015a; Liu *et al.* 2018). Different rhizobial genera may have different nodulation genes that can only be recognized by their specific compatible legume partners (Sprent 2009). *Mesorhizobium ciceri* has a very narrow host range and, that can only nodulate the genus *Cicer* (Sharma et al., 1994). However, for chickpea nodulation, *nod* genes are highly specific and only some *Mesorhizobium* species: *M. ciceri* (Nour *et al.* 1994), *M. mediterraneum* (Nour *et al.* 1995), *M. amorphae* (Rivas *et al.* 2007), *M. tianshanense* (Rivas *et al.* 2007), *M. loti* (Laranjo *et al.* 2008), *M. huakuii* (Alexandre *et al.* 2009), *M. opportunistum* (Laranjo *et al.* 2012) and *M. muleiense* (Zhang *et al.* 2012a) carry symbiosis genes required for effective nodulation in chickpea.

To determine the specificity of nod genes in *Mesorhizobium ciceri*, a cross-inoculation study was conducted between *biserrula* and chickpea. It was found that there was no cross-inoculation compatibility between *Biserrula* and chickpea mesorhizobia, which carry different nodulation genes, even though their chromosomal backgrounds are related (Nandasena *et al.* 2004; Nandasena *et al.* 2006). The reciprocal inoculation also showed that *M. ciceri* type strain isolated from chickpea did not nodulate symbiovar *biserrula.* Other legumes do not show the same level of specificity, with some symbiovar *trifolii* able to nodulate clover and *Pisum sativum* (pea) (Marek-Kozaczuk *et al.* 2013). Additionally, some rhizobial strains such as *Sinorhizobium* sp. strain NGR234 can nodulate up to 112 legume genera (Pueppke and Broughton 1999). The *nodD* of *M. ciceri* induced the expression of *nod* gene inducers in response to chickpea root exudates, while the expression of *nod* inducers was inhibited by root exudates of other legumes, indicating the high specificity between *M. ciceri* and chickpea (Kamboj *et al.* 2010).

2.4. Rhizobial inoculation in chickpea

Due to the high specificity of chickpea to nodulate with *Mesorhizobium* spp. (Phyllobacteriaceae) (Kamboj *et al.* 2010), inoculation with a compatible strain is required for nodulation. The nodulation genes for chickpea are not found in most countries of the world. Chickpea has high N requirements for growth and high grain protein and BNF achieved through inoculation with effective rhizobia is an economical means to reduce the cost of N fertilizer in sustainable farming systems.

Chickpea nodulating rhizobia were not present in Australian soils before the introduction of chickpea in 1970s and commercial chickpea inoculant strain CC1192 was applied to optimise the crop productivity through symbiotic nitrogen fixation (Corbin *et al.* 1977). Chickpea grain yields in Australian cropping soils are often suboptimal and chickpea itself is N_2 limited because of poor nodulation and N_2 fixation efficiency, possibly due to high specificity and limited adaptative traits to grow in some soils (Schwenke *et al.* 1998; Herridge *et al.* 2008). It is recommended that chickpea is inoculated in every year that they are sown in Australia to avoid inoculation failure of this important crop.

In Myanmar, the requirement for inoculation is not as vital, as chickpeas appear to be frequently well-nodulated despite virtually no records of widespread inoculation. Although there was a small scale-inoculant production using indigenous strains (YAU45, YAU55, YAU65 and YAU92) selected for chickpea (Than 2010), it only partially covered 25% of the chickpea growing areas until 2019. There were marginal beneficial responses to inoculation with an effective Australian commercial inoculant strain CC1192 at most field experimental sites, despite rather low populations of resident rhizobia (5 to 32,000 cfu/g soil) at 17 out of 20 sampled sites (Denton et al. 2017). However, chickpea is typically well-nodulated in Myanmar with large nodules in most fields.

Rhizobia may be applied in legume cultivation by inoculating seed or soil. Seed coating with peat inoculant slurry is the most common method of inoculating Australian chickpea. It is important to sow the seeds immediately after inoculation as rhizobial cells will die quickly when expose to drying winds, high temperatures and direct sunlight (Deaker *et al.* 2004). Granular and liquid inoculants are also used in some circumstances (GRDC 2018). In Myanmar, the limited awareness of rhizobial inoculation by legume growers may be due to ineffective inoculants caused by poor storage and transportation conditions. To optimise nodulation in N fixation in legumes, access to effective inoculants is vital.

2.5. Diversity and taxonomy of chickpea rhizobia

Chickpea rhizobia were initially assigned into the genus *Rhizobium*, as *R. ciceri* (Nour *et al.* 1994) and *R. mediterraneum* (Nour *et al.* 1995). However, since chickpea rhizobia have an intermediate growth rate compared with fast growing rhizobia (e.g., *Rhizobium*) and slow growing rhizobia (e.g., *Bradyrhizobium*), a new genus, *Mesorhizobium* (meso-, middle, intermediate) was proposed, and these two species of chickpea rhizobia were reclassified as *Mesorhizobium: M. ciceri* and *M. mediterraneum* (Jarvis *et al.* 1997). Later, two other novel species of chickpea rhizobia were proposed, *M. muleiense* (Zhang *et al.* 2012a) and *M. wenxiniae* (Zhang *et al.* 2018). To date, many other *Mesorhizobium* species were identified after isolation from other legume hosts, such as *M. abyssinicae, M. amorphae, M. gobiense, M. huakuii, M. loti., M. opportunistum, M. plurifarium M. tarimense, M. temperatum* and *M. tianshanense, M. tamadayense*. These shared highly similar symbiosis gene sequences with *M. cieri* and *M. mediterraneum* and were reported as potential symbionts able to nodulate chickpea (Rivas *et al.* 2007; Alexandre *et al.* 2009; Laranjo *et al.* 2012; Elias and Herridge 2015; Tena *et al.* 2017; Dekkiche *et al.* 2018; Zaw *et al.* 2021). Some studies also reported that three other genera: *Sinorhizobium* (*Ensifer*) (Dekkiche *et al.* 2018), *Agrobacterium* and *Ochrobactrum* (Naseem *et al.* 2005) were also isolated from chickpea nodules. The current list of symbiotic

Mesorhizobium species isolated from chickpea nodules in different countries are presented in Figure 2.2.

Generally, the diversity of chickpea rhizobia is associated with the geographic locations, the history and crop domestication history. *M. ciceri* and *M. mediterraneum* were considered the cognate chickpea rhizobia found in association with wild relative of chickpea from the centre of origin, southern Turkey (Von Wettberg *et al.* 2018) and the type strains of these two species were initially isolated from domesticated chickpea nodules and Spain (Nour *et al.* 1994; Nour *et al.* 1995). Currently, these two cognate chickpea rhizobia are widely distributed in many countries including Ethiopia, India, Morocco, Portugal, Iran, Spain, and Tunisia (Fig. 2.2). However, some species such as *M. muleiense* and *M. wenxiniae* were found only in chickpea nodules in China and Myanmar (Zhang *et al*. 2018; Soe *et al*. 2020). The most commonly found chickpea nodulating speciesv are *M. ciceri* and *M. mediterraneum*, probably due to co-introduction of these species with seeds imported from the chickpea centre of origin. A diversity study conducted in Indian soils showed low diversity of rhizobial species, with the majority of strains (54%) clustered with *M. ciceri* and *M. mediterraneum*, and the rest of the strains (46%) shared similar 16S rRNA sequence with *M. loti* (Rai *et al.* 2012). Moroccan soils also were also shown to have a low diversity species, with only three major chickpea symbionts *M. ciceri*, *M. mediterraneum* and *M. loti* (Maatallah *et al.* 2002). Soils in Iran (Rouhrazi and Khodakaramian 2015), Spain (Nour *et al.* 1994; Nour *et al.* 1995) and Tunisia (L'taief *et al.* 2007) were shown to contain the least diverse chickpea rhizobial species, with only two cognate chickpea symbionts: *M. ciceri* and *M. mediterraneum* dominating the chickpea growing regions. In contrast, soils in Portugal (Laranjo *et al.* 2004; Rivas *et al.* 2007; Laranjo *et al.* 2008) and Algeria (Dekkiche *et al.* 2018) contained a vast array of chickpea nodulating mesorhizobia, including both cognate chickpea symbionts and other species such as *M. amorphae*, *M. opportunistum*, *M. tianshanense*, *M. tarimense* and *M. tamadayense*.

M. muleiense was found to nodulate chickpea in alkaline Chinese soils, where chickpea has been grown for over 2500 years (Zhang *et al.* 2012a, b, 2014), but very few instances of the cognate chickpea rhizobia have been discovered in China and Myanmar. In Myanmar, only three dominant *Mesorhizobium* species: *M. muleiense*, *M. tianshanense* and *M. plurifarium* have been found, although these strains were isolated from limited sampling locations (Soe *et al.* 2020), In contrast, a low diversity of chickpea rhizobia was detected in countries (e.g. Australia) with more recent introduction of chickpea which was usually inoculated with effective *M. ciceri* inoculants, where the majority of resident strains were related to *M. ciceri* or *M. mediterraneum* (Elias and Herridge 2015; Zaw *et al.* 2021).

Rhizobial strains have been shown to genetically evolve after introduction into the soil (Sullivan et al. 1995), and another study showed that only 6.4 % of isolates recovered from field-grown nodules sharing identical nucleotide sequences with introduced strains (Batista *et al.* 2007). In Western Australian soils, diverse resident mesorhizobial populations nodulating *Biserrula pelecinus*, were found six years after inoculant introduction. As these diverse strains were distantly related to original inoculant strain, it suggests the possible transfer of symbiosis genes from inoculant strain resident *Mesorhizobium* species (Sullivan et al. 1995). In Australian chickpea production,a reduction in symbiotic effectiveness over time of introduced *Mesorhizobium ciceri* CC1192 suggested that there has been genetic alteration among the naturalized and inoculant strains in soils (Elias and Herridge 2015; Zaw *et al.* 2021). Understanding the diversity of rhizobia is important to investigate mesorhizobial species with different background genetics that nodulate chickpea in different environments, and with different inoculation histories.

2.6. Biogeographic distribution of chickpea rhizobia

Cicer arietinum was domesticated from wild chickpea (*C. reticulatum*) in south-eastern Turkey since over 11,000 years ago (von Wettberg *et al.* 2018). Then, chickpea was distributed to the Middle East and Mediterranean basin, Indian subcontinent, and Ethiopia. To date, more than 50 countries including those from the America, Indian subcontinent, Middle East, North Africa, North America, southern Europe and Australia are growing chickpea (Jukanti *et al.* 2012). A study of the impact of chickpea domestication on diversity of chickpea rhizobia revealed that the majority of *Mesorhizobium* species from regions that lacked rhizobial inoculation were phylogenetically distinct from those derived from nodules of wild chickpea. In addition, the genetic diversity of chickpea rhizobial strains had a positive correlation with their geographic origins and environmental factors, particularly soil types and latitude, suggesting that the dominant rhizobial strains in each location may have been adapted to local environments (Greenlon *et al.* 2019).

Inoculation history had an impact on the distribution of chickpea rhizobia, with greater diversity of *Mesorhizobium* species were found in countries where inoculation is absent or rare, following long history of crop introduction, compared to countries with frequent inoculation (Greenlon *et al.* 2019). In Australia, the majority of isolated strains were closely related to *M. ciceri* due to continued inoculation with a commercial inoculant (Elias and Herridge 2015; Zaw *et al.* 2021). In some countries like Pakistan (Zafar *et al.* 2017), Spain (Nour *et al.* 1994; Nour *et al.* 1995), Tunisia (L'taief *et al.* 2007) and Iran (Rouhrazi and Khodakaramian 2015) only cognate chickpea rhizobia were dominantly found. The occurrence of *M. loti* in chickpea nodules was reported in India (Rai *et al.* 2012) and Morocco (Maatallah *et al.* 2002), although the majority of strains were related to *M. ciceri* and *M. mediterraneum*. In contrast, in countries that lack widespread inoculation like China and Myanmar, none of the species were related to the cognate chickpea rhizobia but rarely described chickpea symbionts such as *M. muleiense*,

M. tianshanense and *M. plurifarium* were predominantly found throughout the chickpea growing soils (Zhang *et al.* 2012a; Zhang *et al.* 2012b; Soe *et al.* 2020). A survey in Portugal also revealed that most of the strains isolated from chickpea nodules shared similar sequences with *M. amorphae* and *M. huakuii*, while only 33% of strains were closely related to *M. ciceri* or *M. mediterraneum.* The distribution of these *Mesorhizobium* species in Portuguese soils were associated with geographic locations, soil pH and, probably inoculation history as well since the symbiotically effective strains were more related to *M. ciceri* group (Alexandre *et al.* 2009).

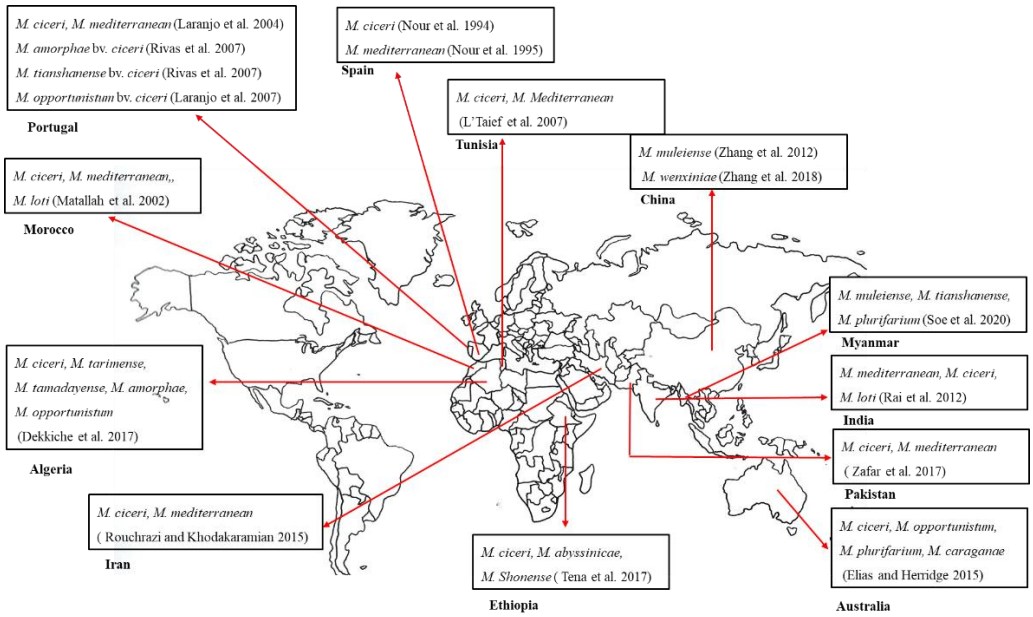

Fig. 2.2 The distribution of chickpea nodulating rhizobia in the world

2.7. General schemes for studying phylogenetic diversity and taxonomy of rhizobia

Determining the diversity and taxonomy of mesorhizobia involves several different mechanisms. Some traditional methods including nodulation tests, and biochemical and biophysical analysis are generally useful in rhizobial diversity studies. The 16S rRNA sequencing, multilocus sequence analysis (MLSA) using housekeeping genes has been used to identify species. A polyphasic approach, including genomic analysis, phylogenetic analysis

using multiple gene sequencings, and phenotyping has generally applied in recent studies of rhizobial diversity.

2.7.1 Molecular characterization

Several molecular methods are available for investigating the diversity of rhizobia at genus, species or strain level. However, some are not widely used since more convenient and accurate methods have been developed in recent years. For example, multilocus sequencing (Martens *et al.* 2008) has been more commonly applied and recommended to replace previously used methods such as multilocus enzyme electrophoresis (Wang *et al.* 1998; Wang *et al.* 1999), PAGE of total bacterial proteins (SDS-PAGE) (Diouf *et al.* 2000), amplified fragment length polymorphism (AFLP) (Gao *et al.* 2001; Terefework *et al.* 2001) and PCR-RFLP using 16S rRNA sequencing alone (Wang *et al.* 1998; Wang *et al.* 1999). However, these methods have some drawbacks such as being labour-intensive, time-consuming, or requiring a large amount of DNA for analysis, thereby creating variable results which are unreliable at low levels of genetic relatedness, with pairwise results that are not suitable for database construction (Goris *et al.* 2007). Therefore, DNA-DNA hybridisation (DDH) methods were replaced with average nucleotide identity (ANI) and digital hybridisation of complete genome for novel species and genera descriptions, following the development of genome sequence analysis (Grönemeyer *et al.* 2017; Safronova *et al.* 2018). Since different molecular approaches may provide similar results in the differentiation of rhizobial genera, species and strains, the choice of suitable molecular techniques to investigate genetic diversity may vary among different studies (Wolde-Meskel *et al.* 2005; Bala and Giller 2006; Yan *et al.* 2014; Jiao *et al.* 2015a).

16S rRNA is widely used in phylogenetic studies of bacteria because of its presence in almost all bacteria (Janda and Abbott 2007) and its size (1500bp) is large enough to identify several bacterial genera as well as to detect novel bacterial strains (Patel 2001). Sequencing of 16S rRNA, which is known as a conserved gene, may provide initial identification of rhizobia at

the level of species and genera (Young and Haukka 1996; Janda and Abbott 2007). However, due to the high similarity of 16S rRNA sequences in *Mesorhizobium* species, the alternative phylogenetic markers should be used to discriminate species within a genus, since the use of 16S rRNA alone cannot differentiate between some *Mesorhizobium* species such *M. mediterraneum* and *M. temperatum* or *M. metallidurans* and *M. gobiense* (Martens *et al.* 2008; Laranjo *et al.* 2012). The analysis of 16S-23S rDNA IGS region is more accurate in identifying closely related rhizobial genera, species or strains. The specificity of 16S-23S rDNA IGS is due to variation in size and sequence of the intergenic spacer (IGS) even within species, allowing precise identification at intraspecies or strains level (Doignon-Bourcier *et al.* 2000; van Berkum and Fuhrmann 2000; Willems *et al.* 2001; Kwon *et al.* 2005).

2.7.1.1 Phylogenetic analyses of housekeeping genes

The combined use of different housekeeping genes that are highly conserved and distributed along the chromosome may provide more precise phylogenetic analysis (Pérez-Yépez *et al.* 2014). Sequence analysis of the housekeeping genes such as *atpD, dnaJ* and *recA* may identify rhizobia at the genus and species levels (Yan *et al.* 2014; Jiao *et al.* 2015b). The strains with identical housekeeping genes can be grouped under a common genus, while the 97% average nucleotide identity (ANI) can be applied as a threshold to differentiate species (Wang *et al.* 2019).

The concatenated phylogenetic analysis using multiple housekeeping genes such as 16SrRNA, *atpD, recA, glnII, dnaK, gap, gln*A, *gltA, gyrB, pnp, rpoB* and *thrC* have been recommended to provide more accurate species affiliation of rhizobial strains (Martens *et al.* 2008). The species identified from the concatenated phylogeny can be applied in calculating diversity index and in analysing principal components using the data geographic locations and soil factors to investigate biogeographic distribution of rhizobia (Han *et al.* 2009). In the genus *Mesorhizobium*, the most recently described species were identified by phylogenetic analysis

using two or three housekeeping genes, mostly *recA* (Chen *et al.* 2009; Chen *et al.* 2011), *atpD* (Han et al. 2008), and *glnII* (Guan *et al.* 2008; Lu *et al.* 2009; Nandasena *et al.* 2009). However, the *atpD* and *recA* sequences of *Mesorhizobium* are highly conserved and may provide low resolution of phylogenetic relationship (Degefu *et al.* 2011; Armas-Capote *et al.* 2014).

2.7.1.2 Phylogenetic analysis of symbiosis genes

Symbiosis related genes (*nod, nif* and *fix)* are specific for rhizobia, as they are responsible for nodulation and N fixation. *Nif* and *fix* genes in rhizobia are required for nitrogen fixation. The rhizobial *nif* genes re monophyletic with the free-living N_2 fixing bacteria, while the *fix* genes can be found only in symbiotic organisms (Long 1989). The symbiosis genes of chickpea rhizobia are highly conserved, and the monophyletic cluster of both *nifH* and *nodC* genes of strains isolated from chickpea indicate the specificity of chickpea-rhizobia, while these are differently clustered from most other rhizobial groups (Laranjo *et al.* 2008).

2.8. Horizontal gene transfer (HGT) of symbiosis islands in legume-nodulating bacteria

The transfer of symbiosis islands from chickpea symbionts to other *Mesorhizobium* spices may results the diverse species able to nodulate chickpea that lacked nodulation genes previously. In *Mesorhizobium,* symbiosis genes are chromosomally encoded, but they can be easily readily transferred as they are mobile genetic materials called on integrative and conjugative elements (ICEs) (Poole *et al.* 2018). ICEs of *Mesorhizobium* species may either contain a single contiguous region (monopartite) (Ramsay *et al.* 2006) or may comprise three separate chromosomal regions (tripartite) that recombine into a single region before exchange before transfer (Greenlon *et al.* 2019; Haskett *et al.* 2016). The rapid genome evolution of *Mesorhizobium* through HGT has been reported in both laboratory (Sullivan and Ronson 1998; Hill *et al.* 2021) and field experiments (Nandasena *et al.* 2007; Sullivan *et al.* 2002). Examples of rapid evolution in *M. australicum* and *M. opportunistum* arose from Australian resident

strains that acquired the capacity to nodulate *Biserrula pelecinus* following lateral transfer of symbiosis genes from inoculant strain, *M. ciceri* bv. *biserrulae in situ* (Nandasena *et al.* 2009).

In New Zealand, following 7 years after inoculant (*M. loti*) introduction, diverse species of resident *Mesorhizobium* were able to nodulate *Lotus* in a field that originally devoided *Lotus* nodulating rhizobia, indicating the possible occurrence of HGT of symbiosis genes from inoculant strain to diverse resident *Mesorhizobium* species (Reeve *et al.* 2014; Sullivan *et al.* 1995). The *in vitro* transfer of symbiosis islands of *Mesorhizobium ciceri* CC1192 to an ICE-cured derivative of *M. japonicum* was demonstrated in a recent genetic evolution study in Australia and suggest the HGT may provide the strains with improved SE than inoculant (Hill *et al.* 2021). In chickpea, HGT has been a driving factor in the change of efficiency of strains in Australian soils. A study in NSW reported that about 53% of chickpea nodules from 26 fields were occupied by strains distantly related to commercial inoculant strain CC1192, and about 41% of these naturalised strains were considered ineffective relative to CC1192 (Elias and Herridge 2015), suggesting HGT nodulation genes may have occurred from inoculant strains to diverse resident strains with poor N fixation ability (Sullivan *et al.* 1995; Hill *et al.* 2021; Colombi *et al.* 2021). There is has been very little investigation into the occurrence of HGT in Myanmar soils, but the diversity of chickpea-nodulating rhizobia with significant genetic divergence from the cognate species of *M. ciceri* and *M. mediterraneum* (Soe et al. 2020) indicates HGT of symbiosis genes from a common source.

2.9. Constraints for nodulation and nitrogen fixation

The chickpea-rhizobial symbiosis can be affected by a variety of environmental stresses. The rhizobial strains must tolerate some abiotic stresses such as high temperature and drought (Lebrazi and Benbrahim 2014) and extremes in soil acidity, salinity and temperature (Zahran 1999; Ruiz-Díez *et al.* 2012) under field conditions. About 4-6 million ha of cropping soils in

southern Australia are acidic with low clay, carbon and moisture contents and are exposed to high temperatures during summer months (Van Gool 2011; Siddique *et al.* 2012). Additionally, limited and erratic rainfall, high temperature and low crop diversity are major constraints in key chickpea growing regions of Myanmar (Cornish *et al.* 2018; Herridge *et al.* 2019). These conditions are unfavourable for survival of rhizobia and nodulation process (Howieson and Ballard 2004; Atieno and Lesueur 2019). Under such conditions, selection of superior rhizobial strains that can tolerate to a variety of abiotic stresses is crucial to optimise the biological nitrogen fixation in legume cropping (Denton *et al.* 2000; Yates *et al.* 2005; Howieson *et al.* 2008). The adaptation of inoculant strains to environmental and cropping conditions may influence the phenotypic and genotypic diversity of rhizobial population in the soils. Therefore, studies on the ecological adaptation of chickpea rhizobia to a variety of environmental stresses such as extreme temperatures, soil pH and heavy metals are required to better understand the potential range of adaptation among strains.

Survival of rhizobial inoculants on seed is one of major factors affecting success of inoculation. Poor survival of rhizobia may decrease nodulation that in turn may decline legume productivity (Roughley *et al.* 1993). The rhizobial cells on seed may rapidly die, particularly when they are exposed to unfavourable environmental conditions such as high temperature and direct sunlight between the time of inoculation and sowing (Brockwell *et al.* 1987). The rapid mortality of rhizobial cells inoculated seeds were reported that only 4.8% ($Log_{10}3.83$) of the inoculated rhizobial cells ($Log_{10}5.15$) survived at 4 h after inoculation and only 0.83% ($Log_{10}3.07$) after 22.5 h in soil (Roughley *et al.* 1993).

2.9.1 Soil pH

During recent decades, about half the agriculturally productive soils in Australia are somewhat affected by acidification and the extent of this problem is increasing due to inadequate management and increased intensification of production (McKenzie *et al.* 2017). The value of

lost in agricultural production due to soil acidification was about $1.6 billion per year in 2001 (NLWRA 2001). Cultivation of legume crops on acidic soil can be limited by toxicity of H+, Al and Mn, and deficiency of Ca, Co, Mg, P, Fe and Mo (O'Hara 2001; Indrasumunar *et al.* 2011). Soil acidity limits the ability of rhizobia to survive and persist in soil, and to nodulate and fix N effectively (Munns 1968; Graham *et al.* 1982; Brockwell *et al.* 1991; Brockwell *et al.* 1995). Soil pH also influences the distribution and abundance of resident rhizobial population. For example, acidic soils (pH <4.6) in pasture farming of south-eastern Australia contains high population *Rhizobium leguminosarum* bv. *trifolii* compared with other rhizobial genera (Slattery and Coventry 1993; Burnett *et al.* 1994). It is possible that the resident rhizobia have survived in acidic soils through colonisation of micro-environments with a higher soil pH (Strong *et al.* 1997) or by showing inherent ability to survive in acid soils. In contrast, *Sinorhizobium meliloti* is sensitive to low pH (pH <6.0) that strongly inhibits the growth and nodulation process (Munns 1968).

A study on chickpea-*Mesorhizobium ciceri* on acidic soils has shown that only 5% of the strains tested could nodulate effectively in these strongly acidic environments (Rai 1991). Under this stress, the rhizobial strains must be saprophytically competent to survive until they can infect the host plant (Chatel 1968). In fact, chickpea itself is typically sensitive to soil acidity and Al_3+ toxicity that may inhibit root hair formation and development causing failure in nodulation (Singh and Raje 2011; Choudhury and Sharma 2014). Therefore, the combined use of acid-tolerant chickpea varieties and rhizobial strains may be a useful strategy for both improving crop productivity but also potentially expending the use of chickpea onto more acidic soils, where lime application is not always practical or economical (Hartel and Bouton 1991; Slattery and Coventry 1999).

2.9.2 Soil salinity

Soil salinity is one the major causes of soil degradation in Australia due to increased concentration of NaCl in soil water. In Australia, soil salinity problems are increasing from extensive land clearing for agricultural purposes (GRDC 2018). In Myanmar, soil salinity is a challenging abiotic stress reducing the crop yield in central dry zone, mostly affecting 6,357 ha in 16 townships of Mandalay region during 2013-2014 (Khin and Ando 2017; Oo *et al.* 2017). Chickpeas are sensitive to saline conditions due to limited access of water and nutrients in saline soils (Vadez *et al.* 2006). While chickpea rhizobia have higher salt tolerance than the host plant, the chickpea-rhizobia symbiosis is adversely affected by salinity through reducing nodulation, nodule size and N fixation (Singh *et al.* 2005; Flowers *et al.* 2010).

2.9.3 Seed applied fungicides or herbicide residues

In intensified cropping, the increased use of herbicides and pesticides are becoming a major issue in many agricultural lands. These chemicals are typically applied as seed-applied fungicides and pre-emergence or post-emergence herbicides. For weed control, herbicides are widely used in crop and pasture farming systems. Some herbicide residues in soil have adverse impact on biological nitrogen in legumes, and such situations are common in areas where soil environments inhibit the rapid degradation of chemical residues (Ferris *et al.* 1992; Douka *et al.* 1995). For example, low rainfall, alkaline soils, and drought can limit the breakdown of soil chemical residues (Unkovich *et al.* 1997). Soil factors such as soil particle size, organic matter and clay content, cation exchange capacity, microbial activity and pH influence the degradation of the active ingredients in herbicides (Aislabie and Lloyd-Jones 1995; Meharg 1996).

Soil chemical residues can interfere the ability of rhizobial strains to survive, grow, nodulate and fix N (Kumar *et al.* 1981; Durgesha 1994; Martinez *et al.* 1996), with varying impacts on the growth of rhizobial strains (Durgesha 1994). In subterranean clover some herbicides

showed phytotoxic impact on plant growth and nodulation under controlled conditions (Eberbach and Douglas 1989; Eberbach and Douglas 1991). The direct and residual effects of some commonly used chemicals on medic-rhizobia symbiosis are major concerns in north-western Victoria, where soils are mostly alkaline (pH 7-9) (Slattery *et al.* 2001). A recent study in South Australia demonstrated that seed-applied fungicide inhibited the rhizobial survival and nodulation in both lab and field experiments (Rathjen *et al.* 2020). These conditions may also apply in chickpea growing regions in Myanmar where limited rainfall, alkaline soil and drought are major constraints for rapid degradation of chemicals.

2.9.4 Temperature and rainfall

Most agricultural regions in southern Australia have variable winter rainfall, and hot and dry summers (Unkovich *et al.* 1997). In Myanmar, chickpea cultivated regions in the central dry zone (CDZ) received very erratic rainfall, with 500 to 1000 mm per year, while the other parts of country receiving 2000 to 5000 mm annually (Tun *et al.* 2015; Herridge *et al* 2019). In the dry season, the average maximum temperature is around 40 ºC (Boori *et al.* 2017; Cornish *et al.* 2018). The erratic rainfall often affects the growth and grain filling of the crops. Therefore, the adaptation of crop varieties to variable weather conditions for specific regions is important to be addressed. Additionally, the availability of rhizobial strains capable of nodulating legumes should be considered when introducing new legumes into specific agricultural regions (Howieson 1995) and strains must also be able to grow and survive under stress conditions in these regions.

Due to dry soil and high temperature, the decline in population of *Rhizobium leguminosarum* bv. *trifolii* was found over the summer period (Chatel and Parker 1973; Evans *et al.* 1988). Effective nodulation requires an adequate population size of effective rhizobia is established in cropping soils. It is possible that smaller populations and slower growth rates of rhizobia may be found in regions with long periods of dry due late opening rains than in regions with

higher early soil moisture (Evans *et al.* 1988). Consequently, drying soil may reduce nodulation and root growth rate (Davey *et al.* 1989). Variable rainfall events, high temperatures and dry soil conditions are major constraints in key chickpea growing regions in both Australia and Myanmar.

2.10. Selection and introduction of new elite rhizobial strains into the field

The size of rhizobial population in soils typically depends on cropping history, time of sampling, soil physicochemical properties and the presence of the particular host legume/s. For example, populations of *Rhizobium leguminosarum* bv. *trifolii* vary with sampling times during growing season (Evans *et al.* 1988), with the highest population in winter and spring (10^5 –10^6 cfu/g soil), with population declining in the hot and dry summers (10^0 –10^3 cfu/g soil). The success of inoculation under field conditions is often dependent on the size of naturalised rhizobial population that can nodulate target legumes, and limit nodulation and establishment of the inoculant strain. Seed inoculation with newly introduced strains is normally successful in soil where there are low naturalized rhizobial population (<50 cfu/ g soil) specific to target legumes. In contrast, inoculation can be difficult and often fail in soils with high rhizobial population (>10^3 cfu/g soil) (Thies *et al.* 1991; Brockwell *et al.* 1995). Another study also reported that rhizobial inoculation is more successful in soils with low naturalised rhizobial population (<10^2 cfu/g soil), than those contain high populations naturalised rhizobia (10^5 cfu/g soil) (Rice and Olsen 1992).

Rhizobial inoculation is required particularly in soils that have insufficient effective rhizobial population for nodulation and N fixation. Success of rhizobial inoculation in the field may be limited by several factors, including the size and effectiveness of naturalised rhizobial populations, host compatibility and soil environmental conditions (Dughri and Bottomley 1984). The failure in nodulation by inoculant strains may be due to poor competitive and

adaptative traits to local soil conditions compared with resident strains (Romdhane *et al.* 2007; Nandasena *et al.* 2007a; Romdhane *et al.* 2008; Thilakarathna and Raizada 2017). Additionally, over time the inoculant strains may share symbiosis genes to resident strains through HGT that may result strains with inferior or superior symbiotic performance (Sullivan *et al.* 1995; Hill *et al.* 2020). After a long period of using a single inoculant strain for chickpea, it has been identified that rhizobia collected from soils and from chickpea nodules vary in infectiveness and effectiveness, relative to CC1192 (Elias and Herridge 2015; Zaw *et al.* 2021). Under such conditions, selection of new elite strains that showed better adaption to specific abiotic and abiotic stresses is crucial to optimise the legume productivity in specific regions (Howieson *et al.* 1988; Denton *et al.* 2003; Yates *et al.* 2021).

Introducing new inoculant strains into Myanmar soils is required since there has been no deliberate widespread application of effective inoculant strains. Chickpea cultivated soils in Myanmar are mostly Vertisols with high clay content and neutral-high pH, while there are some sandy soils found in Magway regions. The key chickpea growing regions (CDZ) experienced high temperature, received limited rainfall and frequent drought during the chickpea growing season. There is limited knowledge regarding the diversity of rhizobial strains in Myanmar soils, but a lack of inoculation and prolific nodulation observed in chickpea crops, these soils may provide the source for collection of new elite rhizobial strains that could be evaluated as chickpea inoculants in Myanmar or Australia.

2.11. Summary

Australian soils originally lacked resident chickpea rhizobia until chickpea was introduced in the 1970s. Consequently, chickpea is usually inoculated at every sowing with commercial chickpea inoculant *M. ciceri* CC1192. Over time, using a single inoculant strain may have led to variable SE if resident rhizobia acquired the symbiotic ability from CC1192 through HGT.

Phylogenetic analyses of core and symbiosis genes may identify whether HGT occurred in Australian soils. Phenotypic assessment of isolated strains in both laboratory and field conditions may provide a better understanding the impact of genetic variation on SE and ecological adaptation of the resident chickpea rhizobia.

In Myanmar, chickpea is grown in the CDZ with limited inputs including N fertilizers and inoculation. In this region, limited and erratic rainfall, high temperature and drought are major constraints in to crop production. Chickpea are grown in soils ranging from Vertisols to course-textured soils with low organic matter and water holding capacity. There is a very narrow crop diversification where chickpea is mostly rotated with rice in lowlands and with sesame, coarse grains, pearl millet, sorghum, and maize in uplands (Herridge *et al.* 2019). Diversity of chickpea rhizobia in Myanmar soils may not be closely related to the cognate chickpea rhizobia as found in other countries where commercial chickpea inoculants are extensively used. Studies on phenotypic and phylogenetic diversity of Myanmar strains from a large-scale soil collection are required to investigate the relationship of biogeographic distribution of chickpea rhizobia with cropping and inoculation history.

This literature review has considered important information on the process of biological nitrogen fixation through symbiotic association between legumes and rhizobia that serves as useful background to this thesis. It has discussed the major factors affecting the chickpea-rhizobial symbiosis in farming systems. In addition, some useful techniques to study rhizobial diversity and their evolution have been discussed.

There are some limitations in our understanding of the genetic diversity, particularly the role genetic exchange between chickpea inoculant strains and resident rhizobia that could contribute the variability in strain effectiveness and ecological adaptation in different soil environments in Australia and Myanmar. There is a paucity of knowledge regarding inoculation in Myanmar

that requires investigation into strains diversity, both phenotypically and genetically. Our understanding on how the symbiosis between chickpea and rhizobia under acidic or alkaline soils is limited.

The purpose of this thesis is, therefore, to address the questions:

Does a large-scale Australian soil collection provide genetically diverse chickpea rhizobial strains with varying symbiotic and ecological adaptation traits, relative to the commercial inoculant strain? (Chapter 3)

How diverse are rhizobial strains from chickpea growing soils from the central dry zone (CDZ) of Myanmar? Additionally, does the diversity of chickpea rhizobia in Myanmar differ with rhizobial diversity in Australia? (Chapter 4)

Do locally adapted acid tolerant and symbiotically effective strains from Australia and Myanmar improve chickpea nodulation and N fixation under acid and alkaline soils in field experiments in southern Australia? (Chapter 5)

2.12. References

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Chapter 3. Symbiotic effectiveness, ecological adaptation and phylogenetic diversity of chickpea rhizobia isolated from a large-scale Australian soil collection

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3.1. Statement of authorship

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Contribution to the Paper	Planned and conducted all experiments, analysed and interpreted the data, and wrote the manuscript		
Overall percentage (%)	80%		
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ii. permission is granted for the candidate in include the publication in the thesis; and

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3.2. Abstract

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ORIGINAL ARTICLE

Symbiotic effectiveness, ecological adaptation and phylogenetic diversity of chickpea rhizobia isolated from a large-scale Australian soil collection

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Abstract

Aims To investigate phylogenetic and phenotypic diversity of resident chickpea-nodulating rhizobia from Australian cropping soils.

Methods Eighty *Mesorhizobium* strains collected from across Australian cropping regions were analysed in a pot experiment to evaluate nodulation and symbiotic effectiveness (SE%) in chickpea plants. In vitro testing of these strains tolerant to specific stresses (pH, temperature, antibiotics, heavy metals and NaCl) was performed. In addition, phylogenetic analyses were carried out using16S-23S rRNA, *atpD*, *recA*, *nodC* and *nifH*.

Results All strains were members of the genus *Mesorhizobium* based on phylogenies of 16-23S rRNA IGS and core genes. Strains with diverse 16S-23S rRNA IGS carried symbiosis genes that had high similarity to those in chickpea symbionts, suggesting the resident rhizobia in Australian soils may have acquired symbiotic traits from inoculant strains through horizontal gene transfer (HGT). Inoculation of chickpea revealed that variation in SE% among isolated strains was correlated with phylogenetic relatedness to the commercial inoculant strain *Mesorhizobium ciceri* CC1192. Strain A47 collected from Queensland gave the highest shoot biomass and two strains (A78 and A79) from Western Australia grew under acidic conditions (pH 4.4). In a field experiment, inoculation with four selected strains or strain CC1192 all increased shoot biomass compared with an uninoculated control; chickpea inoculated with strain A47 had the highest nodulation.

Conclusion Australian cropping soils contain resident chickpea rhizobial genotypes that are genetically and phenotypically diverse, with some genotypes having equal or superior SE compared with the inoculant strain CC1192. This study provides evidence to support HGT of symbiosis genes in Australian soils, and has identified strains adapted to different environmental conditions.

Keywords Chickpea · Diversity · *Mesorhizobium* · Phenotypic · Phylogenetic · Symbiotic

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3.3. Introduction

Chickpea (*Cicer arietinum*) is a globally important pulse crop, second only to common bean (*Phaseolus vulgaris*) in production. Australia is the world's leading exporter of desi chickpea and chickpea is the most commonly grown pulse crop in Australia (ABARES 2019). In Australia, chickpea is mainly grown in New South Wales (NSW) and Queensland with cultivation extending across southern Australia to Western Australia.

Chickpea can fix up to 140 kg N $ha^{-1}yr^{-1}$ through a highly specific symbiotic relationship with *Mesorhizobium* (López-Bellido et al. 2011; Unkovich et al. 2010). However, symbiotic effectiveness depends on multiple factors such as the size and composition of the soil rhizobial population as well as previous and current inoculation (Denton et al. 2000; Drew and Ballard 2010; Furseth et al. 2012; Mothapo et al. 2013; Slattery et al. 2001). Over time and without continued inoculation, the effectiveness of rhizobia residing in the soil can decline from that of the original inoculant strain (Batista et al. 2007; Elias and Herridge 2015; Sullivan et al. 1995). A study of soils from chickpea growing areas in northern NSW observed that strains isolated from these soils had low to high symbiotic N fixation compared with the commercial strain, but none of the isolated strains exceeded the symbiotic efficiency of the commercial inoculant *Mesorhizobium ciceri* CC1192 (Corbin et al. 1977), possibly because they were obtained from a small sampling region (Elias and Herridge 2015). Another survey of rhizobia from 50 pulse fields in southern Australia identified that only 7% of these fields contained sufficient resident rhizobia for effective nodulation of chickpea (Slattery et al. 2004).

Successful establishment of inoculant strains in soil is influenced by several factors including the size of the resident rhizobial population, the presence of a compatible host, and favourable soil conditions (Brockwell et al. 1995). The introduction of inoculant strains is usually successful in soils where the resident compatible rhizobial populations are small (< 50 rhizobia

g^{-1} soil), whereas soils rhizobia (> 10^3 rhizobia g^{-1} soil) generally lead toa low recovery of the inoculant strain (Brockwell et al. 1995; Denton et al. 2002; Thies et al. 1992). Despite this, the resident rhizobia can acquire the capacity to nodulate, although with generally lower SE than the inoculant strain (Batista et al. 2007; Chen et al. 2002; Ferreira and Hungria 2002; Laguerre et al. 2003). The resident rhizobia may become efficient symbionts after acquisition of a symbiosis island from inoculant strains through horizontal gene transfer (HGT) (Brockwell et al. 1995; Hill et al. 2021), while maintaining adaptation to the local soil environment.

HGT in *Mesorhizobium* occurs with the transfer of nodulation and nitrogen fixation genes as a complete element in a symbiosis island to a recipient cell. Symbiosis genes may be chromosomally encoded but are easily exchanged between or within species because these genes are located on integrative and conjugative elements (ICEs). ICEs in *Mesorhizobium* spp. either span a single contiguous region (monopartite) of approximately 500 kb (Ramsay et al. 2006) or may exist as complex structures such as tripartite ICEs, with three separate regions on the chromosome that recombine into a single element before transfer (Greenlon et al. 2019; Haskett et al. 2016). In New Zealand, Lotus nodulation by genetically diverse *Mesorhizobium* genotypes occurred 7 years after a single inoculation with *Mesorhizobium loti* in a field that originally lacked rhizobia able to nodulate Lotus, suggesting that some resident non-symbiotic rhizobia acquired the capacity for symbiosis from the inoculant strain through HGT of the ICE symbiosis island (Reeve et al. 2014; Sullivan et al. 1995). A recent study of Australian chickpea rhizobia demonstrated the transfer of the symbiosis island of *Mesorhizobium ciceri* CC1192 in vitro to an ICE-cured derivative of M. japonicum (Hill et al. 2021). This shows that HGT is likely to occur in chickpea-nodulating rhizobia in Australian soils through the transfer of nodulation genes from symbiotically capable *Mesorhizobium* inoculants, with strain CC1192 the only commercial inoculant for over 40 years.

Nod and *nif* genes required for nodulation and nitrogen fixation in *M. ciceri* strain CC1192 are located in the ICE region (Hill et al. 2021). Generally, *nod* genes are more specific for a symbiotic relationship with a particular plant species compared with *nif* genes that occur in many species of bacteria (Laranjo et al. 2008). The *nod* genes in *Mesorhizobium* are considered highly specific, and all chickpea-nodulating rhizobia share a similar set of symbiosis genes that are monophyletic (Greenlon et al. 2019; Laranjo et al. 2008). It is possible that *Mesorhizobium* with different chromosomal backgrounds contain similar symbiosis genes that are highly conserved across multiple species of *Mesorhizobium* (Laranjo et al. 2008; Nandasena et al. 2006, 2007).

Successful symbiosis relies on the capacity of the nodulating rhizobia to express symbiotic traits and to compete with resident rhizobia (Remigi et al. 2016). Mutations may occur in bacteria at the rate of 8.9×10^{-11} synonymous substitutions per base pair generation (Wielgoss et al. 2011), and this rate may increase under stressful conditions (Bjedov et al. 2003). Genetic variation in resident rhizobial populations may also be influenced by soil physicochemical properties, temperature and the host legume itself, which can promote the diversification of compatible rhizobial strains (Bottomley 1992; Brockwell et al. 1995; Thies et al. 1992). The abundance and distribution of compatible rhizobia can be influenced by the soil environment, such as acidity, salinity, chemical residues, soil type, high temperature and long periods of drought (Davey and Simpson 1990; Lauter et al. 1981; Slattery et al. 2001, 2004; Zhang et al. 2017). Rhizobial diversity may also be affected by agricultural management practices (Ferreira and Hungria 2002; Kaschuk et al. 2006; Torabian et al. 2019) and environmental stresses such as high temperatures that may accelerate genetic recombination events including HGT, deletion, insertion, integration, mutations and rearrangements of DNA elements (Galli-Terasawa et al. 2003; Sawada et al. 2003; Sullivan and Ronson 1998). It is also possible that the distribution and symbiotic capabilities of native legumes may influence the presence of

Mesorhizobium species capable of nodulating chickpea (Dekkiche et al. 2018). Over time the genetic makeup of an original inoculant strain can be modified by the resident rhizobial population, either through HGT or adaptation of descendants of the inoculant. Resident rhizobia are typically more adapted to local soils than non-native inoculant strains (Batista et al. 2013; Rai et al. 2012). Therefore, resident rhizobia in diverse Australian cropping soils may include genotypes with tolerance to specific environmental constraints (Howieson and Dilworth 2016; Zahran et al. 2003) and with variable symbiotic efficiency.

Due to insufficient numbers of suitable mesorhizobia in Australian soils, it is strongly recommended that chickpea crops are inoculated at sowing with the commercial inoculant, strain CC1192 (GRDC 2017), a strain that has been used for over 40 years (Unkovich et al. 1997). HGT has been shown to occur in soils with a history of chickpea inoculation (Hill et al. 2021), yet the background population of resident soil bacteria into which the symbiotic genes have transferred are often unknown. Therefore, HGT in chickpea strains provides an opportunity to test adaptation and diversification of strains. It is likely that there is a wide diversity of chickpea rhizobia in Australia, through the transfer of symbiosis island from CC1192 to *Mesorhizobium* species previously unable to nodulate and fix nitrogen with chickpea (Hill et al. 2021).

While sequence analysis of highly conserved genes such as 16S rRNA, *nodC* and *nifH* is useful to define key interspecies variation, it may provide low resolution analysis of intraspecies diversity (Laranjo et al. 2012; Martens et al. 2008). In *Mesorhizobium* species, the use of alternative phylogenetic markers other than 16S rRNA is important to distinguish the species within this genus. The intergenic gene spacer (IGS) regions are known for their variability in size and sequence (Doignon-Bourcier et al. 2000), and analysis of 16S-23S IGS is a useful method to identify bacterial populations at the intraspecific level, especially in rhizobial

isolates (Laguerre et al. 1996; Laranjo et al. 2012; van Berkum and Fuhrmann 2000; Willems et al. 2001). In *Mesorhizobium*, sequence analysis using a number of house-keeping genes throughout the genome such as *atpD*, *recA*, *gyrB*, *rpoB*, *gltA*, *dnaJ*, *glnA*, *dnaK*, *truA* and *thrA*, has provided more accurate analysis of interspecies and intraspecies phylogenetic relationship (Degefu et al. 2011; Laranjo et al. 2012; Perez-Yepez et al. 2014).

The objectives of this study were (1) to evaluate symbiotic and ecological adaptive traits of chickpea rhizobia from diverse cropping regions across Australia, where a single inoculant strain CC1192 has been used previously, (2) to investigate the diversity and evolutionary relationships among chickpea rhizobia relative to the commercial inoculant strain CC1192, using sequencing of the 16S-23S rRNA IGS region, *atpD* and *recA* and selected symbiotic genes, and (3) to evaluate the field performance of selected strains that showed high symbiotic effectiveness in a glasshouse experiment.

3.4. Materials and Methods

3.4.1 Soil sampling

Soil samples were collected from 73 Australian National Variety Trials (NVT) sites in New South Wales, Queensland, South Australia, Victoria and Western Australia during early 2017; detailed site information is presented in supplementary data (Table S1). Soils were sampled to 100 mm depth at nine arbitrarily selected points per field using a clean spade and mixed thoroughly to make a composite sample. A 1 kg composite soil sample was collected from each site and soil samples were stored at 4 °C until used. The information on chickpea cropping history of soils in the past 10 years was obtained via NVT from farmers at those sites. To determine the soil physicochemical properties, 10 g each of soil sample were oven-dried at 65 °C for 24 h and mixed with 50 ml of distilled water, then shaken 1 h. After allowing sedimentation for 30 min, pH was measured using electrodes at room temperature (24.5 °C).

Total soil N, P, K and Ca concentrations were measured by APAL, Thebarton, South Australia using Inductively Coupled Plasma (ICP) analysis (Rayment and Lyons 2011).

3.4.2 Isolation, culture and authentication of rhizobial strains

Rhizobia were isolated from chickpea plants grown in sterile pots containing 400 g of sterilised coarse sand (2–3 mm diameter) moistened with 75 ml of N-free nutrient solution (McKnight 1949) and covered with a layer of field soil (20 g), followed by a shallow layer of sterile sand (Howieson and Dilworth 2016). Chickpea cv. Hattrick seed was sterilized in 70% ethanol for 30 s and rinsed five times with sterile water before sowing three seeds per pot (Howieson and Dilworth 2016). After germination, the seedlings were thinned to one plant per pot and a 20 mm layer of sterile plastic beads was added to the surface to minimize the risk of cross-contamination. Twenty ml of sterile McKnight's nutrient solution were added approximately every 3 days (McKnight 1949). The plants were kept at 20 to 30 °C in a glasshouse exposed to natural sunlight.

The plants were removed from the pots after 5 weeks, and nodules were collected and stored at − 20 °C. Three to five nodules were selected from each plant and were surface sterilized in 70% ethanol for 30 s and 6% NaOCl for 1 min and rinsed six times in sterile distilled water. Nodules were then crushed aseptically in 100 μL of water with a sterile plastic rod. A loopful of the nodule suspension was streaked on yeast mannitol agar (YMA) plates. Single colonies of bacterial strains were selected after incubation at 28 °C for 5 d and purified through subculturing (selecting and streaking from single colonies).

Authentication tests to confirm that strains were able to nodulate chickpea were performed by inoculating chickpea seedlings after 7 days of growth in sterile 180 × 165 mm growth pouches (CYG seed germination pouches, Mega International, Minneapolis, MN, USA). The seedlings were supplied with sterile McKnight's nutrient solution. Three weeks after inoculation,

nodulation was assessed, rhizobia were re-isolated and pure cultures were stored in 25% glycerol solution at − 80 °C (Howieson and Dilworth 2016).

3.4.3 Symbiotic effectiveness

Authenticated strains obtained from soils collected from 20 field sites out of the total of 73 sampled (80 strains in total) were streaked on YMA plates and incubated at 28 °C for 5 days. A loopful from a single colony was transferred to yeast mannitol broth (YMB) and incubated on a rotatory shaker (200 rpm) for 48 h to obtain approximately 109 cells ml^{-1}. One ml of inoculum was added at the base of 5-day-old seedlings growing in sterilized sand in 120-mm diameter plastic pots. Plants were supplied weekly with 20 ml of nitrogen-free McKnight's nutrient solution and 20 ml of sterile water. Inoculation with strain CC1192 and an uninoculated treatment were included as positive and negative controls respectively. The experiment was laid out in a randomised complete block design with two replicates, with two plants included in each replicate. Plants were harvested 50 days after sowing. Nodules were removed from the roots and counted. Leaf chlorophyll content was measured using a chlorophyll meter (SPAD-502 Plus, Konica Minolta Optics, Inc., Osaka, Japan). Nodule, root and shoot material was oven-dried at 70 °C for48 h and dry weights per plant were recorded. Symbiotic effectiveness (SE%) of the strains was calculated by comparing the shoot dry biomass of each treatment with the shoot dry biomass of the positive control (*M. ciceri* CC1192) (Denton et al. 2000; Elias and Herridge 2015; Howieson and Dilworth 2016) using the formula:

SE% = x/y X 100, where x is shoot dry weight of plants inoculated with test strains and y is shoot dry weight of positive control plants inoculated with CC1192. Finally, the symbiotic effectiveness of strains was rated as effective (> 80%), poorly effective (60–80%) and ineffective (< 60%), relative to the commercial inoculant.

3.4.4 Physiological characterisation

Phenotypic assessments were performed on cultures grown on YMA plates divided into 20 equal segments. Each square was spot-inoculated with 10 µL of rhizobia suspension (10^9 cells ml^{-1}) previously grown in YMB. All assessments were done with three replicates in a completely randomised design. The plates were incubated at 28 °C and bacterial growth was assessed 7 days after inoculation. Temperature tolerance was assessed by incubating cultures at 5, 10, 15, 20, 30, 35, 40 and 45 °C. Salinity tolerance was assessed on YMA supplemented with 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% NaCl, and pH tolerance on YMA adjusted to pH 4.3, 4.4, 4.5, 4.6, 4.7, 4.8, 4.9, 5, 5.5, 6, 8, 8.5, 9, 9.5 and 10 with potassium citrate (16.2 g L^{-1}) buffer and adjusted with 30% HCl or KOH solution. Extra agar was added into acidic YMA media (pH 4.3–5.0) to ensure solidification of media after autoclaving. Stock solutions of antibiotics or metals were sterilised using a 0.2 µm membrane filter before addition to molten YMA at 50 °C. The antibiotics were obtained from Glentham Life Sciences Ltd (Corsham, Wiltshire, UK). The antibiotics used were ampicillin (Batch: 223CSN) (50 µg ml^{-1}), chloramphenicol (054WLC) (5 and 15 µg ml^{-1}), erythromycin (593ICU) (10 and 20 µg ml^{-1}), kanamycin (934RYJ) (50 µg ml^{-1}), lincomycin (082EYR) (100 µg ml^{-1}), neomycin (609GQU) (5 and 20 µg ml^{-1}), novomycin (5 µg ml^{-1}), spectinomycin (085LRM) (250 and 500 µg ml^{-1}), streptomycin (575BUT) (40 and 80 µg ml^{-1}) and trimethoprim (433DQK) (200 µg ml^{-1}). The metals assessed were $MnSO_4$. H_2O (500 µg ml^{-1}), $AlCl_3.6H_2O$ (500 µg ml^{-1}), Pb $(CH_3COOH).3H_2O$ (500 µg ml^{-1}), $CoSO_4.7H_2O$ (100 µg ml_{-1}), $CuCl_2$ (100 µg ml^{-1}), $ZnCl_2$ (500 µg ml^{-1}) and $NiSO_4.6H_2O$ (500 µg ml^{-1}). The concentrations used were chosen based on published methods (Howieson and Dilworth 2016; Maatallah et al. 2002; Tena et al. 2017). Strains were classified as tolerant or intolerant based on the presence or absence of growth after 7 days of incubation.

3.4.5 Field experiment

A field experiment was conducted to investigate the inoculation response of four selected strains (A47, M009, M075 and M082) at Angas Valley, South Australia (34.7546° S, 139.3085° E) between June and November 2020. Strains were selected based on their high symbiotic effectiveness in previous pot experiments. Peat inoculants were made by growing the selected strains and CC1192 in YMB for 2 days until the solution became opaque, then 65 ml of the YMB culture was injected into 150 g gamma irradiated peat bags (New-Edge Microbials, North Albury New South Wales, Australia). The YMB culture was massaged through the peat bag to ensure even moisture distribution and incubated for 2 weeks to allow the rhizobia to colonise the peat. Before sowing, the peat inoculant was mixed to a slurry with 5 g peat / 20 ml sterile water, with 1 ml of the slurry added to 100 g chickpea seeds in a ziplock bag. The seeds and peat slurry were shaken until all seeds were evenly coated and were allowed to air dry in the bag. The inoculated chickpea seeds were sown in six rows per plot (12 m × 1.68 m) at the rate of 180 g seeds/plot. Inoculation with commercial strain CC1192 and no inoculant treatments were included as positive and negative controls, respectively. The experiment was laid out in a randomised complete block design with three replicates. Twelve plants were randomly collected from central four rows at 80 cm intervals at 12 weeks from sowing to measure nodulation, while shoot material was collected at peak biomass (4.5 months after sowing). Grain was harvested around 6 months after sowing. Nodules were counted for each plant and a nodulation rating on a 0–5 scale was applied based on several parameters particularly of number, size, position and distribution of nodules on the crown and lateral roots (Corbin et al. 1977; Hartley et al. 2005). Shoot and nodule samples were oven-dried at 65 °C for 48 h and dry weights were recorded.

To estimate the amount of N fixed by inoculation treatments at Angas Valley, the N15 natural abundance method was applied (Unkovich et al. 2008). Dried whole shoot samples were finely

ground and analysed for δ^{15}NAir (%) and total shoot N (µg g^{-1}) (Stable isotope facility, University of California, Davis, USA). Chickpea N derived from fixation (%Ndfa) was calculated by comparing the δ^{15}N in inoculated plants (δ^{15}N legume) with that of non-nodulating canola reference plants (δ^{15}N canola). The following formula was applied to calculate the %Ndfa of chickpea inoculated with rhizobial strains.

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N canola} - \delta^{15}\text{N legume}}{\delta^{15}\text{N canola} - \text{B}} \text{X} \frac{100}{1}$$

The B value, -2.1 (Unkovich et al. 2008), is the δ^{15}N of chickpea that are fully dependent upon N_2 fixation and sampled at the same growth stage as the field grown plants.

The amount of N fixed (mg N $plant^{-1}$) was calculated using the following formula:

N fixed (mg fixed N $plant^{-1}$) = (%Ndfa/100) × (Total shoot N/sample weight in 15 N analysis) x SDW $plant^{-1}$.

3.4.6 PCR amplification and sequencing of rhizobial genes

The genomic regions studied were the16S-23S rRNA IGS, the conserved genes (*atpD* and *recA*) and the symbiosis genes (*nodC* and *nifH*). A single colony of each strain was used as a source of DNA for PCR. Rhizobial cells were added by selecting a single colony freshly grown on YMA with a sterile pipette tip and transferring it into a PCR reaction tube. Each tube had 12.5 µl MyFi™ Mix added, 1 µL each of primers and 10.5 µL sterile MQ water following manufacturer's instruction (Bioline, A Meridian Life Science Company, NSW, Australia). PCR cycles were performed using a thermal cycler (Bioer Version 1.10, GeneWorks, Hindmarsh, SA, Australia). The primers used for 16S-23S rRNA were FGPS1490 (5′-TGC GGC TGG ATC ACC TCC T-3′) and FGPL132' (5′-CCG GGT TTC CCC ATT CGG -3′) (Laguerre et al. 1996). The PCR cycle was 95 °C for 5 min, followed by 35 cycles of 30 s of denaturation at 95 °C, 30 s of annealing at 56 °C, 2 min of extension 72 °C, followed by a final extension for 5 min at 72 °C. The primers *nodCMeso*F (5′-CGA(CT) CG(AG)AG(AG)

TTCAA(CT)TTC-3′) and *nodCMeso*R (5′-CT(CT) AAT GTA CACA(AG)(GC)GC-3′) (Rivas et al. 2007) were used to amplify the *nodC* gene under the following conditions: preheating at 95 °C for 1 min, 35 cycles of denaturing at 95 °C for 30 s, annealing at46.5 °C for 30 s and extension at 68 °C for 2 min, and final extension at 68 °C for 5 min. The *nifH* gene was amplified using the primers *nifH*-1 (5′-AAG TGC GTG GAG TCC GGT GG-3′) and *nifH*-2 (5′-GTT CGG CAA GCA TCT GCT CG-3′) (Eardly et al. 1992). The amplification steps for *nifH* were 2 min at 95 °C, and 35 cycles of 30 s at 95 °C, 30 s at 61 °C, 2 min at 68 °C, and final extension at 68 °C for 5 min.

To verify the robustness of 16S-23S rRNA-based phylogeny, nine strains from different groups and strain CC1192 were selected for further phylogenetic analysis using two conserved housekeeping genes (*atpD* and *recA*). PCR amplification and sequencing of partial *atpD* and *recA* genes of the selected strains and CC1192 were undertaken as described by Gaunt et al. (2001). The amplified PCR products were submitted to AGRF (Adelaide, SA, Australia) for housekeeping and symbiosis gene sequencing using Sanger sequencing.

3.4.7 Phylogenetic data analysis

Sequences were checked and edited using Molecular Evolutionary Genetics Analysis version 7 (MEGA7) (Kumar et al. 2016). The sequences were compared with NCBI GenBank databases (https://blast.ncbi.nlm.nih.gov/Blast.cgi) using nucleotide BLAST for preliminary identification. The sequences of reference strains (28, 22, 27 and 24 reference strains for 16S-23S rRNA IGS, *nodC*, *nifH* and *atpD-recA* genes, respectively) available in the GenBank database were also included for comparison with test strains. Multisequence alignments for all test strains and reference strains were generated using ClustalW method in MEGA7 (Kumar et al. 2016). Poorly aligned positions and divergent regions of nucleotide sequences were eliminated. A maximum likelihood phylogenetic tree was constructed using kimura 2-parameter model in MEGA7 and positions with sequence gaps were deleted. The relatedness

of genes was estimated using a two-parameter model (Kimura 1980). The robustness of the tree topology was computed by bootstrap analysis with 1000 replicates for maximum likelihood analysis. Pairwise average nucleotide identity (ANI) of test and reference strains was calculated using OrthoANIu tool (Yoon et al. 2017). Using 95% ANI as a lower boundary for species delineation (Goris et al. 2007), the type strains or undefined species of *Mesorhizobium* available in GenBank database were selected. Only the reference and type strains which shared greater than 95% ANI with our test strains were included in the phylogenetic analyses. The aligned sequences of *atpD* and *recA* were concatenated in MEGA7. The concatenated phylogenetic tree of *atpD-recA* genes was constructed using the best fitting model (Tamura-Nei model with discrete Gamma distribution) of nucleotide substitution to improve the bootstrap values (Laranjo et al. 2012; Tamura and Nei 1993).

Mantel tests were performed using the R package 'vegan' (Oksanen et al. 2007) to determine the associations between genetic pairwise distances (16S-23S rRNA IGS) of the rhizobial strains and pairwise geographical distances, i.e., pairwise differences in symbiotic and ecological adaptation traits of the rhizobial strains, and soil factors of sampling sites. For genetic distance, kinship matrices were calculated using MEGA-7 software.

3.4.8 Nucleotide sequence accession numbers

The nucleotide sequences derived from 77 test strains and CC1192 were deposited to NCBI GenBank. The accession numbers are MW584722-MW584799 for 16S-23S rRNA IGS, and MW67689-MW676943 for *nodC* and *nifH* genes (Table S2).

3.4.9 Statistical analysis

All quantitative data for symbiotic traits (nodulation, shoot dry weights, SE% and leaf chlorophyll content) were analysed by one-way analysis of variance using general linear model in Statistix 8.0 software (USDA 2007) and mean values were compared at LSD ($p = 0.05$)

level. The associations between symbiotic traits of inoculated plants were determined using linear regression analysis. Data were normalised to $\log_{10}$ and the homogeneity of variances was tested using Bartlett's test. A two-sample t-test was performed to determine the variation of symbiotic traits of inoculated plants (normalised data) between the two groups of origin (with and without history of chickpea cropping in the past 10 years). The graphs and box plots were generated using SigmaPlot Version 14.0 (Systat, Software In., San Jose CA, USA).

3.5. Results

3.5.1 Symbiotic effectiveness of chickpea rhizobia

Chickpeas were grown in soils sourced from 73 sites across Australia, and 80 strains collected from their nodules were authenticated as chickpea rhizobia. From this number, 65 test strains (81%) were able to induce a shoot dry weight (SDW) that was statistically equivalent to CC1192. In contrast, 15 strains (19%) exhibited lower performance in SDW accumulation than CC1192 (Fig. 1a). Although the ability of rhizobial strains to form nodules varied among the test strains, SDW accumulation of inoculated plants was positively correlated with leaf chlorophyll content (Fig. 1, Fig. 2b).

SE% was computed by comparing the SDW of plants inoculated with test strains with the SDW of positive control treatments inoculated with CC1192. Strains varied considerably in their SE% on chickpea ($p < 0.01$, Fig. S1). The majority of the strains (81%) were considered to be effective based on their SE% (> 80%) being equivalent to that of CC1192 (100%). Strain A47, collected from chickpea cropped fields in Queensland provided the highest SE% (110%), that was statistically higher ($p < 0.01$) than 50% of the test strains in this study. In contrast, 15 strains had lower SE% than CC1192, ranging from 53 to 75%. Four strains (A14, A17, A35 and A36) with the lowest SE% (< 60%) were obtained from soils where chickpea had not been cultivated in the past 10 years (Table S1).

Regression analysis of the symbiotic traits revealed that nodule number was strongly correlated with nodule dry weight ($p < 0.001$) (Fig. 2a). However, neither nodule numbers nor nodule dry weight were correlated with shoot biomass.

3.5.2 Physiological characterisation of chickpea rhizobia

There was considerable variation in physiological characteristics among the test strains (Fig. 3). All strains grew at pH between 6.0 and 8.5, however the percentage of tolerant strains declined outside this range (Fig. 3a). No strains were able to grow at a pH lower than 4.4. Three strains (A14, A78 and A79) were more acid tolerant (grew at pH 4.4) than CC1192 (pH 4.7). Most strains (80%) showed high alkaline tolerance and were able to grow in media up to pH 10, although they were isolated from soils with pH that ranged from 6.5 to 8.3. Nineteen tested strains (23%) had higher temperature tolerance (40 °C) than CC1192 (35 °C). Although all strains were particularly sensitive to high NaCl concentrations, four strains had greater tolerance (up to 2% NaCl) than CC1192 (up to 1.5% NaCl) (Fig. 3c). Additionally, some strains had greater tolerance to some metals and antibiotics, namely, Pb, Co, Ni, Al, ampicillin and streptomycin than CC1192 (Fig. 3d, e).

3.5.3 Influence of chickpea cropping history on nodulation and symbiotic effectiveness of rhizobial strains

A total of 70 strains, 39 from fields with a history of chickpea cropping and 31 from fields with no chickpea history in the last 10 years, were analysed using a two-sample t-test. Ten strains were omitted because they were collected from sites with an unknown cropping history and soil factors (Table S1). Generally, strains isolated from fields without a history of chickpea cropping induced higher nodule numbers ($p < 0.01$) and nodule dry weights ($p < 0.05$) than

those from chickpea cropped fields (Fig. 4a, b). For example, strains isolated from chickpea fields (e.g., A44, A45 and A57) induced lower nodulation (average nodule number of 5) than strains A02, A16, A17 (average nodule number of 17), that were isolated from soils with no

history. However, strains isolated from fields with a history of cultivated chickpea generated higher shoot dry weights and had greater symbiotic effectiveness than those obtained from soils without chickpea cropping in the past 10 years ($p < 0.05$, Fig. 4c, d). The most effective strains were from fields with a history of chickpea cropping, regardless of nodule numbers (Fig. 4).

3.5.4 Symbiotic performance of selected strains under field conditions

Four test strains and CC1192 were tested under field conditions to evaluate their symbiotic effectiveness on chickpea. Inoculation significantly enhanced shoot dry weights compared with the uninoculated control (Fig. 5b). The most effective strain identified in the pot experiment (A47, Fig. 1) showed more effective nodulation, based on a 0–5 nodulation rating scale, than other strains including CC1192 in the field (Fig. 5a). Strain A47 also had the highest nitrogen fixation (%Ndfa), followed by M082 (Fig. 5c), while the amount of N fixed per plant was not significantly different among inoculation treatments (Fig. 5d). Inoculation did not have a significant impact on chickpea grain yield (data not shown).

3.5.5 Phylogenetic analysis of 16S-23S rRNA IGS region

Phylogenetic analyses of 77 test strains (selected on 16S-23S rRNA IGS sequence quality), CC1192 and reference sequences from GenBank revealed that all strains belonged to the genus *Mesorhizobium and* could be subdivided into four distinct clusters (Fig. 6).

Group I contained 31 strains that were closely related to common chickpea symbionts, *M. ciceri*, *M. loti* and *M. opportunistum*, with 95 to 100% ANI. Among these, 20 strains were closely related (99–100% ANI) to CC1192 and most of these strains were isolated from soils with a history of chickpea cropping in the last 10 years. The 16S-23S rRNA IGS sequences of the remaining 11 strains were more closely related to *M. ciceri* LMG14989 and *M. opportunistum* WSM2075, with 98 to 100% ANI. All strains in this group, except A22, were as effective as CC1192 in fixing N. The most effective strain based on SDW, strain A47

obtained from a soil with a history of chickpea cropping in Queensland, also belonged to this group. In contrast, the 16S-23S rRNA IGS sequence of a less effective strain (A22) collected from a field with unknown cropping history in NSW was closer to that of *M. opportunistum* WSM2075, with 99.65% ANI.

In Group II, 10 out of 11 strains were closely related to *Mesorhizobium temperatum* and *M. mediterraneum* with ANI that ranged from 96–100 and were as effective as CC1192, being classified in the group with the highest SE% (> 80%). The sequence of the remaining strain, A32, was more similar to other species of *Mesorhizobium* including *M. tianshanense*, *M. gobiense* and *M. amorphae*, with 96–100% ANI. The ten strains in this group were isolated from soils with a history of chickpea cropping (Table S1). Strain A32 was isolated from a Queensland field with no history of chickpea cropping in past 10 years. However, this strain had high symbiotic efficiency (97%) relative to CC1192 (Fig. S1) and produced 10 nodules per plant, which was less than that produced by six tested strains (A02, A14, A16, A17, A25 and A28) from group III (Figs. 6, 7). The least effective strain A60, belonged to Group II and was isolated from NSW soil and produced just seven nodules per plant, despite originating from a soil with a history of chickpea cropping. The 15 strains belonging to group III were most closely related to *Mesorhizobium huakuii*, *Mesorhizobium* sp. strain N33 and M. sp. S792 with 97–99% ANI. Most of the group III strains were obtained from soils that lacked chickpea cultivation in past 10 years and were considered to be ineffective (A14) or poorly effective (e.g. A26, A27) strains (Fig. 6). Group IV comprised 20 strains that were more closely related to *M. silamuruense*, *M. tamadayense*, *M. plurifarium* and other (undetermined) species of *Mesorhizobium*, with 95–97% ANI.

The symbiotic effectiveness of the strains was significantly associated with their genetic relatedness. The majority of the strains belonging to group I were related to common chickpea

symbionts, especially *M. ciceri* CC1192, and 97% of these strains were considered to have high SE. Only one strain (3%) was considered as poorly effective. In contrast, among group IV strains, which were only distantly related to CC1192, 45% had low SE and only 55% were considered to have adequate SE (Fig. 6).

3.5.6 Phylogenetic analysis of symbiosis-related genes

Nucleotide sequences obtained for *nodC* and *nifH* were 644 bp and 540 bp long respectively, after checking and editing the quality of sequence in MEGA7 program. The phylogenetic grouping of the strains revealed zero genetic distance and shared 100% ANI with a number of known chickpea nodulating symbionts (Fig. 7a, b).

3.5.7 Phylogenetic analysis of *atpD* and *recA* genes

Nine selected strains from different groups in 16S-23S rRNA IGS-based phylogeny and CC1192 were further phylogenetically analysed using two housekeeping genes (*atpD* and *recA*). The partial sequences of these genes, *atpD* and *recA*, had sequence lengths of 468 and 527 bp, respectively. The grouping of the selected strains using these house-keeping genes was consistent with 16S-23S rRNA IGS-based phylogeny (Figs. 6, 8). For example, all three test strains from group I (A47, A78 and A79) belonged to same cluster with CC1192. Strain A72 was grouped separately from other test strains in both phylogenies.

3.5.8 Relationships between geographic origins and phenotypic traits of test strains

Mantel tests were performed to determine the association between the genetic distances separating rhizobial strains and their geographical origin and phenotypic traits, and soil factors (Table 1). A strong positive association was found between 16S-23S rRNA IGS phylogenetic relatedness of *Mesorhizobium* and geographical distances of strain collection sites ($p < 0.01$). The genetic distance among rhizobial strains was also positively correlated with their symbiotic quality, such as nodulation ($p < 0.05$), shoot biomass accumulation or SE% ($p < 0.01$), leaf

chlorophyll content ($p < 0.01$), and ecological adaptation traits, such as tolerance to high ($p < 0.05$) or low ($p < 0.01$) temperature, Pb, Zn ($p < 0.01$) and neomycin ($p < 0.05$) (Table 1). Soil physicochemical properties analysed in this study were not associated with the genetic distance of the strains (Table 1).

3.6. Discussion

3.6.1 Phylogenetic diversity of chickpea rhizobia: occurrence and potential of horizontal gene transfer (HGT) in cropping soils

The genetic and physiological attributes of 80 strains collected from the nodules of chickpea grown in soils sampled from across Australian cropping regions were analysed. The majority (81%) of test strains had SE% that was not significantly different ($p > 0.05$) from the inoculant strain CC1192 (77–110%). There was considerable variation in tolerance to low pH, high temperature, various metals and antibiotics among the tested strains. A Mantel test revealed that the genetic distances of the strains were positively correlated with geographic distances of collection sites, symbiotic effectiveness and some physiological characteristics.

Phylogenetic analysis of 16S-23S rRNA IGS placed the 77 selected rhizobial strains into four distinct groups of *Mesorhizobium* species. Historically, chickpea was considered to be a highly specific host that could be nodulated by either *M. ciceri* or *M. mediterraneum* (Broughton and Perret 1999). However, a recent global collection of *Mesorhizobium* revealed 28 species that were classified as chickpea symbionts, including 20 previously undescribed species (Greenlon et al. 2019). Previous phylogenetic studies of chickpea rhizobia have similarly demonstrated that geographically diverse species of *Mesorhizobium* including *M. amorphae*, *M. tianshanense, M. plurifarium*, *M. opportunistum* and *M. huakuii* are chickpea symbionts (Benjelloun et al. 2019; Laranjo et al. 2004; Maatallah et al. 2002; Tena et al. 2017). In the present study, based on 16S-23S rRNA IGS sequences, some strains were also related to rarely isolated chickpea symbionts such as *M. temperatum*, *M. gobiense*, *M. loti* and other

(undetermined) *Mesorhizobium* strains (e.g., *Mesorhizobium* sp. N33 and *M*. sp. ORS3359). The range of *Mesorhizobium* species that have been found to nodulate chickpea is possibly due to HGT of symbiotic genes from the commercial inoculant species to the resident soil rhizobia (Batista et al. 2007; Greenlon et al. 2019; Sullivan and Ronson 1998). HGT of nodulation genes from an inoculant strain to other resident *Mesorhizobium* species may allow these species to nodulate chickpea (Hill et al. 2021; Andrews et al. 2018; Nandasena et al. 2007; Sullivan et al. 1995).

There was significant variation in SE% between the strains that could be explained in part by genetic relatedness. For example, 27% of strains had nearly 100% similar 16S-23S rRNA IGS sequences, and equal or superior effectiveness to CC1192. Strain A47 has 100% identity to CC1192 in 16S-23S rRNA, *atpD* and *recA* genes. Previous studies have isolated highly effective rhizobia that were assumed to be genetically identical to the original inoculant (Batista et al. 2007; Santos et al. 1999), however, strain A47 had superior performance in both pot and field experiments and was able to grow on slightly lower pH media (4.5) than CC1192 (4.7) *in vitro*.

The integration of genetic elements from the inoculant strain to resident soil rhizobia via HGT may result in ineffective but infective rhizobia that can compete for successful occupancy of nodules, reducing the efficiency of nodulation and N fixation of the inoculant strain (Nandasena et al. 2007). The incongruence between core (16S-23S rRNA IGS) and symbiosis (*nodC* and *nifH*) gene-based phylogenies observed in tested rhizobial strains (Figs. 6, 7a), suggests the possibility that HGT of symbiotic genes into resident soil rhizobia that have different genetic backgrounds has occurred. In this study, some strains (e.g., A44, A48 and A49) had lower SE% relative to CC1192, although they were isolated from soil with a history of chickpea cropping. In contrast, the poorly effective strain A22 isolated from soil with

unknown history of chickpea cropping, was closely related to the chickpea symbiont *M. opportunistum* WSM2075. These results suggest that depending on the chromosomal background of the receiving strain, HGT from the inoculant strain to the resident soil rhizobia may produce symbiosis with chickpea plants of variable effectiveness (Nandasena et al. 2007; Haskett et al. 2016). Since most cultivated legumes have been inoculated in their origins, it is also possible that the rhizobia may have been introduced on the chickpea seedcoat.

Phylogenetic analysis of *atpD* and *recA* genes revealed that the grouping of nine selected strains together with CC1192 was consistent with 16S-23S rRNA-based phylogeny (Figs. 6, 8). For example, all three test strains from group I (A47, A78 and A79) belonged to same cluster as CC1192 in both phylogenies. This result confirms the robustness of 16S-23S phylogeny of our strains. It has recently been recognised that phylogenetic congruence of core genes and incongruence of accessory genes is common and is considered as evidence of HGT in many studies (Andrews et al. 2018).

The Mantel analysis indicated a positive association between genetic distance and geographic distance of the strains, suggesting that the diversity and biogeography of the strains were influenced by soil characteristics (pH), climate (temperature and rainfall), altitude and geology (Bala et al. 2003; Bontemps et al. 2010). For example, most of the strains that were more tolerant to high temperatures *in vitro* were isolated from soils from Queensland, which has warm to hot conditions in summer. Despite this, two strains (A78 and A79) that grew under acidic conditions (pH 4.4) were isolated from soils with neutral pH (6.89 in H_2O), even though the pH of the soil of origin is often related to the pH tolerance of rhizobial strains (Shamseldin and Werner 2005; Slattery et al. 2001). In this study, soil pH and total soil nutrient contents of sampling sites were not associated with the genetic distance of the strains (Table 1). A study on global *Mesorhizobium* diversity, however, revealed a strong association between soil pH,

soil type and genetic relatedness, although the physiological attributes of the strains were not investigated (Greenlon et al. 2019; Lemaire et al. 2015). The poor association found in the present study between low pH tolerance and soil origin pH, may have been due to limited variation in pH values for all 20 soils tested (neutral to slightly alkaline, Table S1). There was no significant relationship between antibiotic resistance of strains and their geographical origins, consistent with previous reports for chickpea rhizobia (Alexandre et al. 2006; Benjelloun et al. 2019).

Despite the genetic diversity among strains collected in this study, there was a positive correlation between strains with a similar 16S-23S rRNA IGS sequence to CC1192, and SE (Table 1). It is possible that the symbiotically effective strains have descended from CC1192 through a combination of mutation and/ or natural selection (Brockwell et al. 1995). In this study potential descendant strains mostly had equal or higher SE than the inoculant strain CC1192. Other studies have also shown that inoculation with strains distantly related to *M. ciceri* have equal to or lower SE with chickpea plants compared with inoculant strains more closely related to *M. ciceri* (Elias and Herridge 2015; Gunnabo et al. 2020). Although this study has not directly measured HGT, one possible explanation is that nodulation genes have been transferred from CC1192 into diverse *Mesorhizobium* species present in the soil, which has resulted in strains with generally lower SE compared with descendant strains closely related to CC1192 that had high SE%. In future, selection of strains better adapted to Australian soils with equal or superior SE than CC1192 may be possible through genetic analysis and SE testing of rhizobia closely related to the inoculant strain.

3.6.2 Symbiotic effectiveness of chickpea rhizobia

Within the 80 *Mesorhizobium* strains that were evaluated in a greenhouse pot experiment, there was a significant positive correlation between nodule numbers and nodule dry weight ($p < 0.01$), and leaf chlorophyll content and SDW (Fig. 2). It is accepted that SDW provides a good

estimate of SE% in legume-rhizobia symbioses in the absence of available nitrate (Howieson and Dilworth 2016; Peoples et al. 2002). In this study, SE% was calculated by comparing the shoot dry biomass of the treatments with that of the Australian commercial inoculant CC1192 (Unkovich et al. 1997) and SE% varied significantly among the tested strains varying from less effective (53%) to more effective (110%) than CC1192. Although most strains had similar symbiotic effectiveness to strain CC1192, some strains (around 19%) had inferior effectiveness, varying from SE% 53 to 75% relative to CC1192. These rhizobia may have acquired nodulation genes from CC1192 but also lost other symbiotic traits (Aserse et al. 2012).

Chickpea cropping history of the sampled fields generally influenced strain effectiveness (Fig. 4). Strains isolated from fields without a history of chickpea cropping generally had higher nodule numbers ($p < 0.01$) and nodule dry weights ($p < 0.05$) than those from fields previously cropped with chickpea (Fig. 4). However, strains isolated from fields with a history of cultivated chickpea generated larger shoot weights and greater symbiotic effectiveness than those obtained from soils without chickpea cropping in the past 10 years. The most effective strains were from fields with a history of chickpea cropping, regardless of nodule numbers; these strains are possibly descendants of commercial strain CC1192 or resident rhizobia that shared similar symbiotic traits with inoculant strains through HGT (Hill et al. 2021). In the present study, the majority of highly ineffective strains (e.g., A14, A17 and A35; Fig. 7a) were obtained from soils without a known history of chickpea cropping in the past 10 years, indicating that these strains may not have the capacity to express mutualistic traits, even though they are able to nodulate. It is possible that chickpea had been grown in some of those fields prior soil sampling and that inoculant rhizobial numbers have declined in the soil, leading to the proliferation of resident strains that have adaptive traits to the soil conditions, and contain symbiotic genes but with low SE%. Continued inoculation of chickpea is required to maintain

symbiotic effectiveness, even if the soil contains resident rhizobia that are capable of nodulating chickpea.

In the field experiment, inoculation with all four selected strains and CC1192 increased shoot dry matter accumulation (Fig. 5). There were no significant differences in nodule numbers among inoculated treatments, but nodule rating varied significantly. The results suggest that the amount (size and number) of nodules may not be an effective indicator of a strains' effectiveness under field conditions. Due to variation in chickpea nodule numbers and morphologies, it has been recommended that a nodulation score on a 0–5 scale be applied, based on several parameters including number, size, position, colour and distribution of nodules on the crown and lateral roots (Corbin et al. 1977; Hartley et al. 2005). Nitrogen fixation (%Ndfa) was significantly increased in plants inoculated with strain A47 compared with CC1192. This strain was identical (nearly 100% ANI) to CC1192 in all selected gene-based phylogenies but consistently had increased SE. It is likely that strain A47 has improved performance in the field environment, through adaptive traits compared with CC1192. In contrast, strain M009 had low efficiency in N fixation, although it was effective in the pot experiment, suggesting that symbiotic performance under field conditions can be constrained by a strain's ability to adapt and proliferate in local soil environments or that the environment has an overriding influence. This study confirmed the importance of field evaluation of strains, following laboratory assessment, to identify suitable and improved inoculant strains (Howieson et al. 1988).

3.6.3 Physiological characteristics of chickpea rhizobia

Environmental factors, including soil physicochemical properties, may limit nodulation, N fixation, persistence and saprophytic competence of rhizobia in the field (Jaiswal et al. 2016; Ndungu et al. 2018). Selection of symbiotically effective chickpea rhizobia adapted to local soil and climatic conditions may enhance nitrogen fixation in farming systems. Chickpea-

nodulating rhizobia may have to deal with low pH stress under field conditions, as chickpea roots release protons and carboxylates that result in acidification of the rhizosphere (Veneklaas et al. 2003), while soil microflora actively inhibit the growth of other microorganisms by producing antibiotics (Naamala et al. 2016; Turpin et al. 1992). The ability of rhizobia to grow in the presence of antibiotics is also important in enabling competition with antibiotic producing microflora in the rhizosphere, however only a minority of the test strains showed resistance to ampicillin (50 μg ml^{-1}), neomycin (20 μg ml^{-1}) and streptomycin (80 μg ml^{-1}) (Fig. 3e).

3.7. Conclusion

Based on genetic and physiological investigations, cropping soils in Australia contain resident chickpea rhizobia with stress tolerance and symbiotic efficiency comparable to, or divergent from, the commercial inoculant strain *M. ciceri* CC1192. The incongruence of core and symbiosis gene phylogenies among re-isolated chickpea-nodulating rhizobia suggests that there have been different evolutionary histories influencing these cores and accessory genes, and may indicate the transfer of symbiosis genes from strain CC1192 to resident rhizobia with diverse core genomic backgrounds. The symbiotic effectiveness of strains was positively associated with their genetic relatedness in 16S-23S rRNA IGS. In our study, strain A47, which is nearly identical to CC1192 in its 16S-23S rRNA IGS sequence, provided considerably greater N fixation capacity in a pot experiment than 50% of the test strains and CC1192, with a SE% of 110% relative to CC1192. Two strains (A78 and A79) were acid tolerant and warrant further testing, especially given the potential for adaptation to acidifying Australian agricultural soils. Strain A47 is considered to have potential as an improved commercial inoculant and warrants further evaluation under field conditions.

3.8. References

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3.9. Tables and figures

Table 1. Mantel test between the genetic distance (16S-23S rRNA IGS region) of strains in association with their phenotypic traits, geographic location and soil physicochemical properties of their orgins. *p < 0.05, **p < 0.01, ***p < 0.005, ^{ns}non-significance.

Factors	**R^2**	**P value**
Geographic origin	0.5277	0.005 **
Nodule numbers (plant^{-1})	0.0337	0.029 *
Shoot dry weight (g plant^{-1})	0.0611	0.001 ***
SE%	0.0611	0.001***
Leaf chlorophyll content	0.0467	0.001 ***
low temperature tolerance (10°C)	0.0465	0.003 **
High temperature tolerance (40°C)	0.0479	0.019 *
Pb tolerance	0.0378	0.005 **
Zn tolerance	0.0571	0.001 ***
Neomycin tolerance	0.0328	0.019 *
Soil pH	0.0008	0.839^{ns}
Soil N	0.0145	0.383^{ns}
Soil P	0.0090	0.702^{ns}
Soil K	0.0108	0.487^{ns}
Soil Ca	0.0180	0.308^{ns}

Table S1. Site details for rhizobial strains isolated from the sampled soils; state, chickpea cropping history (most recent crops), and soil pH and total nutrients in soils.

Strains	No. of strains	State	Coordinates	Chickpea cultivation in previous 10 years	pH (H_2O)	Ca $mgkg^{-1}$ soil	N%	P $mgkg^{-1}$ soil	K $mgkg^{-1}$ soil
A01-A05	5	VIC	35 44'15.58"S 142 11'43.87" E	No	6.46	581	0.0378	98	1300
A06-A11	6	VIC	35 47'09.53" S 142 54'30.85" E	Yes	7.08	661	0.0094	88	1250
A12-A15	4	VIC	35 59'54.38" S 142 30'32 14" E	No	7 56	2030	0.0612	185	4610
A16-A17	2	VIC	36 27'53.54" S 142 33'40 35" E	No	7.46	3090	0.1301	297	6560
A18-23	6	VIC	36 27'53.54" S 142 33'40 35" E	unknown	6.84	-	-	-	-
A24-A27	4	VIC	36 22'27.27" S 141 04'57.75" E	No	6.87	2640	0.1643	248	3080
A28-A29	2	VIC	36 15'06.34" S 143 14'44.62" E	No	6 91	3310	0.1307	274	7510
A30	1	SA	34 04'46.10" S 137 54'44 95" E	No	6.88	100000	0.1255	303	5510
A31	1	SA	31 55'50.12" S 133 06'32 58" E	No	8 32	100000	0.0694	279	3590
A32-A38	7	QLD	27 02'12.51" S 151 04'30 91" E	No	7.47	4280	0.0374	177	2130
A39-A41	3	QLD	27 02'40.58" S 150 08'11.67" E	Yes	7.72	4190	0.0432	155	1840
A42-A45	4	QLD	27 44'49.22" S 149 43'01.62" E	Yes	7.04	2420	0.0362	179	2250
A46-A51	6	QLD	28 02'26.84" S 150 02'22 55" E	Yes	7 19	4320	0.0719	185	2840
A52-A55	4	QLD	28 29'56.86" S 149 38'55.82" E	Yes	7 52	4050	0.0594	168	3380
A56-A57	2	QLD	28 48'32.38" S 148 45'48 31" E	Yes	7 52	5510	0.0326	284	6920
A58-A60	3	NSW	29 32'39.28" S 149 04'45 33" E	Yes	6 94	4930	0.0320	349	6770
A61-A66	6	NSW	29 58'47.77" S 149 49'17.60" E	Yes	8 20	5440	0.0399	514	4090
A67-A70	4	NSW	28 56'23.89" S 150 28'17 30" E	Yes	7.74	-	-	-	-
A71-A75	5	QLD	27 45'27.15" S 151 29'42 31" E	Yes	6.49	2820	0.0454	160	790
A76-A80	5	WA	31 04'42.50" S 117 19'23.03" E	No	6.89	621	0.0201	217	2020
Total	**80**								

Table S2. GenBank accession numbers of chickpea nodulating rhizobia tested in this study. ND indicates not determined.

Strains	Accession numbers		
	16S-23S rRNA IGS	*nodC*	*nifH*
A01	MW584722	MW676789	MW676867
A02	MW584723	MW676790	MW676868
A03	MW584724	MW676791	MW676869
A04	MW584725	MW676792	MW676870
A05	MW584726	MW676793	MW676871
A06	MW584727	MW676794	MW676872
A07	MW584728	MW676795	MW676873
A08	MW584729	MW676796	MW676874
A09	MW584730	MW676797	MW676875
A10	MW584731	MW676798	MW676876
A11	MW584732	MW676799	MW676877
A12	MW584733	MW676800	MW676878
A13	MW584734	MW676801	MW676879
A14	MW584735	MW676802	**ND**
A15	MW584736	MW676803	MW676880
A16	MW584737	MW676804	MW676881
A17	MW584738	MW676805	MW676882
A18	MW584739	MW676806	MW676883
A19	MW584740	MW676807	MW676884
A20	MW584741	MW676808	MW676885
A21	MW584742	MW676809	MW676886
A22	MW584743	MW676810	MW676887
A23	MW584744	MW676811	MW676888
A24	MW584745	MW676812	MW676889
A25	MW584746	MW676813	MW676890
A26	MW584747	MW676814	MW676891
A27	MW584748	MW676815	MW676892
A28	MW584749	MW676816	MW676893
A29	MW584750	MW676817	MW676894
A30	MW584751	MW676818	MW676895
A31	MW584752	MW676819	MW676896
A32	MW584753	MW676820	MW676897
A33	MW584754	MW676821	MW676898
A34	MW584755	MW676822	MW676899
A35	MW584756	MW676823	MW676900
A37	MW584757	MW676824	MW676901
A38	MW584758	MW676825	MW676902
A39	MW584759	MW676826	MW676903
A40	MW584760	MW676827	MW676904
A41	MW584761	MW676828	MW676905
A42	MW584762	MW676829	MW676906
A43	MW584763	MW676830	MW676907
A44	MW584764	MW676831	MW676908
A45	MW584765	MW676832	MW676909
A46	MW584766	MW676833	MW676910
A47	MW584767	MW676834	MW676911
A48	MW584768	MW676835	MW676912
A49	MW584769	MW676836	MW676913
A50	MW584770	MW676837	MW676914
A51	MW584771	MW676838	MW676915
A52	MW584772	MW676839	MW676916
A53	MW584773	MW676840	MW676917
A54	MW584774	MW676841	MW676918
A55	MW584775	MW676842	MW676919
A56	MW584776	MW676843	MW676920
A57	MW584777	MW676844	MW676921
A58	MW584778	MW676845	MW676922
A59	MW584779	MW676846	MW676923
A60	MW584780	MW676847	MW676924
A61	MW584781	MW676848	MW676925
A62	MW584782	MW676849	MW676926
A63	MW584783	MW676850	MW676927
A65	MW584784	MW676851	MW676928
A67	MW584785	MW676852	MW676929
A68	MW584786	MW676853	MW676930
A69	MW584787	MW676854	MW676931
A70	MW584788	MW676855	MW676932
A71	MW584789	MW676856	MW676933
A72	MW584790	MW676857	MW676934
A73	MW584791	MW676858	MW676935
A74	MW584792	MW676859	MW676936
A75	MW584793	MW676860	MW676937
A76	MW584794	MW676861	MW676938
A77	MW584795	MW676862	MW676939
A78	MW584796	MW676863	MW676940
A79	MW584797	MW676864	MW676941
A80	MW584798	MW676865	MW676942
CC1192	MW584799	MW676866	MW676943

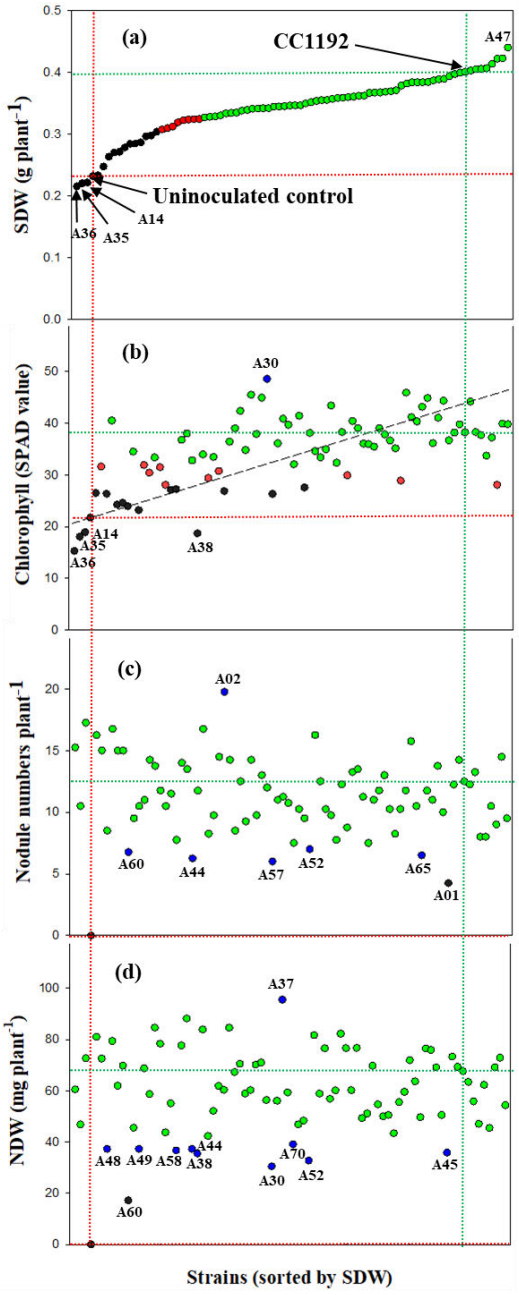

Figure 1. Distribution of a shoot dry weight (SDW), b leaf chlorophyll content, c nodule numbers and d nodule dry weight (NDW) induced by 80 test rhizobial strains and CC1192. Strains were sorted by their ability to enhance SDW in ascending order. Different colours of dots represent statistically different groups based on LSD (0.05) values, where black dots are strains which had a similar response to the uninoculated control, green dots represent the strains similar to positive control (CC1192), red dots are overlapping between positive and negative (uninoculated) controls, and blue dots are significantly different from both groups (red and green). The intercepts of green and red dotted lines indicate CC1192 and uninoculated controls, respectively (colour figure online)

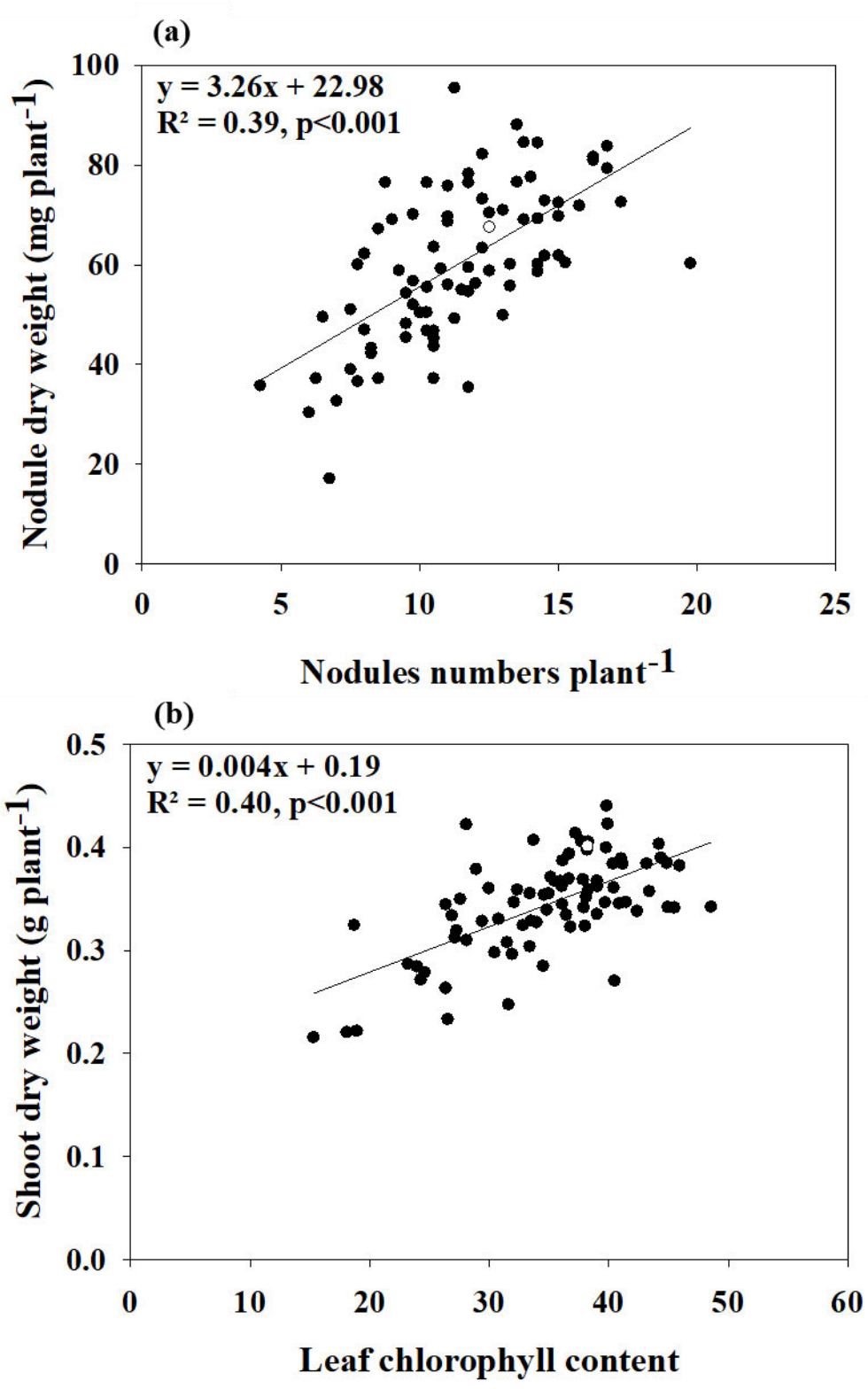

Figure 2. Effect of 80 freshly isolated rhizobia on chickpea nodulation and growth in a pot experiment. Correlation between a nodule number and nodule dry weight, and b leaf chlorophyll content and shoot dry weight. Open circle represents CC1192 and closed circles represent test strains

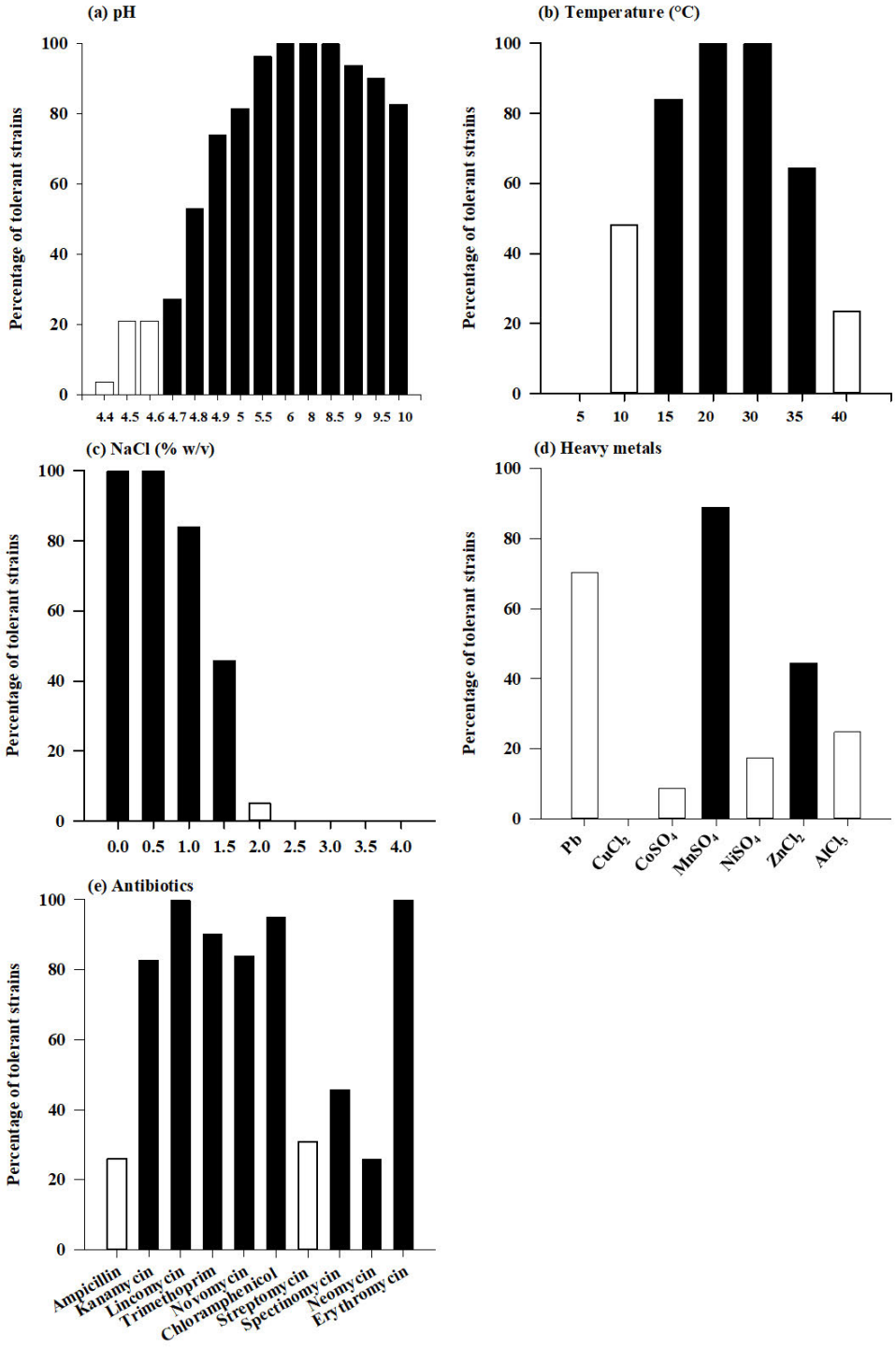

Figure 3. Percentage of tolerant strains (80 test strains and CCll92) to a pH, b temperature (°C), c NaCl (% w/v), d metals and e antibiotics. Filled bars show the percentage of tolerant strains including CC1192; empty bars indicate the percentage of strains but where CC1192 was unable to grow

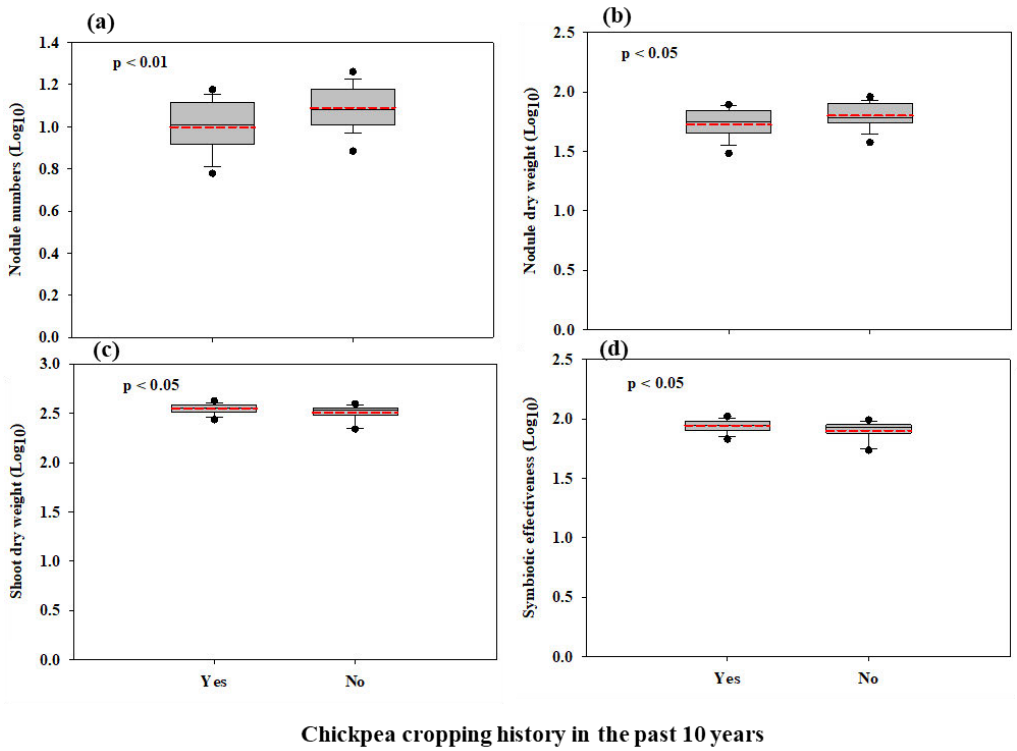

Figure 4. The impact of chickpea cropping history in the past 10 years on a nodule number, b nodule dry weight, c shoot dry weight, and d symbiotic effectiveness (%) produced by 70 selected rhizobial strains. Two categories of strains; n = 39 and 31 from fields with (Yes) and without (No) a history of chickpea cropping, respectively were included in the analysis. P values from two-sample t-tests are provided in both cases. The black line in the box indicates the median, the red dotted line indicates the mean, the ends of the box indicate upper and lower quartiles, the error bars represent 5th/95th percentile values, dots represent the extreme values above the 95th percentile (colour figure online)

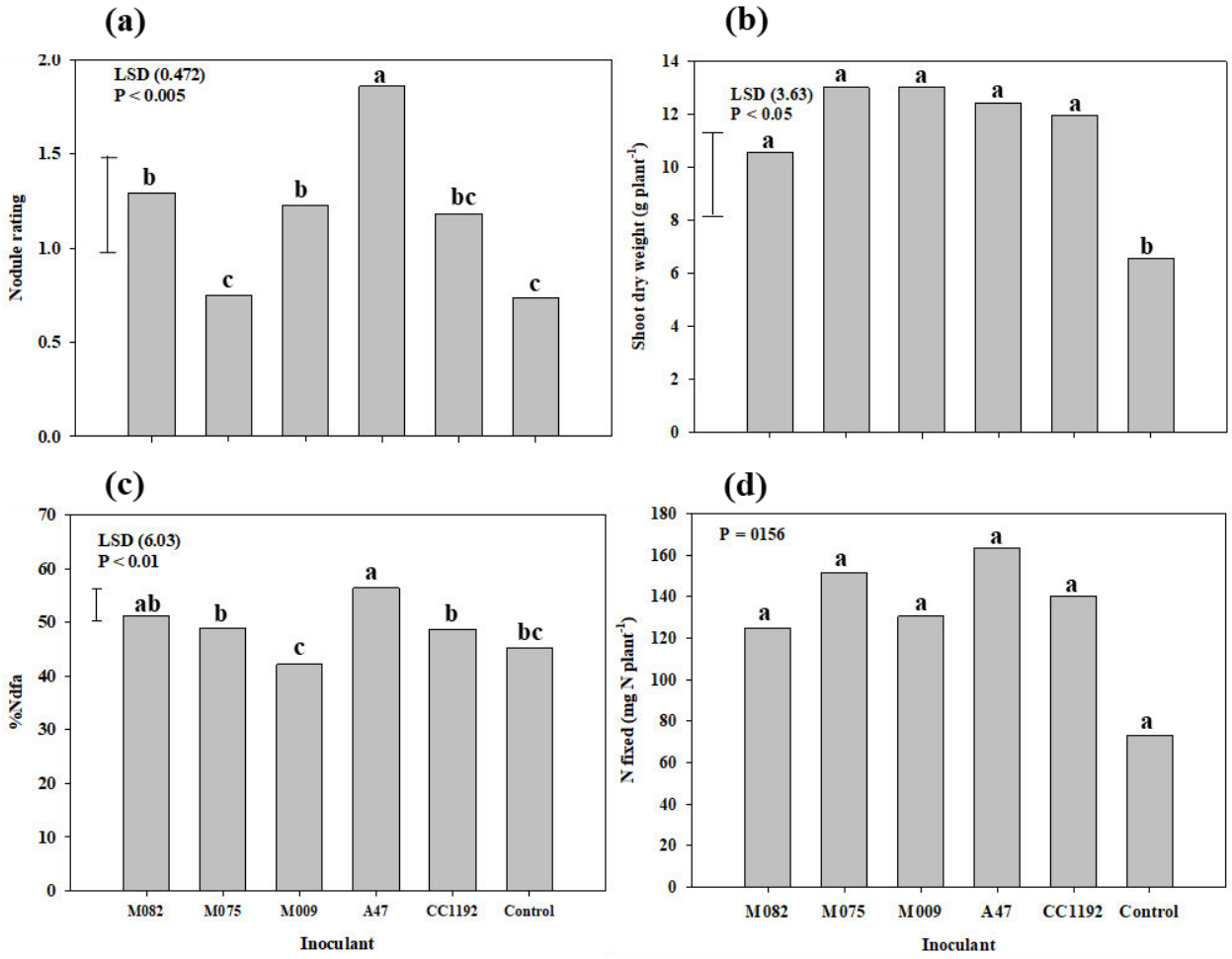

Figure 5. Field performance of selected chickpea rhizobial strains in a nodule rating, b shoot dry weight, c %Ndfa and d N fixed (mg N plant^{-1})

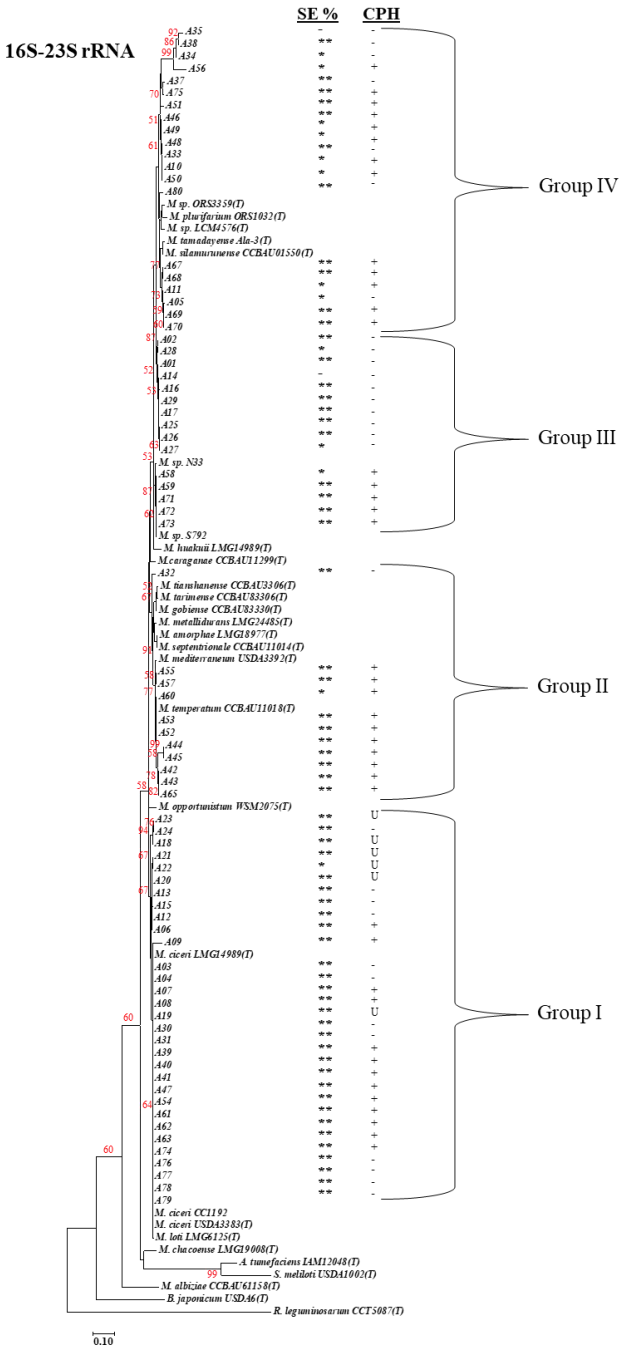

Figure 6. Maximum likelihood phylogenetic tree based on 16S-23S rRNA sequences showing the relationship among chickpea rhizobia strains and reference strains. Bootstrap values were computed based on 1000 replications. Bootstrap values > 50% are indicated at the internodes. The scale bar (0.10) indicates the percentage of nucleotide substitutions per site. *A, Agrobacterium, B, Bradyrhizobium, M, Mesorhizobium, S, Sinorhizobium, R, Rhizobium*, T, type strain SE, symbiotic effectiveness, **effective, *poorly effective, – ineffective, CPH, chickpea cropping history, with (+), without (−) and unknown (U) chickpea cropping in the past 10 years

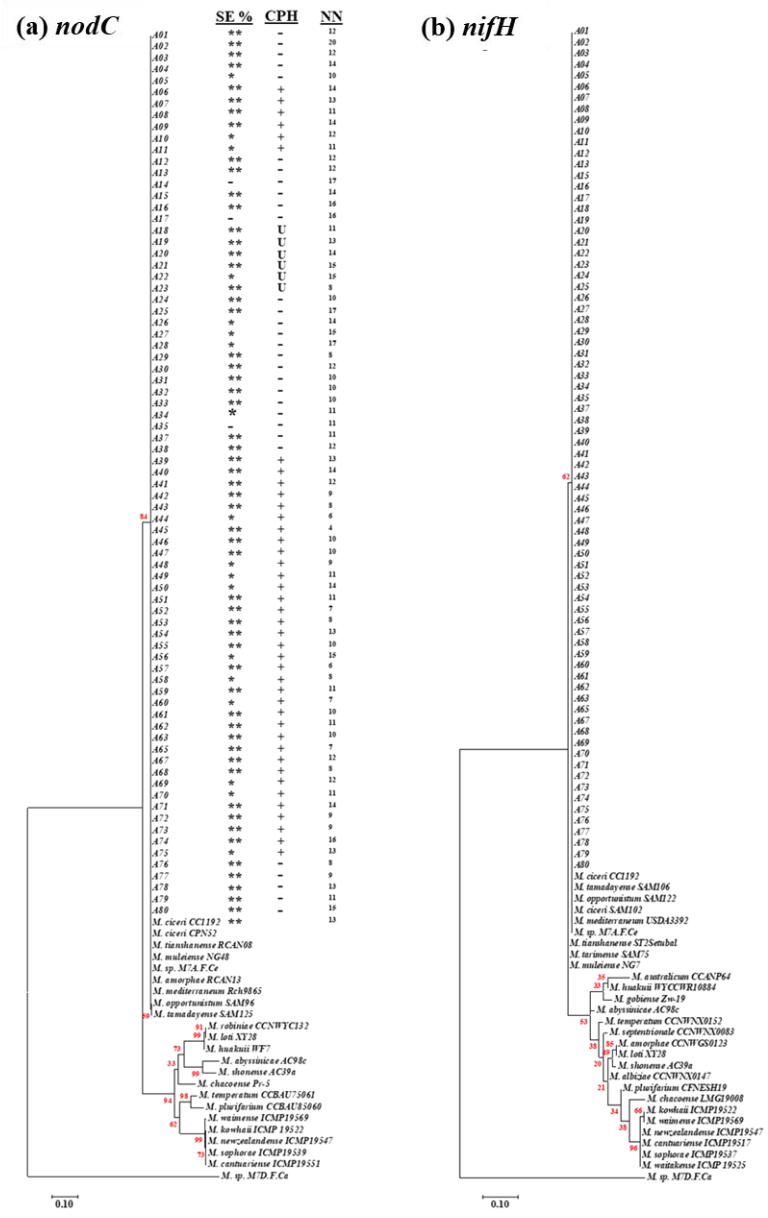

Figure 7. Maximum likelihood phylogenetic tree based on a *nodC* and b *nifH* gene sequences. Bootstrap values are computed based on 1000 replications and indicated above the branches which shows the percentages of trees in which the closely related taxa clustered together. Scale bar (0.10) indicates the percentage of nucleotide substitutions per site. Evolutionary analyses were conducted in MEGA7 in which all missing and gap nucleotide sequences were deleted during phylogenetic tree construction. *M*, *Mesorhizobium*, SE, symbiotic effectiveness, **effective, *poorly effective, – ineffective, CPH, chickpea cropping history; with (+), without (−) and unknown (U) chickpea cropping in the past 10 years, and NN, nodule numbers per plant

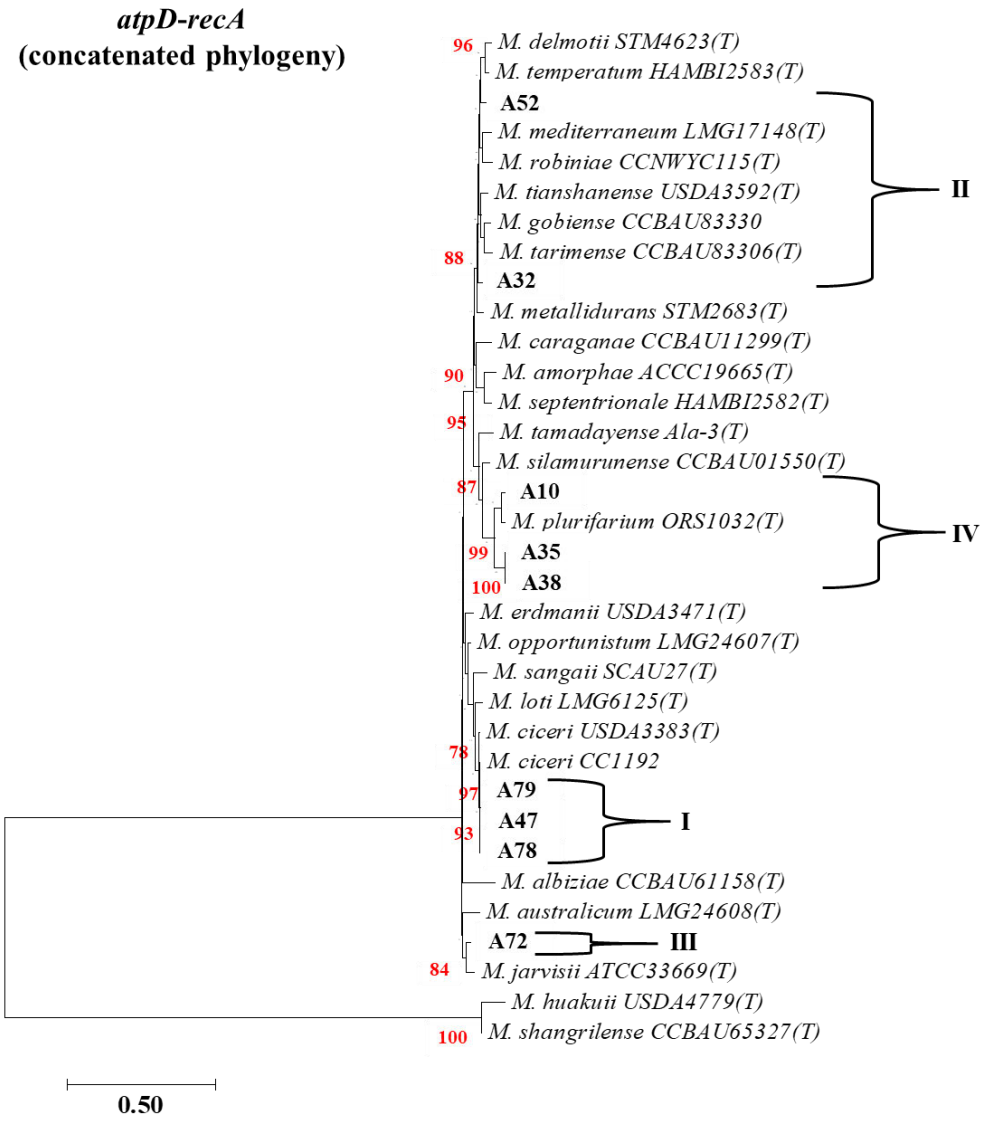

Figure 8. Maximum likelihood phylogenetic tree based on concatenated sequences of *atpD* and *recA* of 9 selected test strains and CC1192 (*M*, *Mesorhizobium*, T, type strain). The concatenated phylogenetic tree of *atpD-recA* genes was constructed using the best fitting model (Tamura-Nei model with discrete Gamma distribution) of nucleotide substitution (Laranjo et al. 2012; Tamura and Nei 1993). The groups I-IV were generated based on phylogeny of 16S-23S rRNA. Bootstrap values were computed based on 1000 replications and the values > 70% are indicated above the branches which shows the percentages of trees in which the closely related taxa clustered together. Scale bars (0.05) indicate the percentage of nucleotide substitutions per site. Evolutionary analyses were conducted in MEGA7 in which all missing and gap nucleotide sequences were deleted during phylogenetic tree construction

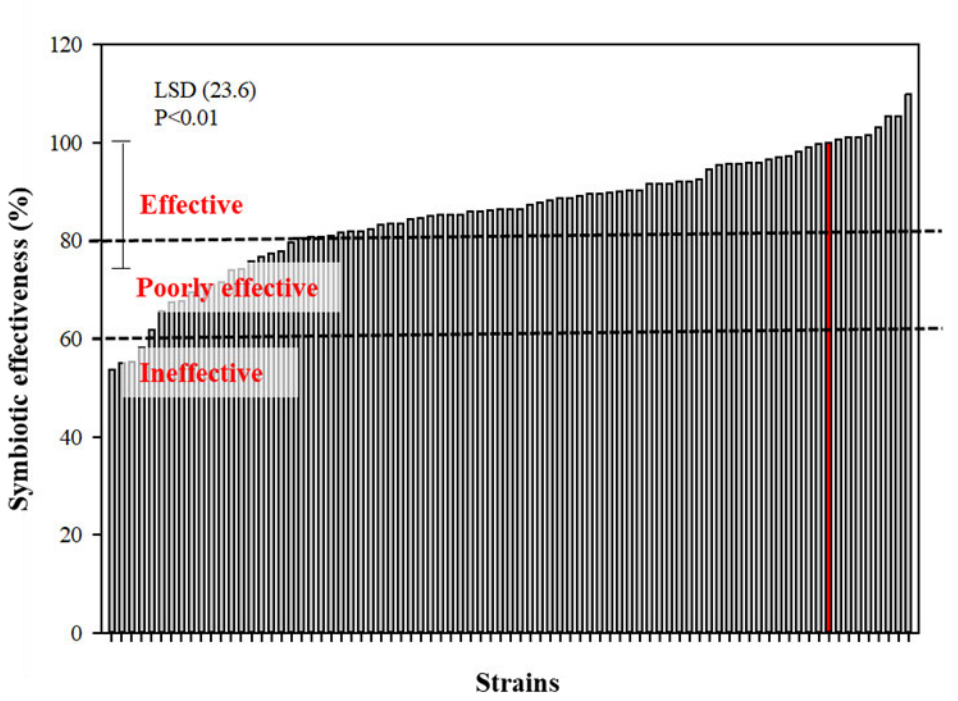

Fig. S1 Mean symbiotic effectiveness (%) based on shoot dry weight of chickpea inoculated with 80 tested strains as compared with that of positive control, commercial strain CC1192, set at 100% (red bar). Effective, >80%; poorly effective, 60-80%; ineffective, <60%, relative to commercial inoculant.

Chapter 4. Rhizobial diversity is associated with inoculation history at a two-continent scale

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4.1. Statement of authorship

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Principal Author

Name of Principal Author (Candidate)	Myint Zaw		
Contribution to the Paper	Planned and conducted all experiments, analysed and interpreted the data, and wrote the manuscript		
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	29/01/2022

Co-Author

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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Contribution to the Paper	Supervised development of work and reviewed the manuscript and corresponding author		
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Signature		Date	31/01/2022

4.2. Abstract

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Rhizobial diversity is associated with inoculation history at a two-continent scale

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One sentence summary: Mesorhizobia isolated from Myanmar soils, where there has been little inoculation, show more genetic diversity compared to Australia where there is frequent inoculation of chickpea crops.
Editor: Angela Sessitsch
‡Co-corresponding author.

Abstract

A total of 120 *Mesorhizobium* strains collected from the central dry zone of Myanmar were analyzed in a pot experiment to evaluate nodulation and symbiotic effectiveness (SE%) in chickpea plants. Phylogenetic analyses revealed all strains belonged to the genus *Mesorhizobium* according to 16–23S rDNA IGS and the majority of chickpea nodulating rhizobia in Myanmar soils were most closely related to *M. gobiense*, *M. muleiense*, *M. silamurunense*, *M. tamadayense* and *M. temperatum*. Around two-thirds of the Myanmar strains (68%) were most closely related to Indian strain IC-2058 (CA-181), which is also most closely related to *M. gobiense*. There were no strains that were closely related to the cognate rhizobial species to nodulate chickpea: *M. ciceri* and *M. mediterraneum*. Strains with diverse 16S–23S rDNA IGS shared similar *nodC* and *nifH* gene sequences with chickpea symbionts. Detailed sequence analysis of *nodC* and *nifH* found that the strains in Myanmar were somewhat divergent from the group including *M. ciceri* and were more closely related to *M. muleiense* and IC-2058. A cross-continent analysis between strains isolated in Australia compared with Myanmar found that there was little overlap in species, where Australian soils were dominated with *M. ciceri*, *M. temperatum* and *M. huakuii*. The only co-occurring species found in both Myanmar and Australia were *M. tamadayense* and *M. silamurunense*. Continued inoculation with CC1192 may have reduced diversity of chickpea strains in Australian soils. Isolated strains in Australian and Myanmar had similar adaptive traits, which in some cases were also phylogenetically related. The genetic discrepancy between chickpea nodulating strains in Australia and Myanmar is not only due to inoculation history but to adaptation to soil conditions and crop management over a long period, and there has been virtually no loss of symbiotic efficiency over this time in strains isolated from soils in Myanmar.

Keywords: central dry zone, *nodC*, inoculation, *Mesorhizobium*, Myanmar, *nifH*

Introduction

In Myanmar, pulses are currently grown on over 4 × 10^6 hectares (21% of agricultural lands) with an annual production of 4,648,000 MT (MOALI 2020). Myanmar accounts for 5% of the world pulse cultivated area and 6% of their production (Soe and Kyaw 2016). Chickpea is mainly grown in the central dry zone (CDZ) of Myanmar by small-holder farmers and is sown on residual moisture as a second crop grown after rice in the postmonsoon season from October to November and harvested in February–March (Herridge et al. 2019). Chickpea has been cultivated in Myanmar for an unknown period of time and was likely introduced historically from India, where chickpea cultivation has been traced back to the Bronze Age (Redden and Berger 2007).

Although chickpea is an important crop in Myanmar, there are very few, if any, inputs into the growth of this crop, including inoculation. Currently, there is no established supply chain for rhizobial inoculants in Myanmar (Denton et al. 2017), although some attempts have been made to produce inoculants on a very small scale using resident strains (Than 2010). Despite the lack of inoculation, chickpea plants in Myanmar can be well-nodulated.

Chickpea is a highly specific host that can be nodulated by members of a single genus, *Mesorhizobium*, strains of which contain highly specific symbiosis genes (Laranjo et al. 2008). These strains were probably not present in Myanmar before the introduction of chickpeas. The original species of chickpea-nodulating rhizobia (*M. ciceri* or *M. mediterraneum*) that evolved in south-eastern Turkey have spread throughout the world (Nour et al. 1995, Greenlon et al. 2019) and may have come to Myanmar with seeds/soil mixture during chickpea introduction from India (Rai et al. 2012). A study has investigated the diversity of a limited number of strains isolated from chickpea nodules in Myanmar and found three main *Mesorhizobium* species: *M. muleiense*, *M. tianshanense* and *M. plurifarium* (Soe et al. 2020). It is possible that Myanmar soils already had high populations of resident rhizobial strains that have acquired symbiotic ability through horizontal gene transfer (HGT) with exotic chickpea symbionts (Bouznif et al. 2019). These resident rhizobia have likely evolved with chickpea plants over a long period of cultivation and may have adapted to the Myanmar environments.

Mesorhizobium species contain strain-specific symbiosis islands that have different structures depending on the geography and host plants (Sullivan et al. 2002, Uchiumi et al. 2004, Kasai-Maita et al. 2013, Greenlon et al. 2019, Perry et al. 2020). The symbiosis islands contain conserved regions with symbiotic genes (*nod* or *nif*; Greenlon et al. 2019, Perry et al. 2020). In particular, the *nodC* gene is essential for nodulation in all rhizobia (Laranjo et al. 2008). In

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4.3. Introduction

In Myanmar, pulses are currently grown on over 4 $\times 10^6$ hectares (21% of agricultural lands) with an annual production of 4,648,000 MT (MOALI 2020). Myanmar accounts for 5% of the world pulse cultivated area and 6% of their production (Soe and Kyaw 2016). Chickpea is mainly grown in the central dry zone (CDZ) of Myanmar by small-holder farmers and is sown on residual moisture as a second crop grown after rice in the postmonsoon season from October to November and harvested in February–March (Herridge *et al.* 2019). Chickpea has been cultivated in Myanmar for an unknown period of time and was likely introduced historically from India, where chickpea cultivation has been traced back to the Bronze Age (Redden and Berger 2007).

Although chickpea is an important crop in Myanmar, there are very few, if any, inputs into the growth of this crop, including inoculation. Currently, there is no established supply chain for rhizobial inoculants in Myanmar (Denton *et al.* 2017), although some attempts have been made to produce inoculants on a very small scale using resident strains (Than 2010). Despite the lack of inoculation, chickpea plants in Myanmar can be well-nodulated.

Chickpea is a highly specific host that can be nodulated by members of a single genus, *Mesorhizobium*, strains of which contain highly specific symbiosis genes (Laranjo *et al*. 2008). These strains were probably not present in Myanmar before the introduction of chickpeas. The original species of chickpea-nodulating rhizobia (*M. ciceri* or *M. mediterraneum*) that evolved in southeastern Turkey have spread throughout the world (Nour *et al*. 1995, Greenlon *et al*. 2019) and may have come to Myanmar with seeds/soil mixture during chickpea introduction from India (Rai *et al.* 2012). A study has investigated the diversity of a limited number of strains isolated from chickpea nodules in Myanmar and found three main *Mesorhizobium* species: *M. muleiense, M. tianshanense* and *M. plurifarium* (Soe et al. 2020). It is possible that

Myanmar soils already had high populations of resident rhizobial strains that have acquired symbiotic ability through horizontal gene transfer (HGT) with exotic chickpea symbionts (Bouznif *et al.* 2019). These resident rhizobia have likely evolved with chickpea plants over a long period of cultivation and may have adapted to the Myanmar environments.

Mesorhizobium species contain strain-specific symbiosis islands that have different structures depending on the geography and host plants (Sullivan *et al*. 2002, Uchiumi *et al*. 2004, Kasai-Maita *et al*. 2013, Greenlon *et al*. 2019, Perry *et al*. 2020). The symbiosis islands contain conserved regions with symbiotic genes (*nod* or *nif*; Greenlon *et al*. 2019, Perry *et al*. 2020). In particular, the *nodC* gene is essential for nodulation in all rhizobia (Laranjo *et al.* 2008). In symbiotic genes, nucleotide insertion events may impact on bacterial evolution including mutations that are deleterious, neutral, or beneficial for effective symbiotic ability of rhizobia (Siguier *et al.* 2014, Vandecraen *et al*. 2017, Perry *et al.* 2020, Consuegra *et al.* 2021, Arashida *et al.* 2022). Given that there have been few inoculation events (or new introductions of symbiosis genes in native rhizobial populations), changes in conserved regions of *nod* genes over time may have occurred in Myanmar.

The diversity of *Mesorhizobium* has been reported within countries, but there has been very little comparison of strains between countries. Previous studies found that *M. ciceri* and *M. mediterraneum* are ubiquitous in most countries including Spain (Nour *et al.* 1994a, 1995), Portugal (Laranjo *et al*. 2004), Morocco (Maatallah *et al.* 2002), Tunisia (L'taief *et al.* 2007), India (Zafar *et al.* 2017) and Australia (Elias and Herridge 2015). In China, *M. ciceri* and *M. mediterraneum* were not identified (Zhang et al. 2012a, b, 2017, 2018a). In countries where chickpea has been introduced relatively recently and grown with frequent rhizobial inoculation (USA, Canada, and Australia), the majority of rhizobial strains isolated from the field are closely related to, but distinct from, the inoculant strains (e.g. *M. ciceri* strain CC1192 in

Australia; Greenlon *et al*. 2019, Zaw *et al.* 2021). In contrast, a broader range of *Mesorhizobium* species was found in countries with a long history of chickpea cultivation such as India, Ethiopia and Morocco where rhizobial inoculation is rare or absent. Resident *Mesorhizobium* strains in Myanmar have experienced unique selective pressure such as cropping, and inoculation history compared with other countries and are likely to have contrasting genetic and symbiotic diversity compared with other countries where rhizobial diversity and ecology have been extensively studied.

The objectives of this study were, therefore, (1) to analyze the phylogenetic diversity of rhizobia in Myanmar chickpea-growing soils to provide a more detailed picture of the diversity of chickpea nodulating rhizobia, particularly in comparison with Australia and (2) to identify highly adapted strains with high N fixation capacity as potential inoculants in Myanmar farming systems for improving crop productivity and soil health.

4.4. Materials and methods

4.4.1 Sample collection and pH assessment

Soil samples were collected from 103 chickpea growing fields in the Mandalay, Sagaing and Magway regions of Myanmar during 2018 (Figure S1 and Table S1, Supporting Information). Soil cores were taken from a depth of 15 cm from five randomly selected points per field using a clean, sterilized spade and mixed thoroughly to make a composite sample. About 200 g of composite soil was collected from each site and placed in separate plastic bags and imported into Australia under a quarantine permit. For soil pH measurements, 5 g of dry soil was mixed with 25 ml 0.01 M $CaCl_2$ and shaken on a bench-top shaker for 1 h. The soil suspension was then left to settle for 30 min to allow sedimentation before conducting pH measurement.

4.4.2 Rainfall and temperature in the CDZ

Weather data for the CDZ of Myanmar (Figure S1, Supporting Information) was obtained from the Department of Meteorology and Hydrology, Naypyidaw, Myanmar. In the CDZ, chickpea is grown after monsoon rice or other lowland crops in late October or early November using residual soil moisture (Cornish *et al*. 2018, Herridge *et al*. 2019). Average monthly rainfall and temperature data for the CDZ during 2018 indicate the environmental conditions for chickpea cultivation from November to March (Figure S2, Supporting Information). The sampling sites received between 100 and 170 mm precipitation in the month of October prior to sowing chickpea and sampling of soils for this study. Rainfall distribution in the CDZ is erratic but typically highest in the May–July monsoon, with further (post-monsoon) seasonal rainfall through to October. The temperature is high (around 30 to 45ºC) throughout the year (Cornish *et al*. 2018). There is very little rain in the winter dry season when chickpeas are grown.

4.4.3 Isolation, authentication, and culture conditions of rhizobial strains

Infectivity of rhizobia was tested by growing chickpea plants in sterile pots containing 400 g of sterilized coarse sand (2–3 mm diameter) moistened with 75 ml of McKnight's N-free nutrient solution (McKnight 1949) and with a layer of collected Myanmar chickpea-growing soil (20 g), covering the sand that was then overlayed with a shallow layer of sterile sand. Chickpea cv. Hattrick seeds were sterilized in 70% ethanol for 30 s and rinsed five times with sterile water before three seeds were sown per pot. After germination, the seedlings were thinned to one plant per pot and a 2-cm layer of sterile plastic beads was added to the surface. Pots without soil were sown with chickpea seeds to check for cross contamination. A total of 20 ml of sterile McKnight's nutrient solution was added every 3 d. The plants were kept at 20–30ºC with regular sunlight in a quarantine glasshouse during winter.

Plants were removed from the pots after 5 weeks and nodules were collected and stored at −20ºC. A total of three to four nodules were selected from each plant and were surface sterilized in 70% ethanol for 30 s and 6% NaOCl for 1 min and rinsed six times in sterilized distilled water. The nodules were then crushed aseptically in 100 µl of water with a sterile plastic rod. A loopful of the nodule suspension was streaked on yeast mannitol agar (YMA) plates. Single colonies of bacterial strains were selected after incubation at 28ºC for 5 d and purified by subculturing through several cycles by selecting and streaking from single colonies.

For authentication as chickpea rhizobia, growth pouches (18 × 16.5 cm CYG seed germination pouches, Mega-International of Minneapolis, USA) were moistened with 20 ml of McKnight's N-free nutrient solution and sterilized by autoclaving. Chickpea seeds were surface sterilized with 70% ethanol for 20 s and three seeds were placed in each pouch. After germination, plants were thinned to one seedling per pouch and inoculated with 1 ml of inoculum (approximately 10^9 cells ml^{-1}). The seedlings were supplied with sterile McKnight's nutrient solution when necessary. A total of 3 weeks after inoculation, nodulation was assessed, rhizobia were reisolated and pure cultures stored in 25% glycerol solution at −80ºC.

4.4.4 Symbiotic effectiveness

The 120 test strains and *M. ciceri* CC1192, the Australian commercial chickpea inoculant strain (as a positive control) were streaked on YMA plates and incubated at 28ºC for 5 d. A loopful from a single colony was transferred to yeast mannitol broth (YMB) and incubated on rotary shaker (200 r/m) at 28ºC for 48 h to obtain approximately 10^9 cells ml^{-1}. A total of 1 ml of inoculum was added at the base of 5-day-old seedlings grown in sterilized sand–vermiculite media in plastic pots (120 × 75 mm). Plants were supplied with an equal amount of N-free McKnight's nutrient solution and supplemented with sterilized distilled water as necessary. Inoculation with strain CC1192 and an uninoculated treatment were included as positive and negative controls. The experiment was laid out in a randomized complete block design with

three replications. Plants were harvested 50 days after sowing. Nodules were removed from the roots and counted. Leaf chlorophyll content was measured using a SPAD meter. Nodule and shoot material were oven-dried at 70ºC for 48 h and dry weights per plant were recorded. Symbiotic effectiveness (SE) was calculated as described previously (Zaw *et al*. 2021).

4.4.5 Temperature, salinity, and pH tolerance of rhizobial strains

Phenotypic assessments were performed on YMA plates divided into 20 equal squares. Each square was spot-inoculated with 10 µl of rhizobia suspension (10^9 cells ml^{-1}) previously grown in YMB. All assessments were done with three replicates in a completely randomized design. The plates were incubated at 28ºC and bacterial growth was assessed 7 days after inoculation, except for the temperature tolerance experiment. Temperature tolerance was assessed by incubating cultures at 5, 10, 15, 20, 30, 35, 40 and 45ºC. Salinity tolerance was assessed on YMA supplemented with 0, 0.5, 1, 1.5, 2, 2.5, 3, 3.5 and 4% NaCl (w/v), and pH tolerance on YMA adjusted to pH 4.2, 4.4, 4.6, 4.8, 5, 5.5, 6, 8, 8.5, 9, 9.5 and 10 with potassium citrate (16.2 g l^{-1}) as the buffer and adjusted with 30% HCl or KOH solution. Strains were classified as tolerant, or intolerant, based on the presence or absence of growth.

4.4.6 Statistical analysis

All quantitative data for symbiotic traits (nodulation, shoot dry weights, and leaf chlorophyll content) were analyzed by one-way analysis of variance (ANOVA) using a general linear model in Statistix software (Version 8) and mean values were compared at LSD (0.05%) level.

4.4.7 PCR amplification and sequencing of rhizobial genes

A single colony of each strain was used as a source of DNA for PCR. Rhizobial cells from a single colony of freshly grown culture on YMA were transferred into a tube using a sterile pipette tip. Each tube had 12.5 µl MyFiTM Mix added, 1 µl of each primer and 10.5 µl sterile water following manufacturer's instructions (Bioline, A Meridian Life Science Company, Sydney,Australia). PCR was carried out using a thermal cycler (Bioer Version 1.10,

GeneWorks, Hindmarsh, South Australia). The primers and PCR conditions for core genes (16S–23S rDNA IGS, *dnaJ*, and *recA*) and symbiotic genes (*nodC* and *nifH*) are described in Table S2 (Supporting Information).

To verify the robustness of the 16S–23S rDNA-based phylogeny, six strains from different groups and strain CC1192 were selected for further phylogenetic analysis using two conserved housekeeping genes (*dnaJ* and *recA*). PCR amplification and sequencing of partial *dnaJ* and *recA* genes of the selected strains were undertaken as described by Alexandre *et al*. (2008) and Gaunt *et al*. (2001), respectively. The amplified PCR products were submitted to AGRF (Adelaide, South Australia) for sequence analysis using Sanger sequencing. A culture of the Indian chickpea rhizobial strain IC2058 (CA-181) obtained from ICRISAT, India was also included for sequencing in this study.

4.4.8 Phylogenetic data analysis

Sequences were checked and edited using Molecular Evolutionary Genetics Analysis version 7 (MEGA7; Kumar *et al*. 2016). The sequences were compared with NCBI GenBank databases using nucleotide BLAST for preliminary identification. The sequences of reference and type strains available in the GenBank database were also included for comparison with test strains. To investigate the phylogeographic diversity, the 16S–23S rDNA IGS sequences of 77 Australian strains from a previous study (Zaw *et al.* 2021) and an Indian strain (IC-2058-CA181) were added to the phylogenetic analysis as compared with those of 114 Myanmar strains in this study. Multisequence alignments for all test and reference strains were generated with ClustalW in MEGA7. In this program, poorly aligned positions and divergent regions of nucleotide sequences were eliminated. The phylogenetic trees of 16S–23S rDNA IGS, *nodC* and *nifH* were constructed using the maximum likelihood method and positions with gaps in any sequence were deleted. The genetic distances were estimated using Kimura's two-parameter model (Kimura 1980). The robustness of the tree topology was computed by

bootstrap analysis with 1000 replications for maximum likelihood analysis. Pairwise average nucleotide identity (ANI) of test and reference strains was calculated using the OrthoANIu tool (Yoon *et al*. 2017). The type strains or undefined species of *Mesorhizobium* available in the GenBank database were selected using a threshold of > 95% ANI for species delineation (Goris et al. 2007). Only reference and type strains which shared > 95% ANI with our test strains were included in the phylogenetic analyses. Approximately 800 bp sequence for *dnaJ* and 500 bp sequence for *recA* were obtained and used for multiple sequence alignment in MEGA 7 software. The sequences of *dnaJ* and *recA* were concatenated in MEGA7 and the phylogenetic tree was constructed using the best fitting model (Tamura-Nei model with discrete Gamma distribution) of nucleotide substitution to improve the bootstrap values (Tamura and Nei 1993, Laranjo *et al.* 2012). To evaluate amino acid changes in the *nodC* gene, the sequences were visually checked using chromatogram files, and aligned with the *nodC* sequence of *M. ciceri* CC1192 using Geneious® v.8.1 (Biomatters, Auckland, New Zealand).

4.4.9 Nucleotide Sequence Accession Numbers

The nucleotide sequences of 16S–23S rDNA IGS, *nodC*, and *nifH* were deposited in the NCBI GenBank database and the accession numbers are presented in Table S3 (Supporting Information).

4.5. Results

4.5.1 Symbiotic effectiveness

Of the 120 test strains, plants inoculated with 112 strains (93%) had shoot dry weight (SDW) not statistically different to those inoculated with strain CC1192, while eight strains were not significantly different from the negative control (uninoculated). Strain M082 produced higher SDW than strain CC1192. In contrast, inoculation with strain M119 led to significantly lower SDW than most strains although it performed better than the negative control (Fig. 1A).

All test strains except M119 had greater leaf chlorophyll than the negative control. The majority of strains (97%) and CC1192 produced statistically similar chlorophyll content, while plants inoculated with strains M055, M073 and M104 had lower chlorophyll content (Fig. 1B).

Mean nodule number per plant varied from 7 (for strain M100) to 27 (for strain M032; Fig. 1C). The highest nodule dry weight (NDW) of 95 mg per plant was found in plants inoculated with strain M119, whereas the lowest NDW of 31 mg per plant was recorded from plants inoculated with M070 (Fig. 1D). Despite strain M119 forming many nodules and having a high NDW, the plants had the lowest SDW and chlorophyll accumulation efficiency (Fig. 1).

4.5.2 pH, temperature and salt tolerance of chickpea rhizobia

There was variation among the strains in ability to grow at extreme pH, temperature and salt conditions (Figure S3, Supporting Information). In general, strains were more tolerant of alkaline than acidic conditions (Figure S3a, Supporting Information). None of the strains were able to grow at > 45ºC (Figure S3b, Supporting Information), but 10 strains grew at low temperature (5ºC). All strains were particularly sensitive to high NaCl concentrations (Figure S3c, Supporting Information).

4.5.3 Phylogeny of the 16S-23S rDNA IGS region of chickpea rhizobia

Based on the quality of sequences, 114 out of 120 strains were selected and subjected to phylogenetic analysis using the MEGA-7 program. Distantly related rhizobial genera such as *Bradyrhizobium*, *Sinorhizobium*, and *Rhizobium* were separated as an outgroup from the *Mesorhizobium* genus.

Approximately half of the test strains (50) were clustered into Group I that had 100% ANI with *Mesorhizobium gobiense* (Fig. 2). This group contained two acid-tolerant strains (M094 and M113) collected from the Magway region. *Mesorhizobium gobiense* was the dominant species in all sampling regions (Sagaing, Mandalay, and Magway) in this study.

A total of 33 strains belonging to Group II had 95%–100% ANI with *M. temperatum*, *M. mediterraneum*, *M. tarimense*, *M. tianshanense*, *M. metallidurans* and an Indian strain IC-2058 (CA-181). Among these, 14 strains shared identical 16S–23S rDNA IGS sequences (100% ANI) with the Indian strain, while seven strains of the broader group were more closely related to *M. temperatum*. Group II also contained strains with the highest SE (e.g. M045 and M082), acid tolerance (M065) and heat tolerance to 45◦C (M021, M043, M061, and M082; Fig. 2; Figure S3, Supporting Information).

Group III contained 33 strains, which shared 95%–100% ANI values between the strains within this group. Among them, four strains (M018, M055, M067 and M069) shared 100% ANI with *M. silamurunense* and *M. tamadayense*. A total of four strains (M016, M074, M075 and M107) were most closely related to *M. plurifarium* and *M. muleiense*, with 95%–97% ANI. The remaining strains in Group III were more closely related to *M. huakuii* and *M. caraganae* with 95%–96% ANI. This group contained strains with varying effectiveness, and slightly more divergent 16S–23S rDNA IGS regions, as compared with Groups I and II. Strains belonging to

Group III were isolated from Mandalay, Sagaing, and Magway regions and had varying SE from inferior (e.g. M055) to equal (e.g. M075) SE, relative to strain CC1192.

4.5.4 Phylogeny of symbiosis-related genes

The grouping of strains in both *nodC* and *nifH* phylogenetic trees was highly similar (Figs 3 and 4). All test strains had similar *nodC* gene sequences (nucleotide position between 490 and 1000) with 99%–100% ANI to other chickpea symbionts (Fig. 3A). Partial sequences of the gene were aligned with the translated *nodC* sequence of *M. ciceri* CC1192 (MT237330.1) and subdivided into five different groups. The groups are outlined in Table 1. Most of the strains (75%) had eight SNPs and one (Group II), two (Group III), or three (Group IV) amino acid substitutions. The strains in group IV had the greatest differences in *nodC* sequences, with three amino acid substitutions: Pro-775-Ser, Glu-981-Asp, and Phe-988-Val relative to CC1192. The *nodC* sequences of Indian strain IC-2058 (CA-181) and *M. muleiense* were closely related to those of Group IV and III, respectively. Analysis of the groupings and SE and nodule numbers showed that there was no difference among the groups according to SNPs and amino acid substitutions in nodulation or SE (Fig. 3A). Group I, which has three amino acid substitutions compared with CC1192, had the most strains with high and intermediate SE and nodule numbers, which has no amino acid substitutions with CC1192. Group IV, which has three amino acid substitutions, showed a profile of SE and NN similar to CC1192.

The *nifH* based phylogeny showed that all test strains carry highly similar nucleotide sequences for this gene and were grouped into a main single cluster with 99%–100% ANI (Fig. 4).

4.5.5 Comparative sequence analysis of 16S-23S rDNA IGS, *nodC* and *nifH* genes of chickpea rhizobial strains from both Myanmar and Australia

Comparative phylogenetic analysis of 16S–23S rDNA IGS sequences of strains isolated from soils in Myanmar and Australia revealed that most Myanmar strains (68%) were closely related to the Indian strain IC-2058 (CA181), which itself has high sequence identity (99%–100%

ANI) to *M. gobiense* and *M. tianshanense* (Fig. 5A). In contrast, most Australian strains (40%) shared highly similar sets of 16S–23S rDNA IGS sequences with the original chickpea symbiont *M. ciceri*, including Australian commercial inoculant strain *M. ciceri* CC1192. Other than *M. ciceri*, about 13% of Australian strains shared 100% ANI with *M. temperatum*. In general, Australian strains were related to well-known chickpea rhizobia (*M. ciceri*), while Myanmar strains were more closely related to rarely described chickpea symbionts such as *M. gobiense*, *M. tianshanense*, *M. tamadayense* and *M. silamurunense*. There was little overlap in species between two countries, where Australian soils were dominated by strains related to *M. ciceri*, *M. temperatum* and *M. huakuii*, while the only species found in both countries were *M. silamurunense* and *M. tamadayense* (Fig. 5A).

Although symbiosis gene-based phylogenies incorporating both Australian and Myanmar strains were highly similar, sequence analysis revealed that most Myanmar strains have some changes in the *nodC* and *nifH* sequences (Fig. 5B and C). Only 21 (20%) and 51 (44%) Myanmar strains had 100% ANI with *nodC* and *nifH* of *M. ciceri* strains including CC1192. Most Myanmar strains (80%) shared highly similar nodC sequences (99%–100% ANI) with *M. tamadayense* and *M. opportunistum, M. mediterraneum, M. muleiense* and Indian strain IC-2058-CA-181 (Fig. 5A). The *nifH* genes of more than half of the Myanmar strains (56%) were most closely related to the *nifH* of *M. muleiense, M. tarimense* and *M. tianshanense* and strain IC-2058-CA181 with 99%–100% ANI (Fig. 5C). In contrast, all Australian strains tested (77) were tightly clustered together with *M. ciceri* CC1192 in both *nodC* and *nifH* phylogenies (Fig. 5B and C).

4.5.6 Phylogeny of concatenated *dnaJ-recA* genes

The concatenated phylogenetic trees *dnaJ* and *recA* revealed that the six selected rhizobial strains from Myanmar fell into three different clusters (Figure S4, Supporting Information). The grouping of strains in concatenated *dnaJ-recA* phylogenetic trees was consistent and in

agreement with that of the 16S–23S rDNA-based phylogeny. For example, the acid-tolerant strain M065 and the highly symbiotically effective strain M082 represented Group II of 16S–23S rDNA IGS-based phylogeny and were also in the same cluster in the concatenated *dnaJ-recA* phylogenetic tree (Fig. 2; Figure S4, Supporting Information). A symbiotically effective strain (M009) and two acid-tolerant strains (M094 and M113) from Group I shared 100% ANI with *M. gobiense* in all phylogenies in this study. Also, a heat-tolerant strain M075 from Group III was consistently assigned together with *M. tamadayense* in both tree topologies of 16S–23S rDNA and concatenated *dnaJ-recA* (Fig. 2; Figure S4, Supporting Information).

4.5.7 Biogeographic distribution of selected rhizobial strains from Myanmar and Australia

A total of 79 Myanmar strains representative of different geographic locations were subjected to scatter plot analysis to investigate the spatial distribution of *Mesorhizobium* species in the CDZ of Myanmar. Figure 6 shows the spatial distribution of test strains under four main species groups representing the closely related seven identified *Mesorhizobium* species. The three main species groups of chickpea-nodulating rhizobia representing *M. gobiense*, *M. huakuii* and *M. muleiense* were distributed throughout Myanmar but *M. temperatum* was not found in the southwestern area of Magway (Fig. 6).

A total of 16 strains from Myanmar and Australia were selected for further phylogenetic analysis based on their potential in SE, tolerance to acid, high temperature, and high salt concentration as well as their divergence in 16S–23S rDNA-based phylogeny (Figs 2 and 7). A total of two acid-tolerant strains from Myanmar (M094 and M113) were closely related to each other, while strains A68 and M062 also shared similar 16S–23S rDNA phylogeny as well as having high salt tolerance. The remaining strains did not show any strong relationship between physiological and phylogenetic relatedness.

4.6. Discussion

Genetic diversity of chickpea-nodulating rhizobia in Myanmar

Phylogeny of 16S–23S rDNA IGS shows that the majority of chickpea nodulating rhizobia isolated from Myanmar soils were most closely related to *M. gobiense*, *M. silamurunense*, *M. tamadayense* and *M. temperatum* (Fig. 2), some of which have also been found in northern China, India and Ethiopia (Table S3, Supporting Information; Zhang *et al*. 2012a, b, 2020, Rai *et al*. 2012, Muleta *et al*. 2022). Some of the chickpea rhizobial species such as *M. temperatum*, *M. huakuii* and *M. plurifarium* in Myanmar have also been found throughout the world (Gao *et al*. 2004, Alexandre *et al*. 2009, Elias and Herridge 2015, Tena *et al*. 2017). Previous studies in India found that most isolated strains were closely related to *M. ciceri* and *M. mediterraneum* (Rai et al. 2012). This is in contrast to the present study where we found the Indian strain IC-2058 (CA-181) was not closely related to those cognate species and is more closely related to the *Mesorhizobium* species found in Myanmar. No strains from Myanmar were closely related to *M. ciceri* and *M. mediterraneum*, which may be due to the lack of inoculation in the chickpea growing regions of Myanmar; it is also possible that the Myanmar strains originally came from India with the historic introduction of chickpea or are resident soil strains.

In total, three of the four main species groups of chickpea nodulating rhizobia, *M. gobiense*, *M. huakuii* and *M. muleiense*, were distributed throughout Myanmar, but *M. temperatum* was not found in the Southwestern area of Magway (Fig. 6). In China, these four species were isolated from alkaline sandy desert soils (Han *et al*. 2008, Zhao *et al*. 2012). The soils in Myanmar range from vertisols, located mainly in the Sagaing and Mandalay regions, to sandy uplands which are commonly found in the Magway region (Herridge *et al*. 2019). The farming systems in Magway differ from those in the lowland central regions of the CDZ, as they are mostly rainfed and do not include rice rotations. It is possible that the *Mesorhizobium* species

found in Magway require specific adaptive traits similar to those of species found in northern China, while the heavy vertisol soils differ in having less stressful conditions that are able to support a greater diversity of *Mesorhizobium* species (Greenlon *et al*. 2019). A study in China showed that the population composition of *M. muleiense* shifted with changing soil nutrient and pH conditions over time (Zhang *et al*. 2018b). Crop rotations and inputs that affect soil chemistry may also be a factor in the regional species differences found in Myanmar.

Although there has been no widespread rhizobial inoculation in Myanmar, chickpea nodulating rhizobia are abundant in cropping soils. During sample collection in Myanmar, we observed that chickpea was well-nodulated in most fields (sometimes with up to 40 nodules per plant) and the sizes of the nodules were much bigger than those observed in our pot experiments. Notably, among the 114 chickpea mesorhizobial strains obtained from geographically diverse Myanmar soils, most established effective symbioses with chickpea. Our strains were extracted from a thin layer of soil in sterilized sand, which may have led to isolation of a greater diversity of strains than those found in nodules in Myanmar, as was observed for bean and soybean in Brazil (Alberton *et al*. 2006) and may be relevant to this study.

Adaptive traits of mesorhizobia in Myanmar

The comparative phylogenetic analysis of the Myanmar and Australian strains that were selected by physiological tolerance to abiotic stresses showed that the physiological attributes of strains are more likely associated with local soil environments than with phylogeny. In a previous study, *M. gobiense* and *M. tianshanense* were acid sensitive strains, and about 50% of the *Mesorhizobium*-like strains including *M. ciceri* were tolerant to acidic pH (Laranjo and Oliveira 2011), but our results suggest that the physiological attributes of rhizobial strains did not cluster according to the classification of the type strains. *M. plurifarium* is reported as acid, heat, and salt tolerant (de Lajudie *et al*. 1998) and was associated with salt and heat tolerance

of strains from Australia and Myanmar (salt) and Myanmar (heat) but not acid tolerance in our test strains. Another two heat-tolerant reisolated strains (M021 and A53) were clustered together with *M. temperatum* (Fig. 7), which was originally isolated from *Astragalus adsurgens* in China (Table S3, Supporting Information; Gao *et al*. 2004) and was sensitive to high temperature (Han *et al*. 2008). Apart from salt and heat tolerances, which were related to genetic similarities, acid tolerance was only present in two closely related strains from Myanmar. Adaptative traits like heat and salt tolerance would explain the success of the isolated strains from their soils of origin, but there was no clear association of these traits with phylogenetic relatedness.

Diversity of symbiotic genes in Myanmar

Although this study shows a diverse array of chickpea nodulating species present in and adapted to Myanmar soils, according to DNA sequence data of core genes, our strains contained sets of both *nodC* and *nifH* genes that were highly similar or identical to those of other chickpea symbionts found in other countries. The most likely explanations are that symbiosis genes were either dispersed to the resident mesorhizobial population through HGT (Sullivan *et al*. 1995, Hill *et al*. 2021), with the original historic introduction of chickpea into Myanmar, or became dispersed more recently through introduction on the seed from neighbouring countries, as found in recent phylogenetic study on peanut *Bradyrhizobium* species (Bouznif *et al*. 2019).

Studies on the *Rhizobium* and *Bradyrhizobium* nod gene have revealed that amino acid substitutions (e.g. from aspartic acid to asparagine) or deletions in the symbiosis genes can cause lower nodule numbers than nonmutant strains, and a failure to fix nitrogen (Burn *et al*. 1989, Go¨ttfert *et al*. 1989, Krause *et al*. 2002, Arashida *et al*. 2022). In this study, we found that amino acid substitutions had no effect on either SE or nodule number per plant, even with

a major amino acid substitution of phenylalanine to valine in groups II, III, and IV compared to the *nodC* protein of *M. ciceri* CC1192. The groups most divergent from the *nodC* gene sequence of CC1192 contained the Indian strain IC-2058-CA181 (Group IV) and *M. muleiense* (Group III). The *nodC* sequence in groups containing the Indian strain and *M. muleiense* were found to be most closely related to *nodC* genes found in rhizobia from chickpea nodules in Ethiopia (Muleta *et al*. 2022). A similar pattern was found with the sequences of *nifH* genes, where genetic divergence among *nifH* sequences of Myanmar strains had no effect on SE. This congruence may indicate a common origin of these symbiosis genes, or that they have acquired the same genetic differences.

A genetic diversity and gene flow study on *Mesorhizobium* strains revealed that the nodulating symbionts have evolved divergently in association with geographic distribution and have been selected by both host plant genotypes and local environments (Ji *et al*. 2015). In mesorhizobial strains of black locust, *Robinia pseudoacacia*, the symbiotic haplotypes were not found in different continents, but were found in multiple locations within China, indicating that there was no recent transmission of symbiotic genes (Liu *et al*. 2019). This finding is in agreement with the results of the present study. The heavy selection pressure by the yearly planting of chickpea crops in Myanmar would maintain symbiotic integrity of the *nodC* gene, even in the absence of HGT from a regularly applied commercial inoculant. Even though the transfer of symbiosis genes to native Myanmar strains was most likely historic and rare, there has been very little divergence relative to the symbiosis genes of inoculant species such as *M. ciceri*, and there is high nodulation of chickpea crops in Myanmar soil.

Comparison of mesorhizobial diversity in Myanmar and Australia

Comparative sequence analysis between the chickpea nodulating strains recently reisolated from soil in Australia and in Myanmar showed that there was a much lower level of diversity

among Australian reisolated strains, with the majority closely related to *M. ciceri*. This was not surprising, as Australia has a much more recent history of chickpea cultivation than Myanmar and frequent inoculation of Australian chickpea crops, leading to a greater prevalence of strains in soil that are related to the inoculant strain. The low degree of chickpea rhizobial diversity in Australia compared to a country with a very different chickpea cropping history such as Myanmar, has been observed in a comparison of strains reisolated in India, Portugal, and Ethiopia with those present in soil in the USA, Canada, and Australia (Greenlon *et al*. 2019).

The occurrence of strains related to species other than *M. ciceri* in Australian soils was also not comparable to the species found Myanmar. For example, a high proportion of Australian strains was closely related to *M. temperatum*, which was only found for relatively few (6%) of the Myanmar strains. The only significant overlap in species reisolated from chickpea soils between the Australia and Myanmar was found with a group related to *M. silamurunense* and *M. tamadayense*. It is likely that this lack of comparability between *Mesorhizobium* species found in Myanmar compared to Australia is not only due to inoculation history but also to crop management, inputs and resident soil species (Hirsch 1996). There may also be an effect of isolated and independent genetic changes throughout the history of chickpea cultivation in Myanmar (Liu *et al*. 2019), which would not be found in Australia due to the much more recent introduction of chickpea (mid-1970s).

Symbiotic genes in Myanmar and Australia

Inoculation history is also reflected in the phylogeny of the symbiosis genes of Myanmar and Australian strains, where the *nodC* and *nifH* in reisolated Australian strains were closely related. The *nodC* sequences from most of the Myanmar strains were in a slightly different phylogenetic group (*M. muleiense* and IC-2058-CA181) from those present in the Australian strains (related to *nodC* from *M. ciceri* CC1192). A similar pattern was found with the *nifH*

genes. These results support the observation that the symbiosis genes found in Myanmar soils are different than in soils where there has been frequent inoculation of commercial strains, as has occurred in Australia, where descendants of the inoculant strain were commonly found among reisolated strains (Zaw *et al*. 2021). A study of Ethiopian strains also uncovered this discrepancy in *nodC*/*nifH* genes, with a majority group closely clustered with *M. ciceri* and a small group also more closely related to *M. muleiense* (Muleta *et al*. 2022). The divergence of the sequence of *the nodC*/*nifH* genes in this study between the Australian and Myanmar strains indicates that the dispersal of symbiosis genes from a common symbiont was probably an historical event, which has not been recently repeated in Myanmar.

Conclusion

This study has generated a detailed analysis of the diversity of chickpea-nodulating rhizobia in Myanmar. The investigation has uncovered that Myanmar is a source of genetic variation of mesorhizobia compared to Australia, where there has been frequent inoculation of the commercial strain *M. ciceri* CC1192. We have provided an insight into the ability of strains that are distantly related, to harbour similar nodulation and fixation genes and form effective symbiosis in genetically diverse backgrounds. The chickpea-nodulating rhizobia in Myanmar are more closely related to species found in India and China, which may be a function of soil adaptation or dispersal over the history of chickpea cultivation. This study has also identified subtle changes in the symbiosis gene *nodC,* which has occurred over time without the constant introduction of these genes through inoculation. Compared with Australia, the symbiosis genes found in Myanmar have some changes in the protein sequence, which at this point have not changed SE, but further mutations may well incur penalties or even improvements in nitrogen fixation over time.

4.7. References

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4.8. Tables and figures

Table 1. Comparison amino acid substitution in highly conserved regions of *nodC* genes of Myanmar chickpea rhizobia relative to that of *M. ciceri* CC1192.

	Amino acid number							
	543	687	775	849	895	981	988	996
	Amino acid							
	Arg	Gln	Pro	Arg	Leu	Glu	Phe	His
	Consensus sequence							
ANI group	CGA	CAG	CCA	CGG	CTG	GAG	TTT	CAT
I (40)	CGG	CAA	TCA	CGA	TTG	GAT	GTT	CAC
II (3)	CGG	CAA	TCA	CGA	TTG	GAT	GTT(Val)	CAC
III (9)	CGG	CAA	TCA	CGA	TTG	GAT(Asp)	GTT(Val)	CAC
IV (20)	CGG	CAA	TCA(Ser)	CGA	TTG	GAT(Asp)	GTT(Val)	CAC
V (24)	-	-	-	-	-	-	-	-

A red colored letter represents a single nucleotide polymorphism compared with the consensus nucleotide sequence of CC1192, and a dash (-) indicates an identical codon to sequence of CC1192. Letters in the bracket (e.g. Val and Asp) indicates the amino acid substitution has occurred. The number in the bracket indicates the number of strains in which specific mutation(s) or amino acid substitution(s) were identified.

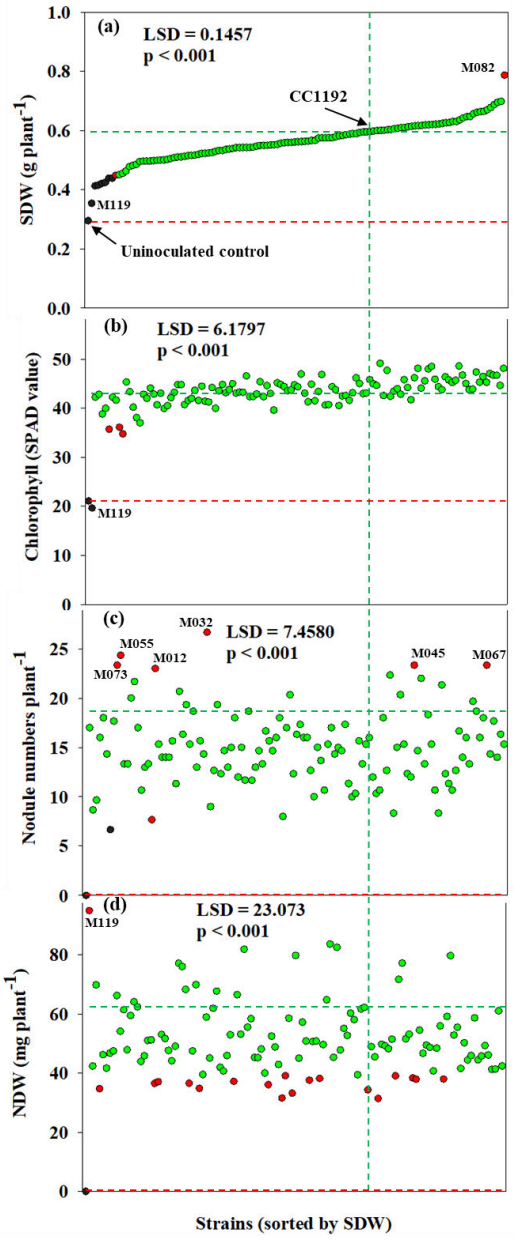

Figure 1. Distribution of (A) SDW, (B) leaf chlorophyll content, (C) nodule number per plant, and (D) nodule dry weight (NDW) induced by 120 test rhizobial strains and commercial strain *M. ciceri* CC1192. Strains were sorted according to their ability to enhance SDW in ascending order. Different colors of dots represent statistically different groups based on LSD (0.05) values, where black dots are strains which had a similar response to the uninoculated control, green dots represent the strains similar to the positive control (CC1192), and red dots are significantly higher or lower than both groups (black and green). The X- and Y-axis intercepts of green and red dotted lines indicate results for strain CC1192 and the uninoculated control, respectively.

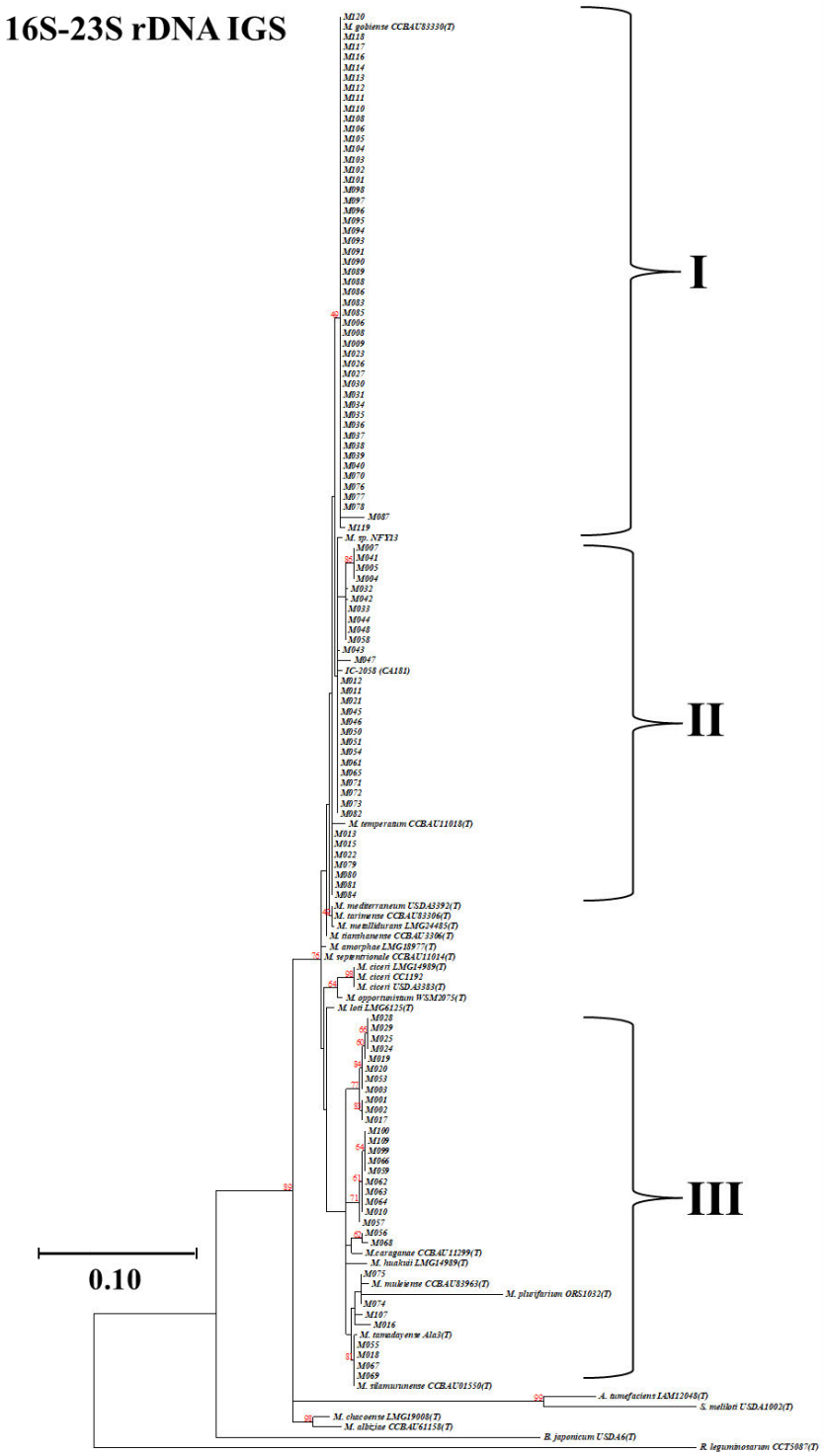

Figure 2. Maximum likelihood phylogenetic tree based on 16S–23S rDNA IGS sequences of 114 Myanmar rhizobial strains and reference strains. Bootstrap values were computed based on 1000 replications. The scale bar (0.10) indicates the percentage of nucleotide substitutions per site. A, *Agrobacterium*, B, *Bradyrhizobium*, M, *Mesorhizobium*, S, *Sinorhizobium*, and R, *Rhizobium*.

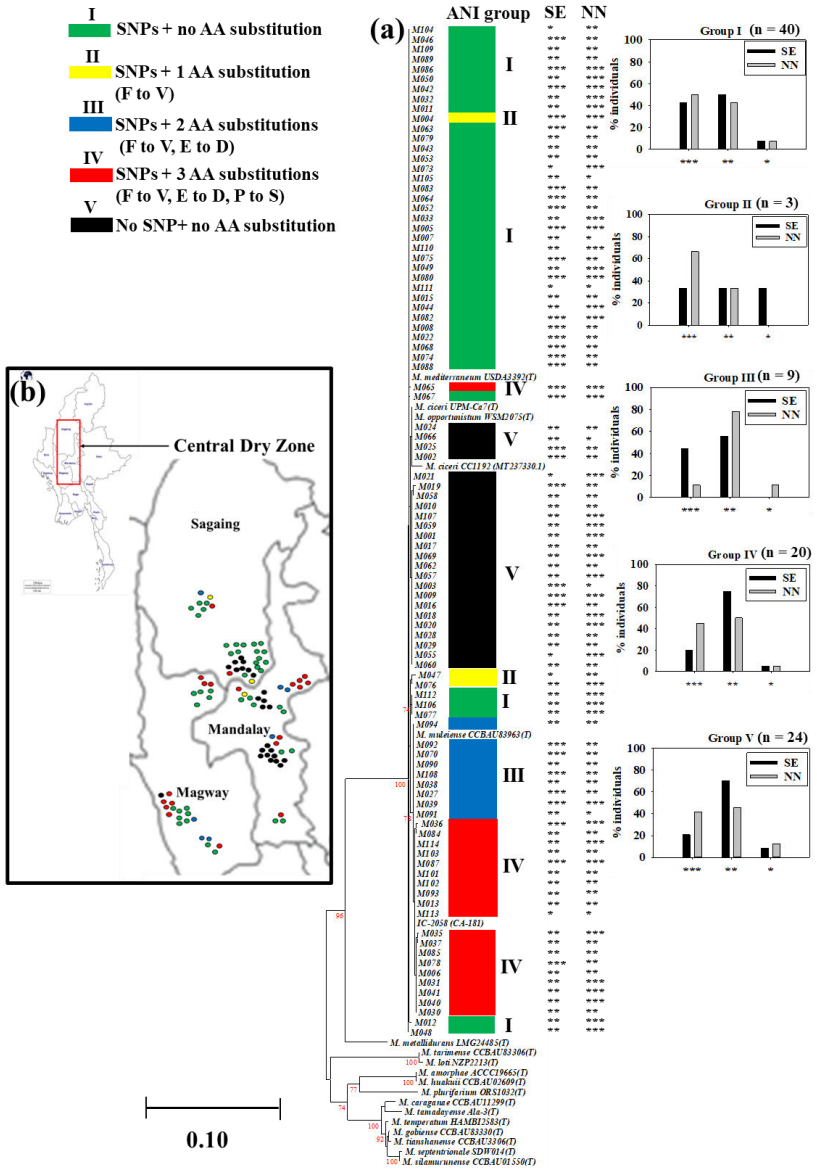

Figure 3. (A) Phylogenetic trees based on partial sequences of *nodC* with different SNPs and amino acid substitutions as compared with the chickpea symbiont, *M. ciceri* strain CC1192, which is the Australian commercial inoculant. The phylogenetic tree was generated using the Maximum Likelihood method based on the Kimura two-parameter model in MEGA7. Bootstrap values were computed based on 1000 replications. The scale bar (0.1) represents the percentage of nucleotide substitutions per site. All positions containing gaps and missing data were eliminated. (B) The distribution of strains from five different ANI groups in association with sample collection sites. M, *Mesorhizobium*, SE relative to CC1192 (∗∗∗ > 100%, ∗∗80%–100%, and ∗< 80%), NN, nodule numbers (∗∗∗ > 15, ∗∗10–15, and ∗ < 10), AA, amino acid, D, Aspartic acid (Asp), E, Glutamic acid (Glu), F, Phenylalanine (Phe), P, Proline (Pro), S, Serine (Ser), V, Valine (Val), and n, number of strains.

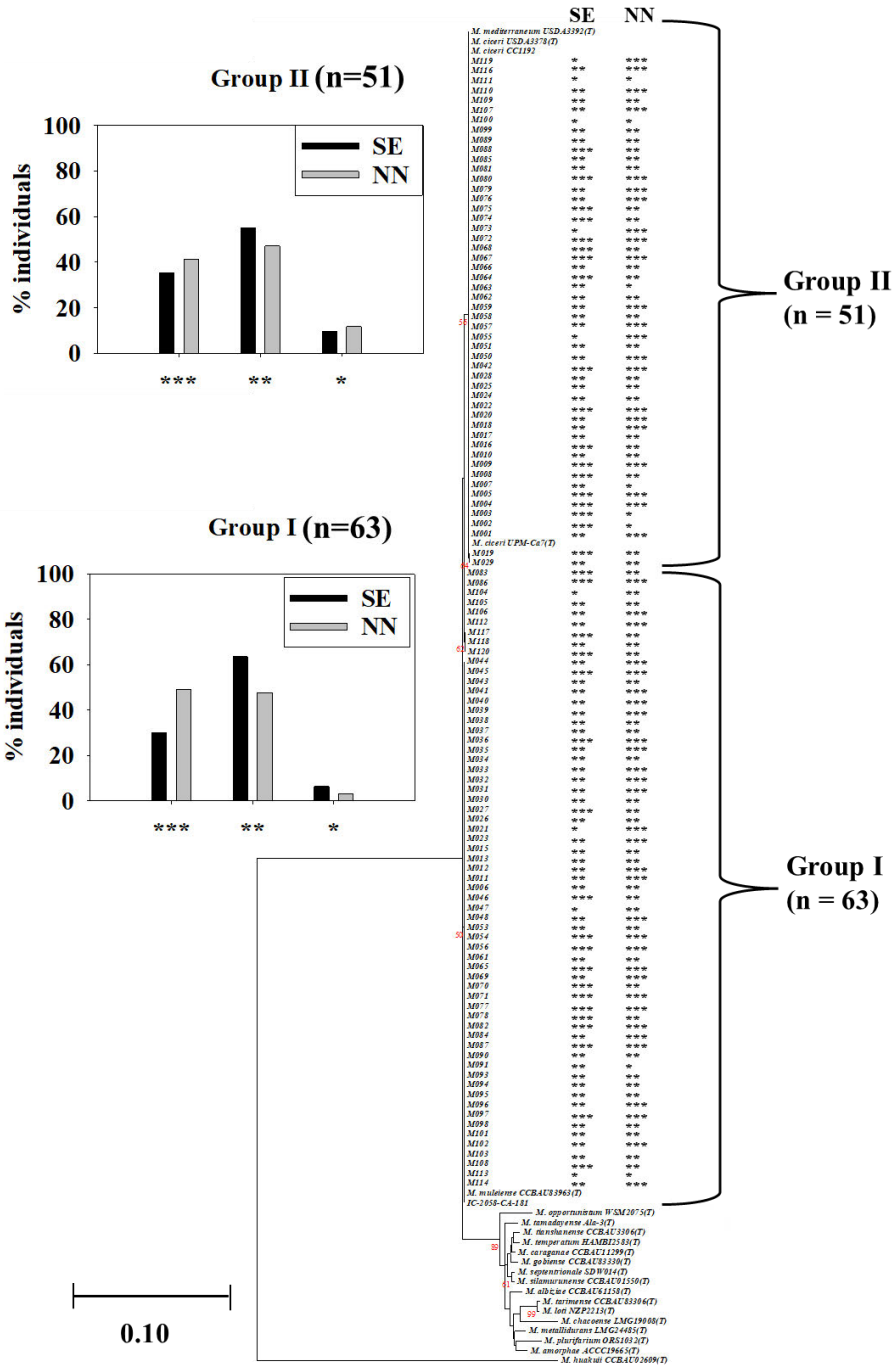

Figure 4. Phylogenetic tree based on partial sequences of *nifH* showing the relatedness of this gene among chickpea rhizobial strains and recognized species of *Mesorhizobium*. The phylogenetic tree was generated using the Maximum Likelihood method based on the Kimura two-parameter model in MEGA7. Bootstrap values were computed based on 1000 replications. The scale bar (0.1) represents the percentage of nucleotide substitution per site. All positions containing gaps and missing data were eliminated. M, *Mesorhizobium*, SE relative to CC1192 (*** > 100%, **80%–100%, and * < 80%), NN, nodule numbers (*** > 15, **10–15, and * < 10), n, number of strains.

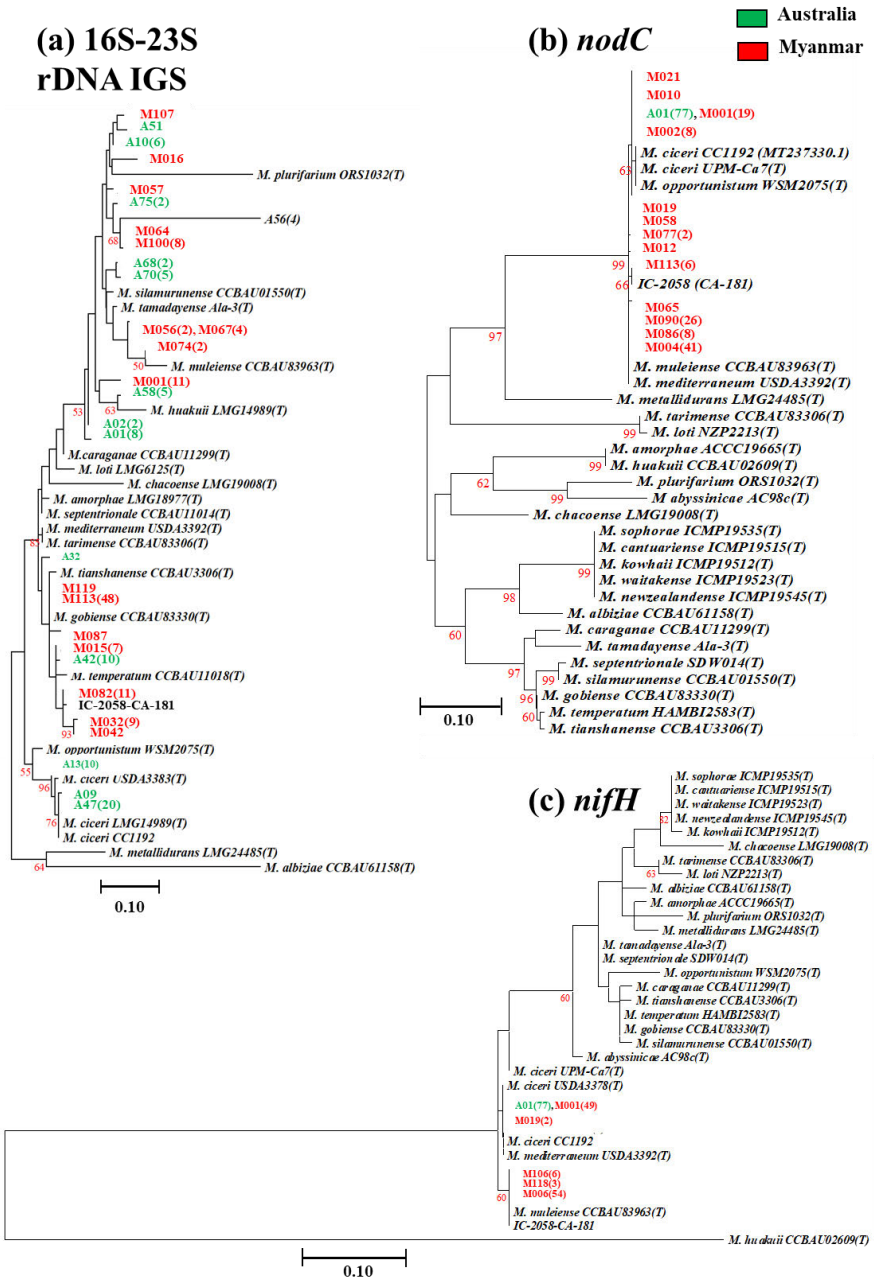

Figure 5. Comparative analysis of (A) 16S–23S rDNA IGS, (B) *nodC*, and (C) *nifH* sequences of 114 Myanmar strains, 77 Australian strains, and reference strains. The maximum likelihood phylogenetic tree was constructed using Kimura two-parameter model in MEGA7. Bootstrap values were computed based on 1000 replicates. The scale bar (0.10) indicates the percentage of nucleotide substitutions per site. A, *Agrobacterium*, B, *Bradyrhizobium*, M, *Mesorhizobium*, S, *Sinorhizobium*, and R, *Rhizobium*. Strains from Australia (green color) and Myanmar (red color) were designated as A and M, respectively (e.g. A01 and M001). The numbers in the brackets indicate the number of strains with identical nucleotide sequences (> 99% ANI).

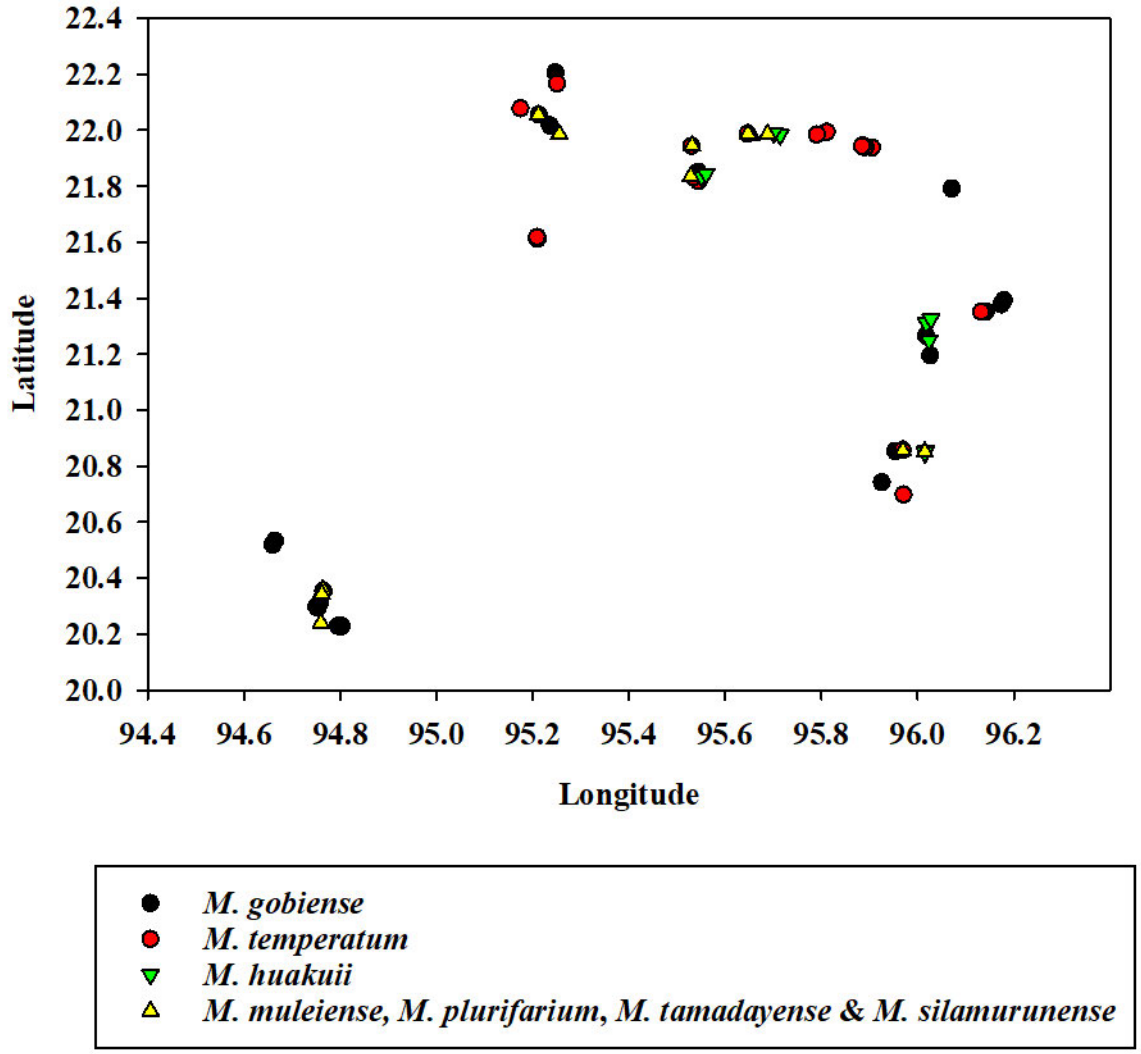

Figure 6. Spatial distribution of *Mesorhizobium* species in CDZ of Myanmar. Different colors and symbols in the scatter plot indicate relative distribution of mesorhizobial species to each other in space. *Mesorhizobium* species were identified based on 16S–23S rDNA phylogeny (see Fig. 5). M, *Mesorhizobium*.

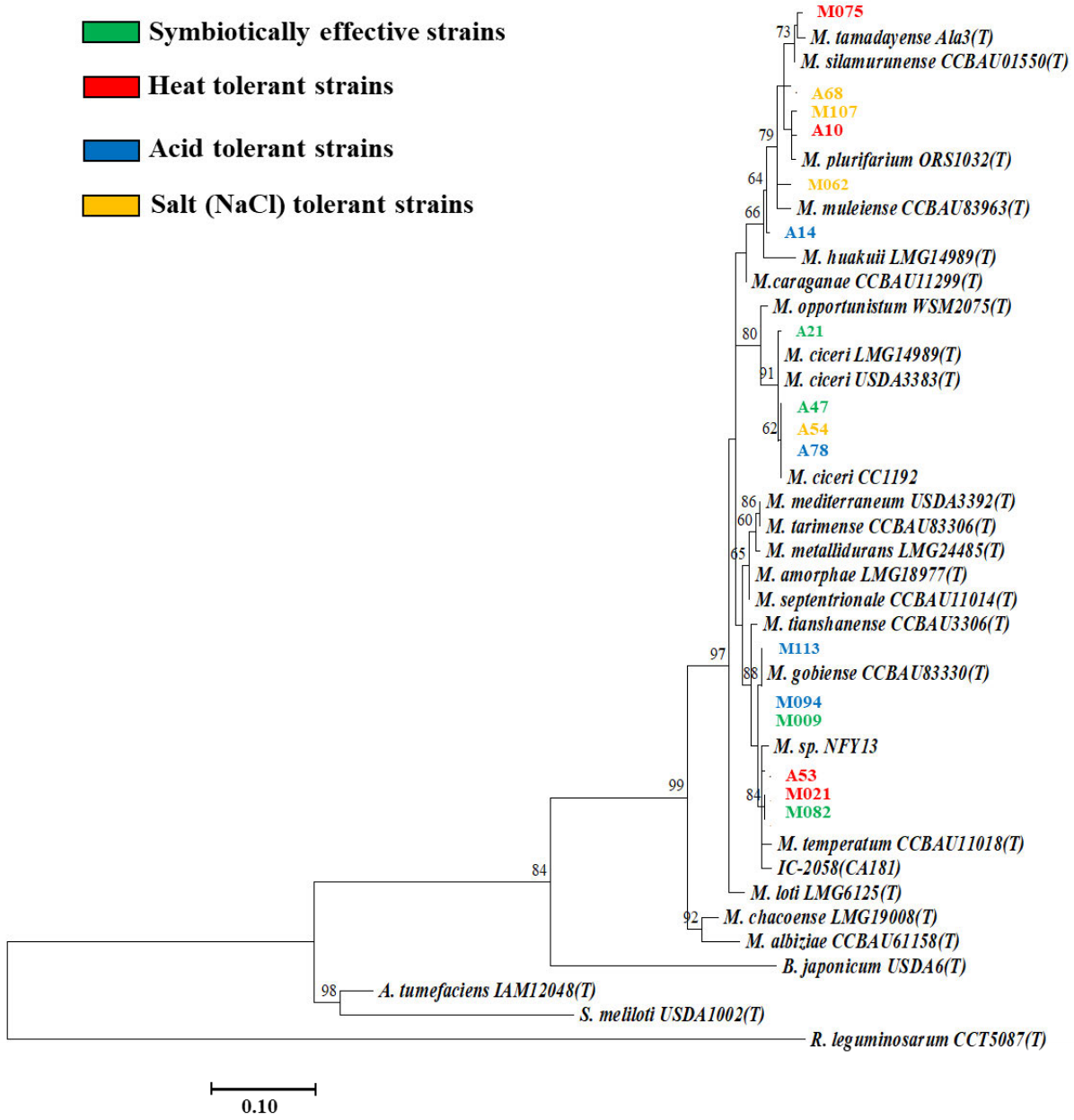

Figure 7. Comparative sequence analysis using16S–23S rDNA IGS of 16 selected strains with potential ecological adaptation and symbiotic traits, and CC1192. Strains from Myanmar and Australia were abbreviated as M (e.g. M113) and A (e.g. A47), respectively. Bootstrap values were computed based on 1000 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*.

Table S1. GPS coordinates, town, region and soil pH of strain collection sites

Strain	Latitude-Longitude	Altitude	Town	Region	Soil pH ($CaCl_2$)
M001, M002	21° 50′ 7 52″ N 95° 33′ 1 55″ E	65m	Ngazun	Mandalay	7 04
M003	21° 50′ 37 09″ N 95° 33′ 37 99″ E	66 m	Ngazun	Mandalay	7 55
M004, M005	21° 49′ 0 91″ N 95° 32′ 40 94″ E	80m	Ngazun	Mandalay	7 57
M006	21° 49′ 48 05″ N 95° 32′ 10 74″ E	62 m	Ngazun	Mandalay	7 63
M007, M008	21° 49′ 50 70″ N 95° 32′ 5 88″ E	61m	Ngazun	Mandalay	7 18
M009	21° 51′ 3 22″ N 95° 32′ 39 20″ E	60m	Ngazun	Mandalay	7 39
M010	21° 50′ 4 33″ N 95° 31′ 43 58″ E	59m	Ngazun	Mandalay	6 81
M011, M012	20° 41′ 53 77″ N 95° 58′ 14 92″ E	177m	Pyawbwe	Mandalay	7 57
M013	20° 44′ 29 98″ N 95° 55′ 32 66″ E	185m	Pyawbwe	Mandalay	7 45
M014, M015	20° 51′ 12 43″ N 95° 57′ 13 83″ E	184m	Meiktila	Mandalay	7 67
M016	20° 51′ 17 81″ N 95° 58′ 9 68″ E	178m	Thazi	Mandalay	6 34
M017	20° 51′ 8 45″ N 96° 1′ 2 92″ E	163m	Thazi	Mandalay	7 22
M018, M019	20° 50′ 59 95″ N 96° 0′ 53 77″ E	157m	Thazi	Mandalay	7 31
M020	20° 50′ 52 20″ N 96° 0′ 53 95″ E	169m	Thazi	Mandalay	6 14
M021, M022	20° 51′ 22 24″ N 95° 58′ 8 75″ E	168m	Thazi	Mandalay	7 31
M023	21° 11′ 43 15″ N 96° 1′ 34 52″ E	123m	Wundwin	Mandalay	7 31
M024, M025	21° 14′ 57 08″ N 96° 1′ 25 59″ E	116m	Wundwin	Mandalay	7 42
M026, M027	21° 15′ 59 34″ N 96° 1′ 2 23″ E	114m	Wundwin	Mandalay	7 43
M028, M029	21° 18′ 39 45″ N 96° 1′ 6 18″ E	113m	Wundwin	Mandalay	7 40
M030, M031	21° 19′ 31 45″ N 96° 1′ 40 44″ E	108m	Wundwin	Mandalay	7 41
M032, M033	21° 21′ 4 15″ N 96° 7′ 55 81″ E	96m	Myittha	Mandalay	7 82
M034, M035	21° 21′ 6 37″ N 96° 8′ 32 41″ E	96m	Myittha	Mandalay	7 67
M036, M037	21° 22′ 39 07″ N 96° 10′ 25 83″ E	97m	Kume	Mandalay	7 88
M038, M039	21° 23′ 32 87″ N 96° 10′ 46 29″ E	97m	Kume	Mandalay	7 82
M040, M041	21° 47′ 27 92″ N 96° 4′ 15 89″ E	73m	Kume	Mandalay	7 71
M042	21° 56′ 16 28″ N 95° 54′ 20 05″ E	66m	Sagaing	Sagaing	7 52
M043	21° 56′ 24 58″ N 95° 53′ 31 35″ E	72m	Sagaing	Sagaing	7 40
M044	21° 56′ 20 66″ N 95° 53′ 27 61″ E	72m	Sagaing	Sagaing	7 36
M045, M046	21° 56′ 40 91″ N 95° 53′ 9 46″ E	73m	Sagaing	Sagaing	7 17
M047	21° 56′ 38 26″ N 95° 53′ 9 39″ E	74m	Sagaing	Sagaing	7 35
M048	21° 56′ 34 06″ N 95° 53′ 5 07″ E	73m	Sagaing	Sagaing	7 48
M049, M050	21° 59′ 37 85″ N 95° 48′ 41 51″ E	84m	Sagaing	Sagaing	7 72
M051	21° 59′ 2 23″ N 95° 47′ 28 30″ E	82m	Sagaing	Sagaing	7 63
M052	21° 59′ 7 90″ N 95° 47′ 28 47″ E	83m	Sagaing	Sagaing	7 68
M053	21° 58′ 57 18″ N 95° 42′ 52 69″ E	70m	Sagaing	Sagaing	7 08
M054	21° 59′ 28 19″ N 95° 42′ 5 56″ E	65m	Sagaing	Sagaing	7 60
M055, M056	21° 59′ 6 67″ N 95° 41′ 17 00″ E	63m	Sagaing	Sagaing	6 75
M057, M058	21° 58′ 58 63″ N 95° 39′ 15 42″ E	69m	Myinmu	Sagaing	6 62
M059, M060	21° 58′ 54 26″ N 95° 39′ 13 48″ E	70m	Myinmu	Sagaing	6 34
M061	21° 59′ 9 77″ N 95° 38′ 48 60″ E	71m	Myinmu	Sagaing	7 05
M062	21° 59′ 11 58″ N 95° 38′ 50 97″ E	70m	Myinmu	Sagaing	6 42
M063, M064	21° 56′ 36 79″ N 95° 31′ 52 73″ E	62m	Myinmu	Sagaing	6 75
M065	21° 56′ 36 48″ N 95° 31′ 48 98″ E	62m	Myinmu	Sagaing	7 14
M066	21° 59′ 4 89″ N 95° 15′ 15 28″ E	72m	Chaung U	Sagaing	6 76
M067, M068	21° 59′ 7 26″ N 95° 15′ 15 91″ E	71m	Chaung U	Sagaing	6 73
M069	21° 59′ 6 12″ N 95° 15′ 19 12″ E	73m	Chaung U	Sagaing	6 46
M070	22° 12′ 20 14″ N 95° 14′ 50 14″ E	127m	Monywa	Sagaing	7 75
M071	22° 9′ 58 53″ N 95° 15′ 2 53″ E	119m	Monywa	Sagaing	7 61
M072, M073	22° 4′ 38 29″ N 95° 10′ 28 82″ E	68m	Monywa	Sagaing	7 72
M074	22° 3′ 14 73″ N 95° 12′ 40 34″ E	69m	Monywa	Sagaing	7 70
M075, M076	22° 3′ 16 73″ N 95° 12′ 42 69″ E	69m	Monywa	Sagaing	7 73
M077, M078	22° 3′ 17 51″ N 95° 12′ 45 40″ E	70m	Monywa	Sagaing	7 75
M079	22° 1′ 0 88″ N 95° 14′ 9 44″ E	67m	Chaung U	Sagaing	7 67
M080, M081	22° 0′ 56 60″ N 95° 14′ 10 08″ E	67m	Chaung U	Sagaing	7 51
M082	21° 37′ 6 72″ N 95° 12′ 32 84″ E	62m	Yesagyo	Magway	7 55
M083, M084	21° 37′ 5 83″ N 95° 12′ 34 99″ E	61m	Yesagyo	Magway	7 68
M085, M086	21° 36′ 45 70″ N 95° 12′ 32 29″ E	63m	Yesagyo	Magway	7 67
M087	21° 36′ 44 24″ N 95° 12′ 35 63″ E	63m	Yesagyo	Magway	7 84
M088, M089	21° 36′ 48 14″ N 95° 12′ 37 69″ E	61m	Yesagyo	Magway	7 81
M090, M091	20° 13′ 38 15″ N 94° 48′ 0 32″ E	45m	Minbu	Magway	6 82
M092	20° 13′ 40 78″ N 94° 48′ 1 14″ E	45m	Minbu	Magway	6 84
M093, M094	20° 13′ 44 65″ N 94° 48′ 3 46″ E	45m	Minbu	Magway	7 30
M095, M096	20° 13′ 43 03″ N 94° 48′ 11 33″ E	45m	Minbu	Magway	7 58
M097, M098	20° 13′ 43 20″ N 94° 47′ 42 70″ E	45m	Minbu	Magway	7 24
M099, M100	20° 14′ 23 32″ N 94° 45′ 34 86″ E	48m	Minbu	Magway	5 53
M101	20° 17′ 39 30″ N 94° 45′ 11 49″ E	47m	Pwintphyu	Magway	6 62
M102	20° 17′ 37 87″ N 94° 45′ 14 25″ E	44m	Pwintphyu	Magway	7 28
M103	20° 17′ 55 59″ N 94° 45′ 5 76″ E	45m	Pwintphyu	Magway	7 08
M104, M105	20° 17′ 53 57″ N 94° 45′ 2 20″ E	45m	Pwintphyu	Magway	6 76
M106	20° 17′ 50 14″ N 94° 45′ 3 49″ E	45m	Pwintphyu	Magway	6 98
M107	20° 21′ 31 69″ N 94° 45′ 49 03″ E	46m	Pwintphyu	Magway	7 22
M108	20° 21′ 14 53″ N 94° 45′ 53 86″ E	45m	Pwintphyu	Magway	7 53
M109, M110	20° 20′ 33 18″ N 94° 45′ 41 83″ E	45m	Pwintphyu	Magway	7 42
M111, M112	20° 18′ 42 68″ N 94° 45′ 26 88″ E	42m	Pwintphyu	Magway	7 38
M113, M114	20° 18′ 44 50″ N 94° 45′ 30 90″ E	42m	Pwintphyu	Magway	7 21
M115	20° 29′ 3 50″ N 94° 39′ 30 28″ E	64m	Salin	Magway	7 25
M116	20° 31′ 7 71″ N 94° 39′ 35 74″ E	67m	Salin	Magway	7 55
M117	20° 31′ 13 30″ N 94° 39′ 34 90″ E	68m	Salin	Magway	7 58
M118, M119	20° 31′ 57 99″ N 94° 39′ 51 21″ E	61m	Salin	Magway	7 63
M120	20° 31′ 56 09″ N 94° 39′ 53 10″ E	59m	Salin	Magway	7 29

Table S2. Primers and PCR conditions for the genes studied

Gene	Primer sequence	PCR condition	Reference
16S-23S rDNA	**FGPS-** (5´TGC GGC TGG ATC ACC TCC T3´) **FGPL-132-** (5´CCG GGT TTC CCC ATT CGG3´)	5 min at 95°C, 35 cycles of 30 s at 95°C, 30s at 56°C, 2 min at 72°C, and final extension at 72°C for 5 min	(Laguerre, et al. 1996: 2029-36)
nodC	***nodC*MesoF-**(5´CGA(CT) CG(AG) AG(AG) TTC AA(CT) TTC3´) ***nodC*MesoR-** (5´CT(CT) AAT GTA CAC A(AG) (GC)GC3´)	1 min at 95°C, 35 cycles of 30 s at 95°C, 30 s at 46.5°C, 2 min at 68°C, and final extension at 68°C for 5 min	(Rivas, et al. 2007: 412-8)
nifH	***nifH*-1-** (5'AAG TGC GTG GAG TCC GGT GG3') ***nifH*-2-** (5'-GTT CGG CAA GCA TCT GCT CG-3')	2 min at 95°C, and 35 cycles of 30 s at 95°C, 30 s at 61°C, 2 min at 68°C, and final extension at 68°C for 5 min	(Eardly, et al. 1992: 1809-15)
dnaJ	***dnaJ*-F-** (5'CAG ATC GAG GTS ACC TTC GAC3') ***dnaJ*-R-** (5'CGT CRY CAT MGA GAT CGG CAC3')	5 min at 95°C, and 30 cycles of 45 s at 94°C, 90 s at 68°C, 2 min at 68°C	(Alexandre, et al. 2008: 2839-49)
recA	***recA*-F1-** (5'CGK CTS GTA GAG GAY AAA TCG GTG GA3') ***recA*-R1-** (5'CGR ATC TGG TTG ATG AAG ATC ACC AT3')	5 min at 95°C, and 30 cycles of 45 s at 94°C, 60 s at 50°C, 90 s at 74°C	(Gaunt, et al. 2001: 2037-48)

Table S3. GenBank accession numbers of rhizobial strains tested in this study

Strain	Accession number			Strain	Accession number		
	16S-23S rDNA IGS	*nodC*	*nifH*		16S-23S rDNA IGS	*nodC*	*nifH*
M001	MW712744	MW736165	MW736279	M063	MW712802	MW736223	MW736337
M002	MW712745	MW736166	MW736280	M064	MW712803	MW736224	MW736338
M003	MW712746	MW736167	MW736281	M065	MW712804	MW736225	MW736339
M004	MW712747	MW736168	MW736282	M066	MW712805	MW736226	MW736340
M005	MW712748	MW736169	MW736283	M067	MW712806	MW736227	MW736341
M006	MW712749	MW736170	MW736284	M068	MW712807	MW736228	MW736342
M007	MW712750	MW736171	MW736285	M069	MW712808	MW736229	MW736343
M008	MW712751	MW736172	MW736286	M070	MW712809	MW736230	MW736344
M009	MW712752	MW736173	MW736287	M071	MW712810	MW736231	MW736345
M010	MW712753	MW736174	MW736288	M072	MW712811	MW736232	MW736346
M011	MW712754	MW736175	MW736289	M073	MW712812	MW736233	MW736347
M012	MW712755	MW736176	MW736290	M074	MW712813	MW736234	MW736348
M013	MW712756	MW736177	MW736291	M075	MW712814	MW736235	MW736349
M015	MW712757	MW736178	MW736292	M076	MW712815	MW736236	MW736350
M016	MW712758	MW736179	MW736293	M077	MW712816	MW736237	MW736351
M017	MW712759	MW736180	MW736294	M078	MW712817	MW736238	MW736352
M018	MW712760	MW736181	MW736295	M079	MW712818	MW736239	MW736353
M019	MW712761	MW736182	MW736296	M080	MW712819	MW736240	MW736354
M020	MW712762	MW736183	MW736297	M081	MW712820	MW736241	MW736355
M021	MW712763	MW736184	MW736298	M082	MW712821	MW736242	MW736356
M022	MW712764	MW736185	MW736299	M083	MW712822	MW736243	MW736357
M023	MW712765	MW736186	MW736300	M084	MW712823	MW736244	MW736358
M024	MW712766	MW736187	MW736301	M085	MW712824	MW736245	MW736359
M025	MW712767	MW736188	MW736302	M086	MW712825	MW736246	MW736360
M026	MW712768	MW736189	MW736303	M087	MW712826	MW736247	MW736361
M027	MW712769	MW736190	MW736304	M088	MW712827	MW736248	MW736362
M028	MW712770	MW736191	MW736305	M089	MW712828	MW736249	MW736363
M029	MW712771	MW736192	MW736306	M090	MW712829	MW736250	MW736364
M030	MW712772	MW736193	MW736307	M091	MW712830	MW736251	MW736365
M031	MW712773	MW736194	MW736308	M093	MW712831	MW736252	MW736366
M032	MW712774	MW736195	MW736309	M094	MW712832	MW736253	MW736367
M033	MW712775	MW736196	MW736310	M095	MW712833	MW736254	MW736368
M034	MW712776	MW736197	MW736311	M096	MW712834	MW736255	MW736369
M035	MW712777	MW736198	MW736312	M097	MW712835	MW736256	MW736370
M036	MW712778	MW736199	MW736313	M098	MW712836	MW736257	MW736371
M037	MW712779	MW736200	MW736314	M099	MW712837	MW736258	MW736372
M038	MW712780	MW736201	MW736315	M100	MW712838	MW736259	MW736373
M039	MW712781	MW736202	MW736316	M101	MW712839	MW736260	MW736374
M040	MW712782	MW736203	MW736317	M102	MW712840	MW736261	MW736375
M041	MW712783	MW736204	MW736318	M103	MW712841	MW736262	MW736376
M042	MW712784	MW736205	MW736319	M104	MW712842	MW736263	MW736377
M043	MW712785	MW736206	MW736320	M105	MW712843	MW736264	MW736378
M044	MW712786	MW736207	MW736321	M106	MW712844	MW736265	MW736379
M045	MW712787	MW736208	MW736322	M107	MW712845	MW736266	MW736380
M046	MW712788	MW736209	MW736323	M108	MW712846	MW736267	MW736381
M047	MW712789	MW736210	MW736324	M109	MW712847	MW736268	MW736382
M048	MW712790	MW736211	MW736325	M110	MW712848	MW736269	MW736383
M050	MW712791	MW736212	MW736326	M111	MW712849	MW736270	MW736384
M051	MW712792	MW736213	MW736327	M112	MW712850	MW736271	MW736385
M053	MW712793	MW736214	MW736328	M113	MW712851	MW736272	MW736386
M054	MW712794	MW736215	MW736329	M114	MW712852	MW736273	MW736387
M055	MW712795	MW736216	MW736330	M116	MW712853	MW736274	MW736388
M056	MW712796	MW736217	MW736331	M117	MW712854	MW736275	MW736389
M057	MW712797	MW736218	MW736332	M118	MW712855	MW736276	MW736390
M058	MW712798	MW736219	MW736333	M119	MW712856	MW736277	MW736391
M059	MW712799	MW736220	MW736334	M120	MW712857	MW736278	MW736392
M061	MW712800	MW736221	MW736335	CC1192	MW584799	MW676866	MW676943
M062	MW712801	MW736222	MW736336				

Table S4. Differential phenotypic characteristics of 16 selected strains from Australia and Myanmar, and closely related species of *Mesorhizobium.*

Strains	SE	AT	HT	ST	Host	Origin	References
A10	-	+	+	-	chickpea	Australia	This study
A14	-	+	-	-	chickpea	Australia	This study
A21	+	+	-	-	chickpea	Australia	This study
A47	+	+	-	-	chickpea	Australia	This study
A53	+	+	+	-	chickpea	Australia	This study
A54	+	+	-	+	chickpea	Australia	This study
A68	+	+	-	+	chickpea	Australia	This study
A78	+	+	-	-	chickpea	Australia	This study
M009	+	-	-	-	chickpea	Myanmar	This study
M021	-	-	+	+	chickpea	Myanmar	This study
M062	-	+	+	+	chickpea	Myanmar	This study
M075	+	+	+	+	chickpea	Myanmar	This study
M082	+	-	+	+	chickpea	Myanmar	This study
M094	+	+	+	+	chickpea	Myanmar	This study
M107	+	+	+	+	chickpea	Myanmar	This study
M113	-	+	+	+	chickpea	Myanmar	This study
M. *ciceri* CC1192	+	+	-	+	chickpea	Israel	This study
M. temperatum		+	+	+	*Astragalus adsurgens*	China	(Gao, et al. 2004: 2003-12)
M. septentrionale		+	-	+	*Astragalus adsurgens*	China	(Gao, et al. 2004: 2003-12)
M. tianshanense		-	-	+	wild legume	China	(Chen, et al. 1995: 153-9, Tan, et al. 1997: 874-9)
M. amorphae		+	-	+	*Amorpha fruticosa*	China	(Wang, et al. 1999: 51-65)
M. gobiense		-	+	+	wild legume	China	(Han, et al. 2008: 2610-8)
M. tarimense		-	+	+	wild legume	China	(Han, et al. 2008: 2610-8)
M. silamurunense		-	+	-	*Astragalus* spp.	China	(Zhao, et al. 2012: 2180-6)
M. ciceri		+	-	-	chickpea	Spain	(Nour, et al. 1994: 345-54)
M. mediterraneum		-	+	-	chickpea	Spain	(Nour, et al. 1995: 640-8)
M. loti		+	-	-	*Lotus corniculatus*	New Zealand	(Jarvis, et al. 1982: 378-80)
M. opportunistum		+	+	+	*Biserrula pelecinus*	Australia	(Nandasena, et al. 2009: 2140-7)
M. plurifarium		+	+	+	*Acacia senegal*	Senegal	(de Lajudie, et al. 1998: 369-82)
M. tamadayense		+	+	+	*Anagyris latifolia*	Spain	(Ramírez-Bahena, et al. 2012: 334-41)
M. metallidurans		+	+	-	*Anthyllis vulneraria*	France	(Vidal, et al. 2009: 850-5)
M. huakuii		+	+	+	*Astragalus sinicus*	China	(Chen, et al. 1991: 275-80)
M. caragnae		+	-	-	*Caragana spp.*	China	(Guan, et al. 2008: 2646-53)

Note: SE, symbiotic effectiveness (>80%); AT, acid tolerance (≥5); HT, heat tolerance (37, 40 ºC); ST, salt tolerance (≤2% NaCl)

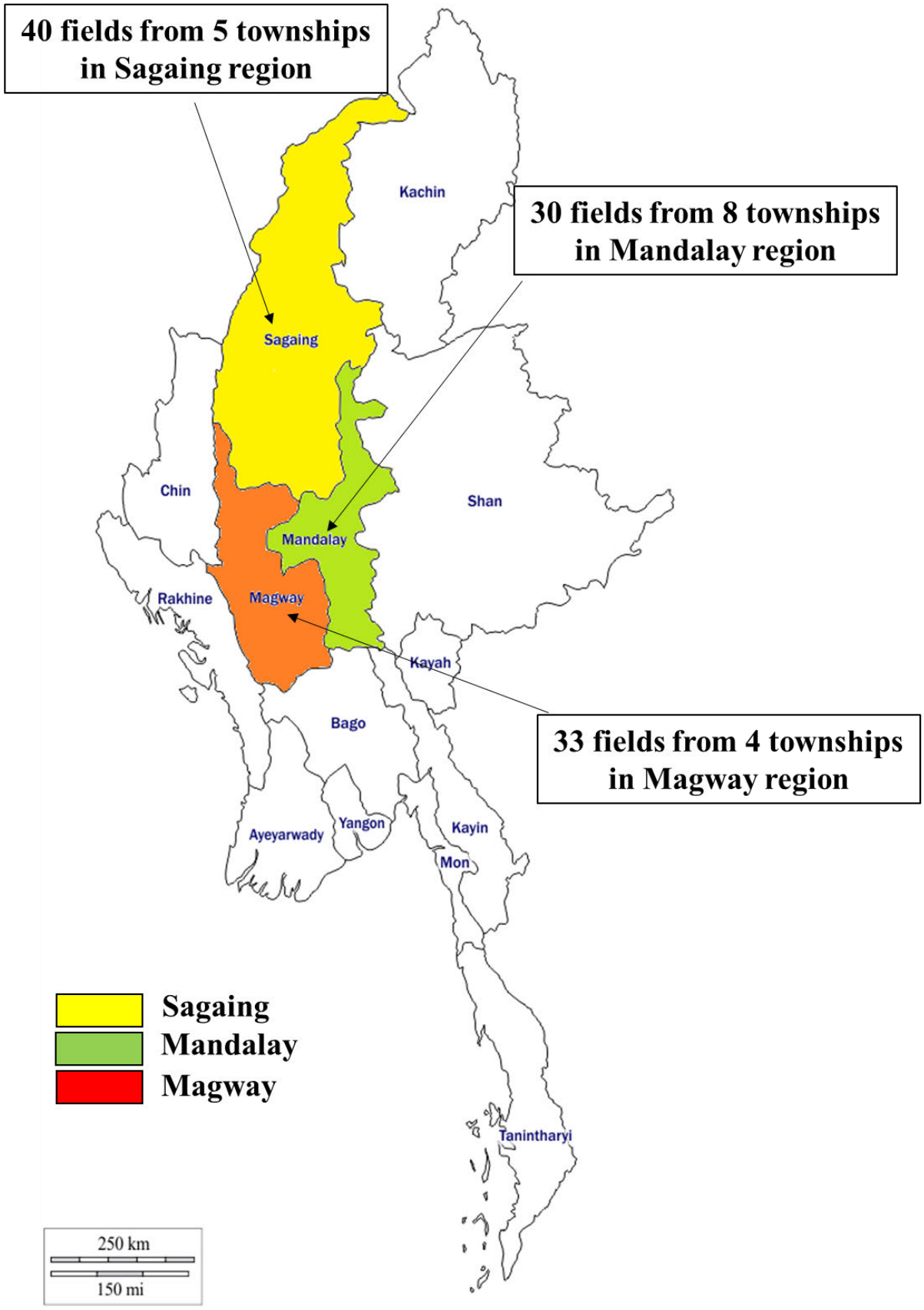

Figure S1. Map showing the sample collection sites in the Central Dry Zone of Myanmar.

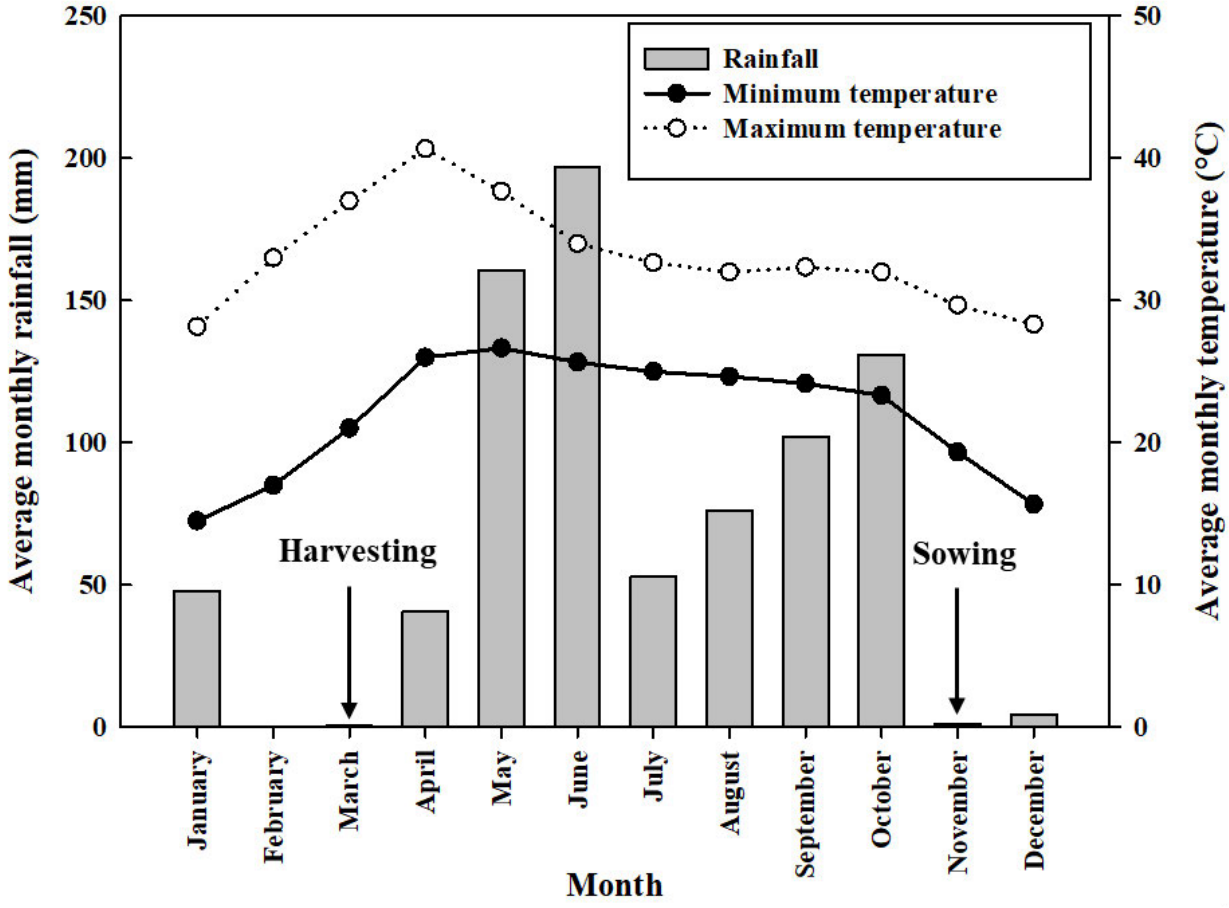

Figure S2. Monthly rainfall distribution and temperatures across soil sampling sites during the 2018 growing season in the Central Dry Zone, Myanmar. (Source: Department of Meteorology and Hydrology,Naypyidaw, Myanmar).

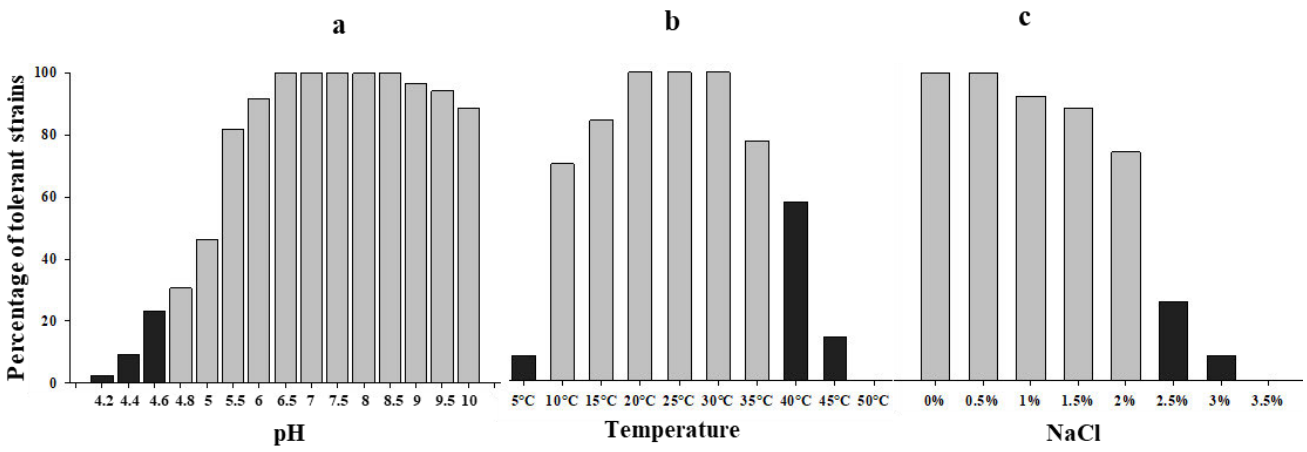

Figure S3. Percentage of tolerant strains (120 test strains and strain CC1192) to (a) pH, (b) temperature (°C) and (c) NaCl (% w/v). Grey bars show the percentage of tolerant strains including strain CC1192; black bars indicate the percentage of strains that were tolerant but where strain CC1192 was unable to grow.

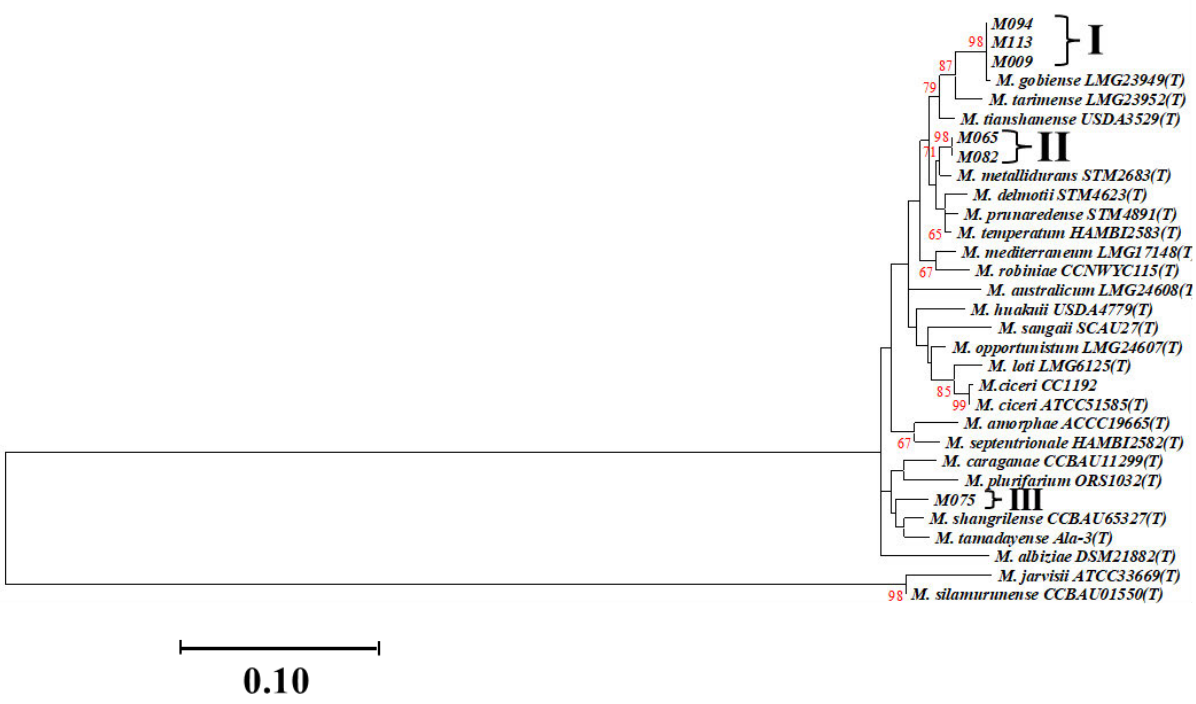

Figure S4. Phylogenetic trees based on partial sequences of *dnaJ* (a) and *recA* (b) showing the relatedness of chickpea rhizobial strains and recognized species of *Mesorhizobium*. The Maximum Likelihood phylogenetic tree was constructed using Kimura 2-parameter model MEGA7. The percentage of trees in which the associated taxa clustered together was presented next to the branches. Bootstrap values were computed based on 1000 replications. The scale bar (0.1) represents the percentage of nucleotide substitution per site. All positions containing gaps and missing data were eliminated. The groups were generated according to 16S-23S rDNA-based phylogeny. *M*, *Mesorhizobium.*

Chapter 5. Optimizing the nodulation and growth of chickpea on acidic soils of South Australia through application of novel chickpea rhizobial strains

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5.1. Statement of authorship

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Publication Status	☐ Published ☐ Accepted for Publication ☐ Submitted for Publication ☑ Unpublished & unsubmitted work written in manuscript style
Publication details	

Principal Author

Name of Principal Author (Candidate)	Myint Zaw
Contribution to the Chapter	Planned and conducted all experiments, analysed and interpreted the data, and wrote the manuscript
Overall percentage (%)	80%
Certification:	This chapter 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 chapter

Signature		Date	29/01/2022

Co-Author

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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Signature		Date	31/01/2022

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Contribution to the Chapter	Comments and reviewed the manuscript		
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Contribution to the Chapter	Supervised development of work and reviewed the manuscript		
Signature		Date	31/01/2022

5.2. Abstract

It has proven that controlled experiments are insufficient to assist in the development of robust rhizobial inoculants for field application. Therefore, we conducted field experiments to identify the potential for rhizobial strains to improve the nodulation, N fixation and biomass accumulation by chickpea. Five field experiments on acidic soils in Australia were conducted to evaluate the symbiotic performance of 12 previously identified strains that had the potential to improve chickpea productivity through symbiotic N_2 fixation. Strains collected from Australian soils were superior in survival on seed, nodulation and increased SDW at most experimental sites with low pH soils, compared with strains isolated from Myanmar soils. At most field sites, the newly isolated strains did not perform better than the commercial strain, CC1192. Strain A47, collected from Australia, was the most effective of all the strains tested in this study, with improved symbiotic N_2 fixation at the Angas Valley site. Strain A78 showed equally good nodulation with CC1192 on a highly acidic soil (pH 4.18) at the Kapunda site, while all other test strains had inadequate nodulation at that site. These strains (A47 and A78) should be tested further in different environments to investigate whether they can perform better than strain CC1192 in Australian farming systems. Although Myanmar strains performed poorly in Australian environments, they have not been tested in Myanmar environments with high pH Vertisol soils and high temperatures, and should be evaluated there in future experiments.

Keywords: Chickpea, nodulation, acidic soil. N_2 fixation, Australia, Myanmar

5.3. Introduction

Australia is one of the largest chickpea exporters in the world, with an estimated market value of $392 million per annum. Chickpea is the second largest grown pulse crop after lupin and is mainly grown in Queensland and NSW although production is increasing in southern states.

Chickpea can fix up to 141 kg N ha^{-1} in shoot biomass through symbiotic association with rhizobia, but this amount is low relative to that fixed by other important legumes in Australian farming systems, such as lupin (19-327 kg N ha^{-1}), lentil (5-191 kg N ha^{-1}) and faba bean (12-330 kg N ha^{-1}) (Unkovich and Pate 2000). N_2 fixation potential of legumes in farming systems could be improved by inoculation with suitable rhizobial strains that show superior performance of nodulation and N fixation in diverse edaphic conditions (Denton *et al.* 2000; Yates *et al.* 2005; Howieson *et al.* 2008; Yates *et al.* 2021).

The commercial inoculant, *Mesorhizobium ciceri* strain CC1192, was originally isolated in Israel (Corbin *et al.* 1977; Bullard *et al.* 2005) and has been widely used in chickpea cultivation in Australia since the 1970s, with adaptation to Australian farming environments. CC1192 is used as an inoculant across Australia through a vast variety of soil and environmental conditions and has good survival characteristics in all types of inoculant formulations, making it ideal for improving N fixation of Australian chickpea crops (Herridge *et al.* 2002; Bullard *et al.* 2005). However, it is generally accepted that this strain could be improved, both in terms of nodulation and N fixation (Hill *et al.* 2021). CC1192 was tolerant to some abiotic stresses such as pH of 4.7 and 1.5% NaCl concentration (w/v), high temperatures of 35 ºC (Zaw *et al.* 2021) and achieves reliable nodulation across a range of environments.

In Myanmar, pulse and oil-seed crop production is concentrated in the central dry zone (CDZ), where black gram, chickpea, green gram and groundnut are commonly grown in rotation with lowland rice. In uplands, cowpea, green gram, groundnut, and pigeon pea are sown after sesame, maize, pearl millet and sorghum (Herridge *et al.* 2019). Low productivity of legume crop production is common in the CDZ (Vaughan and Levine 2015), where unpredictable rainfall, water-stress and high temperature are major constraints in farming systems (Herridge *et al.* 2019). Additionally, CDZ farmers face challenges in managing the coarse-textured soils

with low organic matter and water holding capacity (Guppy *et al.* 2017; Cornish *et al.* 2018; Herridge *et al.* 2019). Low crop productivity in the CDZ is restricted by inputs including N fertilizers and poor crop management, as most CDZ farmers cannot afford or have access to chemical fertilizers. Inoculation of legumes with effective rhizobial inoculants is required to improve farm productivity and profitability in some regions of the CDZ.

Soil acidity is a major constraint to the expansion of chickpea cropping in Australian farming systems (GRDC 2018). During recent decades, about half of the agriculturally productive soils in Australia are affected by acidification and the extent of this problem is potentially increasing due to inadequate treatment and to intensification of land management (McKenzie *et al.* 2017). The value of lost agricultural production due to soil acidification was estimated to be about $1.6 billion per year in 2001 (NLWRA 2001). A survey of Australian cropping systems has shown that due to poor survival of rhizobia in acid soils, there is not only a need to inoculate but also for continued research to find more acid soil adapted rhizobia (Slattery *et al.* 2004). In acid soils, root and shoot growth of the crop are inhibited by high concentrations of available aluminium which limits the elongation of the root through rapid damage of cells in the root apex. Soil acidity may also impair the growth of rhizobia in rhizosphere and inhibit the colonisation of the root (Ryan *et al.* 1993; Kochian *et al.* 2005; Ryan 2018).

Rhizobial strains re-isolated from Australian cropping soils are likely to have adaptation to local cropping conditions such as soil type, low pH, and saline soils. In a previous study, three symbiotically effective strains with 90-109% symbiotic effectiveness (SE), relative to CC1192 isolated from Victoria (A21), Queensland (A47) and Western Australia (A78) grew on acidic media in the laboratory (pH 4.4 to 5) (Zaw *et al.* 2021), and field evaluation in low pH soils was warranted to test the potential for strain field performance. Myanmar strains isolated from Sagaing, Mandalay and Magway regions across the CDZ showed high genetic diversity, SE

and diversity to abiotic stresses (Chapter 4) and may also contain adaptive traits to different soil conditions. Although the chickpea-growing soils in Australia and Myanmar are neutral to high pH, the acid-tolerance of strains and the pH of soil collection sites are not always associated (Wood and Shepherd 1987). The diversity of *Mesorhizobium* species collected from Australia and Myanmar were considered likely to show adaptive traits to Australian field conditions such as low pH and dry soil.

In this study, rhizobial strains were isolated from Australian and Myanmar soils and strains were selected for field evaluation based on their SE and physiological attributes, particularly low *in vitro* pH tolerance. We hypothesised that over time and with inoculation, some strains that were recovered from field soils may have better adaptation and superior symbiotic traits to the original inoculant. The improved tolerance to abiotic stresses may have occurred through horizontal gene transfer (HGT), from an inoculant strain to an adapted resident soil *Mesorhizobium*, or through natural selection of resident strains to soil conditions (Zaw *et al.* 2021). Some studies have reported that selection of highly effective strains was successfully achieved by re-isolating locally adapted strains from cropping soils several years after inoculant introduction (Batista *et al.* 2007; Elias and Herridge 2015). Strains isolated from chickpea-cropping soils may have superior adaptation and survival under Australian conditions, while Myanmar strains are more diverse and may contain traits for an inoculant better than the small genetic pool here in Australia.

In this study, 12 strains were selected from diverse cropping soils from Australia and Myanmar and evaluated for their symbiotic effectiveness at five field sites in Australia including sites with acidic soils (pH_{CaCl2} <5) during the 2020 and 2021 growing seasons. We hypothesised that isolated strains would provide superior effectiveness and would be better adapted to acid soils than the current commercial inoculant strain CC1192 and may also prove valuable as

potential inoculants in Myanmar and Australia. Testing of isolated strains in the field will provide evidence of the ability of these strains to perform as effective inoculants.

5.4. Materials and Methods

5.4.1 Bacterial strains and peat inoculant preparation

A total of 12 strains previously isolated from chickpea nodules grown in Myanmar and Australian soils were selected based on their symbiotic effectiveness in pot experiment and the ability to grow on acidic media *in vitro* (Zaw *et al.* 2021; Chapter 4; Table 1). Growth conditions for the pot experiments in greenhouse were described in chapters 3 and 4. A less efficient strain (M113) was included in field trials because of its ability to tolerate acidic pH in vitro (Table 1). Australian commercial chickpea inoculant *Mesorhizobium ciceri* strain CC1192 was included as a positive control strain and uninoculated controls were used.

For inoculant preparation, a single colony of each bacterial strain was sub-cultured in yeast mannitol broth (YMB) and incubated for 48 hr in a rotatory shaker to achieve approximately 10^9 cfu/ml. Peat inoculants were made by injecting 65 ml of the YMB culture into 150 g gamma irradiated peat bag (New-Edge Microbials, North Albury New South Wales, Australia). The inoculated peat bags were massaged to ensure even moisture distribution and bags were incubated for 2 weeks at 28 ºC to allow the rhizobia to colonise the peat.

5.4.2 Experimental sites

To investigate the potential of chickpea rhizobial strains that had been selected based on symbiotic effectiveness and acid tolerance in controlled conditions, five field experiments were conducted in the 2020 and 2021 growing seasons. The experimental sites were Angas Valley (34.7546° S, 139.3085° E), Kapunda (34.2132° S, 138.8726° E), Loxton (34.3873° S, 140.7712° E), Cockaleechie (34.2213° S, 135.9255° E) and Salter Springs (34.2125° S, 138.6666° E). The experimental sites were previously confirmed to have generally low

background populations of chickpea rhizobia, based on simple unreplicated nodulation pot assessments. The experiments were randomized complete block designs with three replicates. Soil physicochemical properties were measured by APAL, Australia using Inductively Coupled Plasma (ICP) analysis (Rayment and Lyons 2011). Mean monthly rainfall and temperature at the experimental sites were recorded (Fig. 1).

5.4.3 Sowing and inoculation

Before sowing, the peat inoculant was mixed to a slurry with 5 g peat / 20 ml sterile water for Angas Valley and Kapunda, and with 5 g peat / 20 ml sticker (0.5% methyl cellulose) for Loxton, Cockaleechie and Salters Springs. Then, 1.25 ml of the slurry was added to 100 g chickpea seeds in a ziplock bag. The seeds and peat slurry were shaken until all seeds were evenly coated and were allowed to air dry in the bag for 15 min. The inoculated chickpea seeds were sown in six rows (23 cm row spacing) per plot (approximately 20 m^2) to obtain 35 plants / m^2. In the first-year experiments (Angas Valley and Kapunda), sowing was done in late May 2020. During the 2021 growing season, the experiment was initiated in early June at Loxton and mid-June at Cockaleechie and Salter Springs.

5.4.4 Survival of inoculated rhizobial strains on chickpea seeds

To evaluate the survival of rhizobia on inoculated seeds in desiccating environments, nine rhizobial strains in peat sticker were applied onto chickpea seeds at the rate of 5g peat / 20 ml sticker. The inoculated seeds were dried in a laminar flow cabinet. At 24 h (Cockaleechie) and approximately 3-4 hours (Loxton and Salters Springs) after inoculation, three replicates of 10 seeds were added into sterile tube containing 10 ml of sterile water and shaken for 30 s, rested for 30s and shaken again for 30s. Then, 100µl of this solution was taken for serial dilution up to 10^{-4} and three aliquots of 20µl from each dilution were plated onto yeast mannitol agar (YMA) and dried in a laminar flow. Colony forming units (CFU) of rhizobial strains on YMA were counted after 5 days incubation at 28º C.

5.4.5 Field experiment 1: Evaluating symbiotic performance of strains in an infertile sandy and slightly acidic soil (Angas Valley, 2020)

The soil at the Angas Valley site was sandy soil with pH_{Ca} of 5.74 at 0-10 cm. The evaluation of five strains included three Myanmar strains (M082, M075, M009) and an Australian strain (A47) along with commercial inoculant strain CC1192. Chickpea seeds cv. PBA HatTrick were coated with peat inoculant slurry 3 hr prior to sowing on 27 May 2020.

5.4.6 Field experiment 2: Evaluating symbiotic performance of strains in an acidic silty loam soil (Kapunda, 2020)

The soil at Kapunda site was a very acidic silty loam at a depth of 0-10 cm (pH_{Ca} 4.18). Symbiotic performance of five elite experimental strains was tested including three selected Myanmar strains (M094, M113, M065), an Australian srain (A78) and a commercial inoculant CC1192. Peat inoculant slurry was applied to chickpea seeds cv. PBA HatTrick 3 hr prior to sowing on 29 May 2020.

5.4.7 Field experiment 3: Evaluating symbiotic performance of selected potential strains on infertile sandy soil with neutral pH (Loxton, 2021)

The soil at the Loxton site was sandy with pH_{Ca} 6.89 at a depth of 0-10 cm. The evaluation of nine inoculant strains included 8 elite strains from Myanmar (M022, M030, M065, M077 and M082) and Australia (A21, A47 and A78) along with commercial inoculant CC1192 and an uninoculated control. Chickpea seeds cv. PBA Slasher were inoculated with peat-slurry inoculants 4 hr prior to sowing on 4 June 2021 into dry soil. This site received little rain before sowing (April-May) and little rain (<15mm) at sowing time in June and did not receive significant rain until July 2021.

5.4.8 Field experiment 4: Evaluating symbiotic performance of selected potential strains on loamy sand soil with slightly acidic pH (Cockaleechie, 2021)

The soil at Cockaleechie was a loamy sand with acidic pH $_{Ca}$ 5.22 at 0-10 cm depth. Strains were the same as at the Loxton site (Field experiment 3). The peat inoculants were applied to chickpea seeds cv. PBA Slasher 24 hr before sowing. The experiment was sown on 16 June 2021.

5.4.9 Field experiment 5: Evaluating symbiotic performance of selected potential strains on loamy soil with low pH (Salter Springs, 2021)

The soil at Salter Springs was a loam with low pH $_{Ca}$ 5.02 at 0-10 cm depth. Strains were the same as at the Loxton site (Field experiment 3). The peat slurry inoculum was coated onto chickpea seeds cv. PBA Slasher 3 hr before sowing. The experiment was sown on 15 June 2021.

5.4.10 Data collection

Twelve plants were randomly collected from the central four rows (of six sown rows) at 80 cm intervals at around 12 to 13 weeks from sowing, to measure nodulation. Nodules were counted for each plant and a nodulation rating on a 0 to 5 scale was applied based on several parameters particularly of number, size, position, and distribution of nodules on the crown and lateral roots (Corbin et al. 1977; Hartley et al. 2005). Shoot materials from 12 randomly selected plants taken from the central four rows at 80 cm intervals were collected at peak biomass (4.5 months after sowing). Grain was harvested around 6 months after sowing. The plants were not harvested from Kapunda site for grain yield. Leaf chlorophyll content was measured using a SPAD meter during vegetative growth stage in natural field conditions. Shoot and nodule samples were oven-dried at 65 °C for 48 h and dry weights were recorded.

5.4.11 Assessment of N fixation

To estimate the amount of N fixed by inoculation treatments at Angas Valley, the ^{15} N natural abundance method was applied (Unkovich *et al.* 2008). At peak biomass stage, eight above-ground plant samples per plot (20 m^2) were taken from middle rows (Beck 1992 & Sadras *et al*. 2016) and, oven-dried at 70 ºC for 48 hrs and weighted. Total biomass was based on the plant density measures, that were approximately 35 plants per m^2. Samples were finely ground and analysed for $\delta^{15}N_{Air}$ (%) and total shoot N (µg g^{-1}). (Stable isotope facility, University of California, Davis, USA). Canola (cv. HyTTec Trifecta) plants grown in the same experimental plots were collected at the same time with chickpea plants. Chickpea N derived from fixation (%Ndfa) was calculated by comparing the $\delta^{15}N$ in inoculated plants ($\delta^{15}N$ legume) with that of the broadleaf non-nodulating canola reference ($\delta^{15}N$ canola). The following formula was applied to calculate the %Ndfa of chickpea inoculated with rhizobial strains.

$$\%\text{Ndfa} = \frac{\delta^{15}\text{N canola} - \delta^{15}\text{N legume}}{\delta^{15}\text{N canola} - \text{B}} \text{X} \frac{100}{1}$$

Where the B value (-1.26) is the $\delta^{15}N$ of chickpea that are fully dependent upon N_2 fixation and sampled at the same growth stage as the field grown plants (Unkovich *et al.* 2008).

The amount of N_2 fixed were calculated from estimates of legume %Ndfa, legume SDW and %N using following formulae:

$$\text{Legume shoot N} = \frac{\%\text{N}}{\text{Legume SDW}} \text{X} \frac{100}{1}$$

$$\text{Amount shoot N fixed per plant} = \frac{\%\text{Ndfa}}{\text{Legume Shoot N}} \text{X} \frac{100}{1}$$

The amount of N_2 fixed (kg ha^{-1}) were estimated using the plant population (35 plants per m^2) in our experimental plots.

5.4.12 Phylogenetic relationship between 16S-23S rDNA IGS sequences of 12 elite strains selected for field evaluation and commercial chickpea strain CC1192

An abbreviated phylogenetic tree was generated using reference strains and the selected elite strains (Zaw *et al.* 2021; Chapter 4).

5.4.13 Statistical analysis

The symbiotic effectiveness (SE%) of the 12 selected strains was calculated using the following formula.

$$\text{SE (\%)} = \frac{\text{SDW of inoculated treatments}}{\text{SDW of inoculant strain CC1192}} \text{ X } 100$$

Based on this effectiveness scale, strains were considered highly effective (***) when percentage of effectiveness >100%, effective, (**) between 100 and 80% and poorly effective (*) <80%, relative to CC1192.

Data from five field sites were independently subjected to analysis of variance using general linear model in Statistix version 8 software. Mean values were compared using $LSD_{0.05}$ level of significance. To evaluate the consistency of strains performance between pot and field experiments, nodule numbers and normalized SDW provided by individual strains from both experiments were compared.

The relationship of survival on inoculated seed and nodulation efficiency of rhizobial strains was determined using linear regression fitted to the data (R^2 =0.414, p<0.001).

5.5. Results

5.5.1 Rainfall and temperature at experimental sites

Among the five experimental sites, two sites were representative of the Mallee region (Angas Valley and Loxton), where high soil pH and marginal conditions were found. Two other sites (Kapunda and Salter Springs) had low pH soils. Cockaleechie and Salter Springs typically are considered medium to high rainfall. In the 2020 growing season, the Kapunda field site received higher precipitation than Angas Valley before sowing and generally throughout the growing season. Angas Valley experienced hot and dry conditions throughout the growing season and little or no rain around harvesting time. The monthly average maximum temperatures (30 to 33°C) were observed in January and November at both sites (Fig. 1 A, B).

During the 2021 growing season, Cockaleechie and Salter Springs sites received higher rainfall at the time of sowing in June (70-100 mm monthly total) than the Loxton site (< 15 mm). The monthly mean temperatures at all three sites were very similar particularly during the growing season (Fig 1 B-C).

5.5.2 Soil analysis

The analytical results of soil physicochemical properties at the five field sites are presented in Table 2. Soils at Kapunda, Salter Springs and Cockaleechie are very acidic to slightly acidic pH ($CaCl_2$) ranging from 4.52 to 5.22, while the Loxton and Angas Valley sites had slightly acidic to neutral soil with pH ($CaCl_2$) 5.74-6.89. Angas Valley and Loxton soils had low fertility particularly with organic carbon (0.59 and 0.83%), nitrate N (7.6 and 18 mg kg^{-1}) and ammonium N (<1 and 1.5 mg kg^{-1}), respectively. The optimum range of organic carbon in cropping soils is 0.9 to 1.45% in low rainfall areas and 1.45 to 2.9% in high rainfall areas (Peverill *et al.* 1999). Soil textures at Angas Valley and Loxton were sandy, whereas the Kapunda, Salters Springs and Cockaleechie sites have silty loam, loamy and loamy sand soil texture, respectively.

5.5.3 Survival of inoculated rhizobial strains on chickpea seeds

The viable cell numbers of rhizobial strains on seeds were estimated by dilution plating on YMA 3-4 h (Loxton and Salter Springs) and 24 h (Cockaleechie) after seed inoculation. Generally, Australian strains, including CC1192, had higher viable cell numbers on seeds (10^5 cfu/seed) than Myanmar strains, except with M065 at Loxton (Fig. 2). Across those sites, the analysis of the viable cell numbers and nodule rating revealed that strains with higher survival performed better at nodulation ($R^2 = 0.414$, $p < 0.001$) (Fig. 3). An exception was a Myanmar strain (M022) that did not perform well in nodulation regardless of survival on seed, so this strain was excluded from the regression analysis. Two Australian strains (A21 and A47) and CC1192 showed consistently better survival on seed and nodule rating across the sites. In contrast, high survival of Myanmar strain M065 on seed was not associated with better nodulation in the field (Fig. 3).

5.5.4 Field experiment 1: Evaluation of symbiotic performance of strains in slightly neutral pH soil (Angas Valley, 2020)

Inoculation with selected elite strains generally increased nodulation and shoot biomass accumulation and N fixation compared with the uninoculated control (Fig. 4). Inoculation with strain A47 produced the largest nodule dry weight, which was comparable with CC1192 and was statistically higher than other tested strains and uninoculated control (Fig. 4 B). Strain A47 provided the significantly highest nodule rating among the strains including CC1192 and controls (Fig. 4C), while leaf chlorophyll concentration and grain yield were not significantly different among treatments (Fig. 4D, F). In terms of N fixation efficiency, A47 had the best performance (56.1% Ndfa), followed by M082 (48.7% Ndfa) (Fig. 4G). Strain A47 fixed approximately 57 kg shoot N ha^{-1}, whereas the other tested strains and CC1192 fixed a lower amount of shoot N (38.5 to 47.9 kg N ha^{-1}) (Fig. 4H). Both %Ndfa and amount on shoot fixed N were not significantly different between the strains. Total N in shoot biomass (µg per mg)

were not significantly different among treatments (Fig. I). The detailed N fixation data from this study are presented in supplementary table S1.

5.5.5 Field experiment 2: Evaluation of symbiotic performance of strains in acidic soil (Kapunda, 2020)

Only two strains (A78 and CC1192) were able to perform significantly better at nodulation than the uninoculated control in this very acidic soil (Fig. 5 A-C). Strain M094 isolated from Myanmar soil failed to nodulate chickpea under acidic conditions. None of the test strains were superior in shoot biomass accumulation compared with the negative control (Fig. 5C). The shoot biomass of inoculated plants at Kapunda ranged from 4 to 6 g plant^{-1}, which was much lower than at Angas Valley (10 to 13 g plant^{-1}), however, shoot biomass in the negative control plant were not different between two sites. Kapunda site had poor agronomic management particularly weed control and so the plants from this experimental site were not harvested for yield.

5.5.6 Field experiments 3, 4, 5: Evaluation of symbiotic performance of strains in acidic and neutral soils (Loxton, Cockaleechie and Salter Springs, 2021)

The effect of inoculation with nine selected rhizobial strains on nodule numbers and nodule rating were significantly different at Loxton ($p< 0.05$), and at Cockaleechie and Salter Springs ($p< 0.01$) (Fig. 6). Two strains from Australia (A21 and A47) showed significantly better nodulation (4 to 8 nodules plant^{-1}) than the negative control in all three locations but were comparable to CC1192 (1 to 9 nodules plant^{-1}). In contrast, the Myanmar strains did not increase nodule numbers and nodule rating compared with the uninoculated control, except for the nodule rating of M065, M077 and M082 at Salters Springs. The Australian strain, A78, had very poor nodulation at Loxton but not at the other sites. Inoculation with five strains (A21, A47, A78, M022 and M065) significantly increased shoot biomass compared with the uninoculated control at Loxton but were not different to CC1192 (Fig. 6 C), and all strains

except M022 produced significantly higher SDW than the negative control at Salters Springs (Fig 6 J). Although the nodule numbers and nodule rating were not different, Myanmar strains M022 and M065 had significantly higher SDW than CC1192 at Loxton (Fig. 6 C). At Salter Springs, strain A47 was equal to CC1192 in SDW enhancement, which was increased compared with the other test strains (Fig. 6 I).

5.5.7 Comparison of strain performance in controlled environments and field conditions

When symbiotic performances of 12 selected strains and CC1192 between pot and field experiments were compared, the Australian strains showed consistently better nodulation efficiency in field conditions and were almost always comparable with CC1192. In contrast, all Myanmar strains had low nodule numbers under field conditions. This was not comparable to the nodulation and SDW accumulation in the greenhouse experiment, where the Myanmar strains performed better than, or similar to, CC1192 (Fig. 7 A). For example, strain M065 had the highest nodule numbers (18) in the glasshouse but had poor nodulation in the field. In comparison, CC1192 had consistently higher SDW in the field (Fig. 7 B). Two Australian strains (A21 and A47) were consistently superior in SE% across all field sites as well as in the glasshouse experiment, which were comparable with the effectiveness to CC1192 (Fig. 7 C). In contrast, Myanmar strains generally showed inconsistent performances between glasshouse and field experiments. For example, strain M075 and M082 contributed low SE% across all field sites, while they showed superior SE% relative to CC1192 under controlled environments (Fig. 7 C).

5.5.8 Phylogenetic relationships of Australian and Myanmar strains

The Australian strains used for field evaluation (A21, A47 and A78) were in the same group as cognate chickpea rhizobia *M. ciceri* and inoculant strain CC1192, where strain A21 was slightly divergent from A47 and A78 (Fig. 8). Strain A47 performed similarly to CC1192 in

both field and glasshouse (Fig. 7), and these were also genetically closely related (Fig. 8). In contrast, Myanmar strains were more diverse and were distantly related to *M. ciceri*. In Myanmar, *M. gobiense* was the most dominant species across all three sampling regions: Mandalay (M009, M030), Sagaing (M077) and Magway (M094, M113) (Fig. 8). Strain M022 isolated from the Mandalay region was closely related to *M. temperatum*, while strains M065 from Sagaing and M082 from Magway were most closely related to Indian strain IC-2058-CA181. Strain M075 from Sagaing carried 100% identical sequences of 16S-23S rDNA IGS with *M. muleiense*.

5.6. Discussion

This study, conducted over 2 years, has investigated the performance of 12 previously identified strains (Zaw *et al.* 2021; Chapter 4) in the field. These strains improved symbiotic outcomes for chickpea in pot experiments, and had the potential to improve chickpea productivity, particularly in low pH soils. We hypothesised that strains isolated from chickpea-growing soils collected in Australia and Myanmar may have greater symbiotic effectiveness in Australian chickpea farming compared with the current commercial inoculant strain CC1192. Strain CC1192 has been used as an inoculant in Australia continuously since the 1970s and although it is a highly effective strain, it is possible that other strains isolated from local or diverse soils may provide improved nodulation and N fixation for chickpea crops (Herridge *et al.* 1995). In the current study, field analyses of strains collected in Australian soils were vastly superior in survival on seed and increased nodulation and SDW at most experimental sites with low pH soils compared with the strains collected in Myanmar. In most experiments, however, the newly isolated strains did not perform better than CC1192. The Australian strain A47 was the most effective of all the strains analysed, contributing significantly improved symbiotic N_2 fixation at the Angas Valley site. Strain A78 appeared to be adapted to acidic conditions (pH 4.18) at the Kapunda site, as it had equal nodulation with CC1192, while other test strains did not perform well. These strains should be evaluated further across different environments, to determine if they can perform better than strain CC1192 in Australian environments. Although Myanmar strains performed poorly in the Australian field environments, they may perform better in Myanmar conditions such as in high pH Vertisol soils and high temperatures and should be tested there in future experiments.

5.6.1 Survival of rhizobia on seed

The selection of symbiotically effective strains with good survival in desiccating environments is important for efficient nodulation and N_2 fixation (Fletcher *et al.* 2015; 2017; Hunt *et al.*

2019; Porqueddu *et al.* 2016). Desiccation of rhizobia on the seed after inoculation is a major cause of poor survival of rhizobia on seed (Deaker *et al.* 2004; Evans 2005; Deaker *et al.* 2007). To optimise legume nodulation, applying higher rates of inoculant and sowing seed within a few hours into moist soil have been recommended to help maintain rhizobial populations through the critical germination period and before the seedlings establish a rhizosphere (Drew *et al.* 2012a; Herridge *et al.* 2014). However, this is not always possible in practice as there may be a delay between inoculation and sowing, sometimes up to several days, causing high mortality rates of rhizobia before seedling germination (O'Hara *et al.* 2002). Modern farming practices, such as the application of air-seeder machinery in large-scale agriculture, may also accelerate the desiccation of inoculated seeds during sowing (Vriezen *et al.* 2007; Drew *et al.* 2012b). In this study, a strong positive association was found between survival of rhizobia on seed and nodulation. For adequate legume nodulation, the standard minimal requirements of viable cells to deliver on seed are 10^3, 10^4, and 10^5 cfu/seed for small, medium and large seeded legumes, respectively (Deaker *et al.* 2004). The Australian strains and CC1192 maintained higher survival on seed within 3 to 24 hours after inoculation in all three experiments in 2021, compared with most Myanmar strains, particularly strains M030, M077 and M082 (Fig. 2).

Survival of rhizobial strains on seeds is vital for effective nodulation in the field. In this study, Myanmar strains did not produce adequate nodulation under Australian field conditions, as 10 to 30 nodules per plants is considered as adequate nodulation for chickpea in the field. Based on a 0 to 5 nodule rating scale for chickpea, 0 to 1, 2 to 3 and 4 to 5 were considered inadequate, adequate and good nodulation, respectively (GRDC 2018). Myanmar strains were always between 0 and 1.5 scores for nodule rating across the sites. Two Myanmar strains (M065, M022) had better survival on seed compared with other Myanmar strains but they did not give adequate nodulation in the fields, suggesting survival on seeds may not always translate to survival in the soil environment (Mendoza-Suárez *et al.* 2021). In field conditions, aside from

abiotic factors such as desiccation, temperature and humidity affecting rhizobial survival, the inoculated strains may have to deal with biotic factors such as competitive and antagonistic soil microbes (Bottomley 1992; Jack *et al.* 2019). A critical step in successful nodulation is the survival of rhizobia as a free-living bacteria on the seed, then proliferation and eventual nodulation in the rhizosphere (Munns 1965; Robson and Loneragan 1970). Therefore, both survival on the seed and in the soil are essential for adequate nodulation, which were not found to be strong characteristics in the Myanmar strains.

It is also possible that the survival and competition for nodulation in the field may be partly associated with strain genetics. Australian strains that were most closely related *M. ciceri* are more likely to outcompete for chickpea nodulation over a wide range of environments, as *M. ciceri* is the most dominant chickpea symbiont in the world (Zhang *et al.* 2020) and probably had better adaptation both to chickpea genotypes and different environments, compared with other *Mesorhizobium* species (e.g., *M. gobiense*, *M. muleiense*), as found in Myanmar (Chapter 4). Additionally, for effective nodulation in the field, the rhizobial strains need not only to be able to survive but also to compete with the resident soil rhizobia. It is possible that the resident soil rhizobial populations would be very different in Australia and Myanmar, and thus strains isolated from Myanmar soils may be less likely to have sufficient competency for nodulation as well as survival in Australian soils.

5.6.2 Performance of strains across field sites

The two experimental sites (Angas Valley and Loxton) were representative of neutral pH sandy soils and marginal climatic conditions (low rainfall). Myanmar strains showed particularly poor nodulation efficiency at these sites which are considered more difficult farming environments compared with the other more moderate sites (Kapunda, Salters Springs, Cockaleechie). The poor field performance of the Myanmar strains was in direct contrast to the SE obtained under controlled conditions. At Loxton, two Myanmar strains (M022, M065) performed poorly in

nodulation, but they provided a good SDW. The commercial inoculant strain CC1192 showed consistently superior performance in nodulation and SDW accumulation across the sites, which justifies the widespread adoption of this inoculant strain across Australian chickpea growing regions (Bullard *et al.* 2005). Chickpea nodulation and yield can only be improved by inoculation with competitive strains that can tolerate local soil conditions and environmental stresses (Romdhane *et al.* 2008). In the current study, strains A47 improved N_2 fixation in chickpea at the Angas Valley site.

To improve chickpea productivity in acidic soils, it is possible that the re-isolated strains that are able to grow on acidic media *in vitro* would provide greater symbiotic performance on acidic soils. In this study, most of the tested strains performed poorly in a highly acidic soil at the Kapunda site, except for A78 and CC1192, which suggests that acidic conditions (pH_{Ca} 4.18) could be one of the limiting factors for adequate nodulation of chickpea grown in this site, while these strains grew well on acidic media (pH 4.2) *in vitro*. The soil pH where the strains were isolated may influence an ability to symbiotically perform in different soils with contrasting pH (O'Hara *et al.* 1989; Ozawa *et al.* 1999). However, this is not always the case: the acid tolerance of rhizobial strains isolated from acidic soils in Sardinia had no correlation with the pH of soil origin, suggesting the general adaptation of individual strains for inoculation success (Howieson *et al.* 1988; Howieson *et al.* 1991). In this study, there were many strains from Myanmar that had tolerance to low pH in the laboratory (chapter 4), although they originated from neutral-alkaline soils of the CDZ in Myanmar. A potential acid tolerant strain (A78) in this study was isolated from a soil with neutral pH (6.89) in Western Australia (Zaw *et al.* 2021). In the current study, either acid or salt tolerance of the strains *in vitro* did not translate to improved performance in field conditions, confirming the importance of field evaluation of strains following laboratory assessment (Howieson *et al.* 1988).

In this study, the failure to nodulate by most strains at the Kapunda site during the 2020 growing season may be influenced by factors other than soil acidity. This site received poor agronomic management, particularly in lack of effective weed control and had a much higher surface soil nitrate N concentrations (52 mg N kg^{-1}) than other sites (7.6 to 22.5 mg N kg^{-1}) (Table 2), that may have inhibited the nodulation process. The increase in soil nitrate from 20 to 80 mg N kg^{-1} significantly decreased nodulation and N fixation in chickpea (Evans 1982). In soybean, soil nitrate (30 mg N kg^{-1}) delayed nodule initiation and prevented nodule development until 42 days after sowing (Herridge *et al.* 1984). However, high soil nitrate may contribute to the relatively high SDW in uninoculated plants compared with inoculated treatments, as observed at Kapunda (Fig. 5D), possibly because the negative control plants have less biotic stresses such as competition between introduced and resident strains in the rhizosphere. Among our test strains, only A78 and CC1192 were able to survive and nodulate chickpea in the highly acidic Kapunda soil environment. The molecular signalling between legume host and rhizobia in acid soils may have been disrupted, and nodulation compromised when low pH impairs the development of rhizobial strains in rhizosphere and inhibits the ability to colonise the actively growing section of the root zone after sowing. Root and shoot growth may also be inhibited by associated high Al concentrations, which limit the elongation of the root by rapidly damaging the cells in root apex (Ryan *et al.* 1993; Kochian *et al.* 2005; Ryan 2018).

It has been reported that re-isolated native strains were more competitive than exotic commercial inoculant strains and inoculation with non-native commercial strains did not significantly improve nodulation and SDW accumulation in the field, due to poor adaptation of the inoculant to local conditions, as found in Tunisia and Myanmar (Romdhane *et al.* 2007; Romdhane *et al.* 2008; Denton *et al.* 2017). In contrast, the commercial chickpea inoculant strain CC1192 from Israel has been widely used in Australian soils since the 1970s due to its excellent N_2 fixation ability and adaptation across Australian cropping regions (Deaker *et al.*

2004; Bullard *et al.* 2005). In this study, SDW of field-grown plants inoculated with Myanmar strains did not correlate well with nodule numbers or nodule rating particularly at the Loxton site (Fig. 6 A-C). Two Myanmar strains (M022 and M065) had significantly higher SDW than CC1192 in a high pH dry soil at Loxton (Fig. 6C), while these strains performed poorly in nodulation across all experimental sites. Two strains isolated from Australia (A21 and A47) had consistently good symbiotic performance across the sites and A47 fixed more N_2 than other strains tested in the field.

5.6.3 SE of strains in the field vs glasshouse

In this study, there were substantial differences in performance of Myanmar strains between glasshouse and field conditions. For example, strain M065 had the highest nodule numbers per plant (18) in the glasshouse but had poor nodulation (1 to 3 nodules plant^{-1}) in all field sites. However, there was less difference between glasshouse and field performance of re-isolated Australian strains. Two Australian strains (A21 and A47) were consistently superior in SE% across all field sites as well as in the glasshouse experiment. It is possible that the inconsistent performances of Myanmar strains were due to the lack of competition in sterile or mono axenic conditions in the glasshouse compared with microbial competition in the field. The plants were well-watered in the glasshouse experiments, while the plants were sown into rainfed conditions in field environments. Also, rhizobial strains may have suffered less stresses such as desiccation on seed in glasshouse conditions than in the field. For example, rhizobial cells were inoculated directly at the base of seedling in the glasshouse, while seed inoculation was done 3 to 24 h before sowing in the field, which may result in a greater loss of viable rhizobia. Therefore, it is important to evaluate strains in the field as seed-based inoculants, as there appears to be very little correlation between glasshouse and field performance, especially with Myanmar strains.

It was possible that the same saprophytic competence could have been expected from the Myanmar strains, but we did not find that to be the case. They had vastly lower SE in the field compared with the glasshouse experiment. This may be due to genetic diversity from common inoculant strains, or they simply were not able to survive as an inoculant (on the seed) or in Australian soils. However, this may not be the case in Myanmar soils, and the strains should be further tested in Myanmar to evaluate their potential more fully.

5.6.4 Genetic relatedness and origin of selected strains

In this study, based on 16S-23S rDNA sequences, two Australian re-isolated strains (A47 and A78) were identical to *M. ciceri* CC1192 isolated in Israel (Corbin *et al.* 1977), which was widely applied in Australian chickpea growing soil due to its excellent performance across a range of environments (Bullard *et al.* 2005). The re-isolated strains that were potential descendants of CC1192 were found to be symbiotically effective strains, suggesting the SE of the strains were influenced by their genetic relatedness to effective symbionts or inoculant strain with better adaptation traits (e.g., CC1192) (Zaw *et al.* 2021). In contrast, Myanmar strains were more diverse and were most closely related to other *Mesorhizobium* species: *M. gobiense*, *M. temperatum* and Indian strain IC-2058-CA-181, and these species have vastly different adaptative traits compared with strains more closely related to *M ciceri*. It has been reported that the diversity of soybean and chickpea rhizobia was higher in organic farming systems, as are found in Myanmar, where very limited inputs were used compared with conventional farming systems with heavy use of chemical fertilizer, pesticide and herbicide (e.g., Australia) (Grossman *et al.* 2011; Greenlon *et al.* 2019; Muleta *et al.* 2022). The decreased genetic diversity of rhizobia was found in soil amended with commercial fertilizers including $(NH_4)_2SO_4$, NH_4Cl or NH_4NO_3 (Caballero-Mellado and Martinez-Romero 1999). Therefore, the increased diversity of Myanmar strains could mean better adaptation in low input farming systems compared with Australia where farming systems are more intensive.

In this study, strain A78, that is genetically closely related to CC1192, performed well in an acidic soil at Kapunda, whereas Myanmar strains (M094 and M113) were tolerant to low pH *in vitro* were most related to Chinese strains (*M. gobiense, M. temperatum*) and Indian strain IC2058-CA-181 (Fig. 8). Inoculation in Australia has most likely contributed to a lower diversity of chickpea-nodulating rhizobia detected in the soil (Chapter 4) and re-isolated strains are closely related to the inoculant strain and these descendants of those strains are dominant in Australian chickpea cropping soils (Zaw *et al.* 2021). The increased diversity and dominance of other *Mesorhizobium* species that were distantly related to cognate chickpea rhizobia in Myanmar may be due to better adaption of Myanmar strains to local soil conditions and farming systems, as found in India and China. It is also possible that *M. ciceri* would become the predominant strain distributed in Myanmar, if chickpeas were frequently inoculated with effective inoculants of that species.

5.7. Conclusion

Selection of effective rhizobial strains compatible with host genotypes and local environments for specific regions is crucial for optimising legume productivity. The Australian re-isolated strain, A47, provided much greater N_2 fixation than the current commercial strain and could be considered as a potential strain for further testing as alternative inoculant for Australian chickpea production. Even with low nodulation, two Myanmar strains (M022 and M065) produced improvements in SDW on a neutral pH soil at Loxton. Re-isolated strains A21, A47 and A78 performed as effectively as CC1192 in nodulation in both pot and field experiments and could be considered as suitable strains for further development for acidic soils in southern Australia.

5.8. References

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5.9. Tables and figures

Table 1. Symbiotic effectiveness and pH tolerance of selected strains under controlled conditions, compared with inoculant strain CC1192. The strains were identified based on 16S-23S rRNA IGS (accession numbers provided).

Strains	**SE[a]**	**pH tolerance**	**Accession number (16S-23S rRNA IGS)**
M009	***	5.5 - 10	MW712752
M022	***	5.5-10	MW712764
M030	**	4.3-10	MW712772
M065	***	4.2 -10	MW712804
M075	***	5.0 -10	MW712814
M077	**	4.3-10	MW712816
M082	***	5.5 – 10	MW712821
M094	**	4.2 – 10	MW712832
M113	*	4.2 – 10	MW712851
A21	***	5-10	MW584742
A47	***	4.5 -10	MW584767
A78	***	4.4 - 10	MW584796
CC1192	***	4.7 - 10	MW584799

[a]Symbiotic effectiveness (*<80%, **81-100%, ***>100%)

Table 2. The selected physicochemical properties of soil from 0-10 cm depth at experimental sites.

Soil factors	**Angas Valley**	**Kapunda**	**Loxton**	**Cockaleechie**	**Salter Springs**
pH (H_20)	7.04	4.42	7.64	5.93	5.96
pH ($CaCl_2$)	5.74	4.18	6.89	5.22	5.02
CEC (cmol kg^{-1})	6.1	5.5	7.26	11.07	12.8
Organic C (%)	0.83	2.72	0.59	2.53	2.71
Nitrate N (mg kg^{-1})	18	52	7.6	16.5	22.5
Ammonium N (mg kg^{-1})	1.5	5.4	<1	1.45	3.05
K (mg kg^{-1})	386	221	432	284.5	299
Mg (mg kg^{-1})	164	117	149	88.5	220.5
Ca (mg kg^{-1})	723	725	963	1900	1925
Na (mg kg^{-1})	32.9	82.3	29.4	25.2	138
Soil texture	Sand	Silty loam	Sand	Loamy sand	Loam

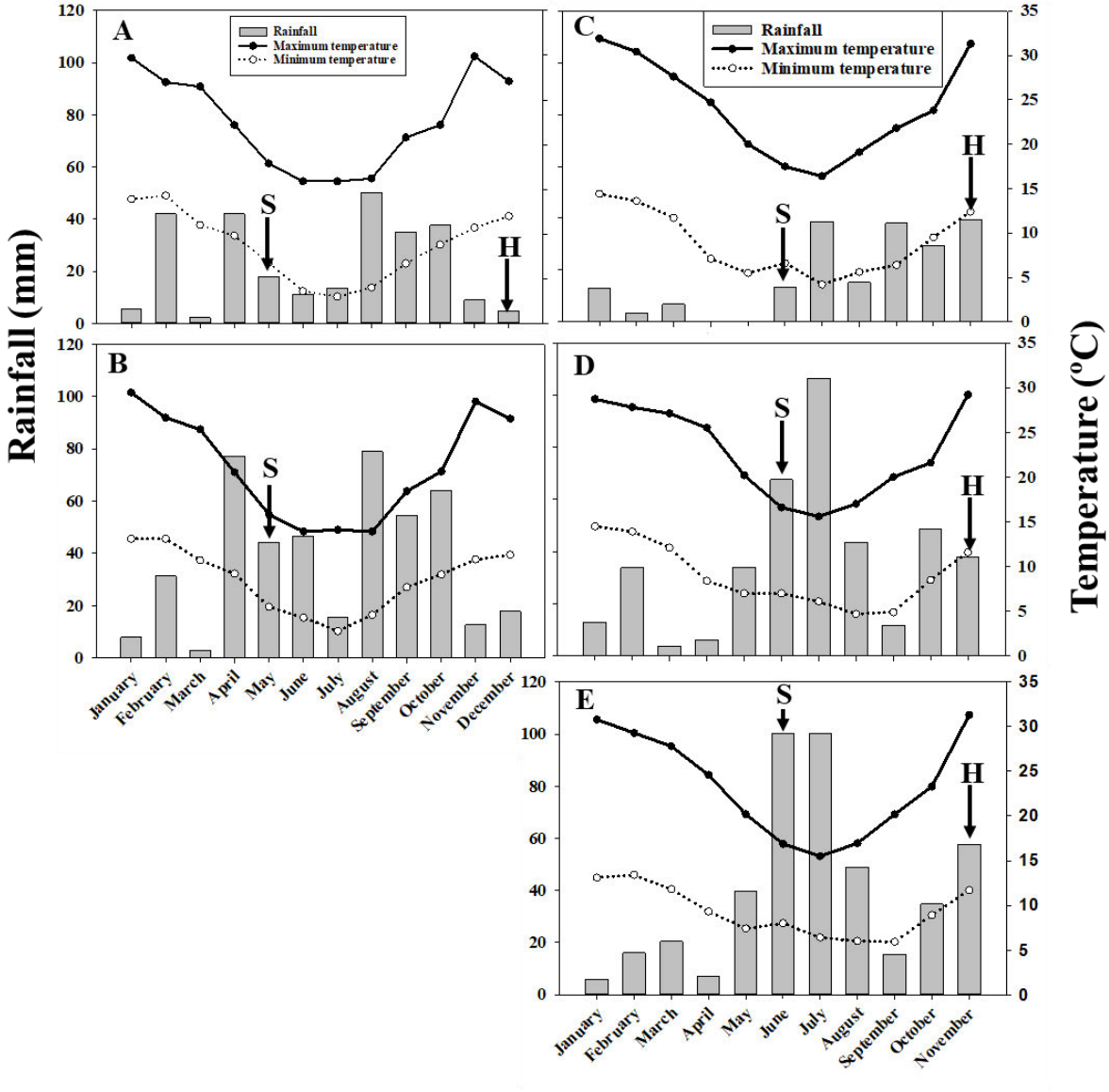

Figure 1 Monthly rainfall and temperature data at (A) Angas Valley and (B) Kapunda during 2020 growing season, and (C) Loxton, (D) Cockaleechie and (E) Salter Springs during 2021 growing season (Bureau of Meteorology, Australia). S, sowing, H, harvesting.

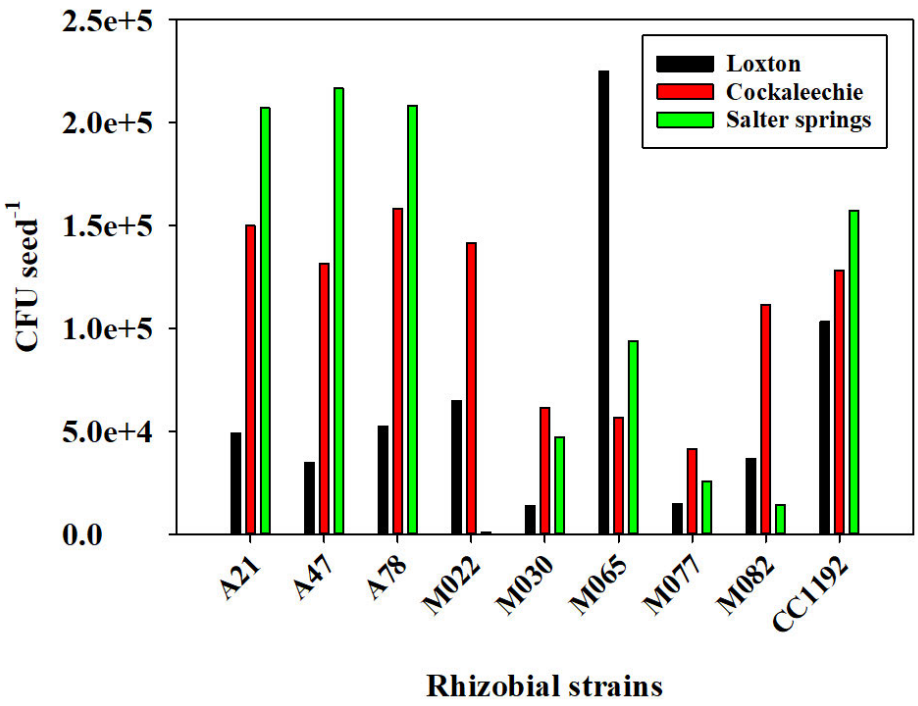

Figure 2 Survival of rhizobial strains on seeds inoculated with a peat slurry prior to sowing at three field experimental sites in 2021: Loxton, Cockaleechie and Salter springs. CFU, colony forming unit. Mean CFU values were from duplicate samples.

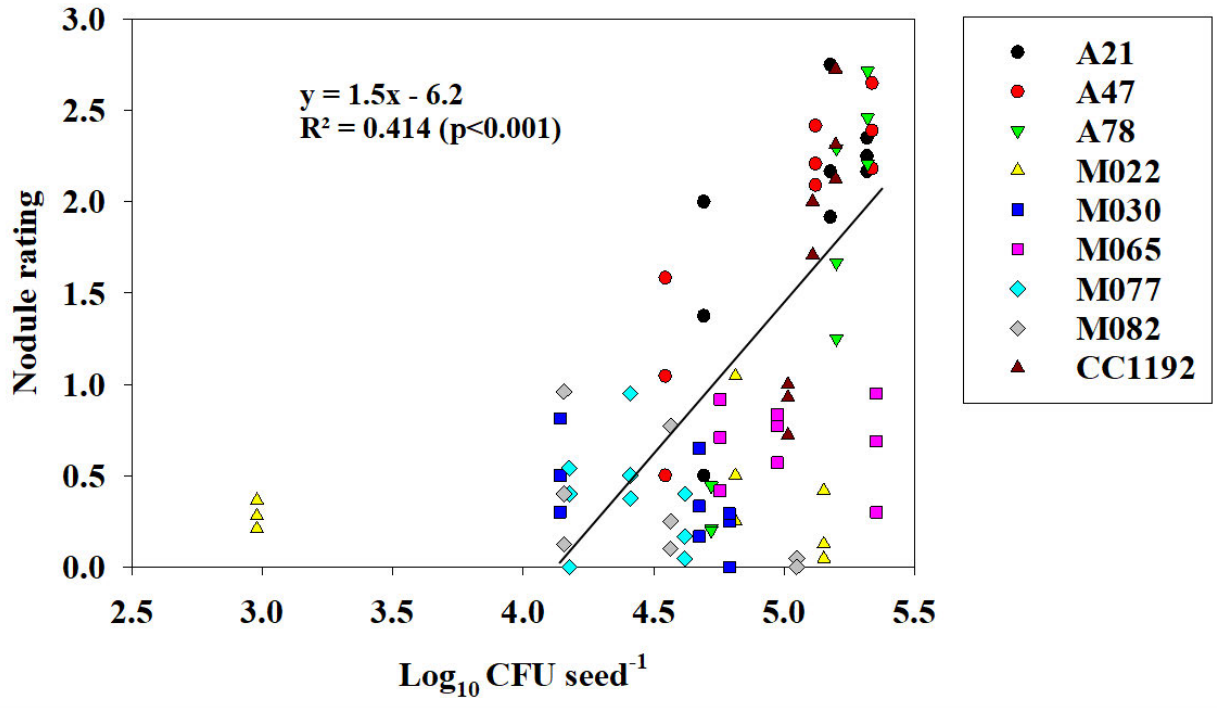

Figure 3 Relationship between survival of rhizobial strains on inoculated seeds (Log_{10} CFU $seed^{-1}$) and nodule rating (n=3) at Loxton, Cockaleechie and Salter springs. A linear regression is fitted to the data ($R^2 = 0.414$, $p < 0.001$). Strain M022 was excluded, as this strain did not fit with this function and performed independently from the other strains.

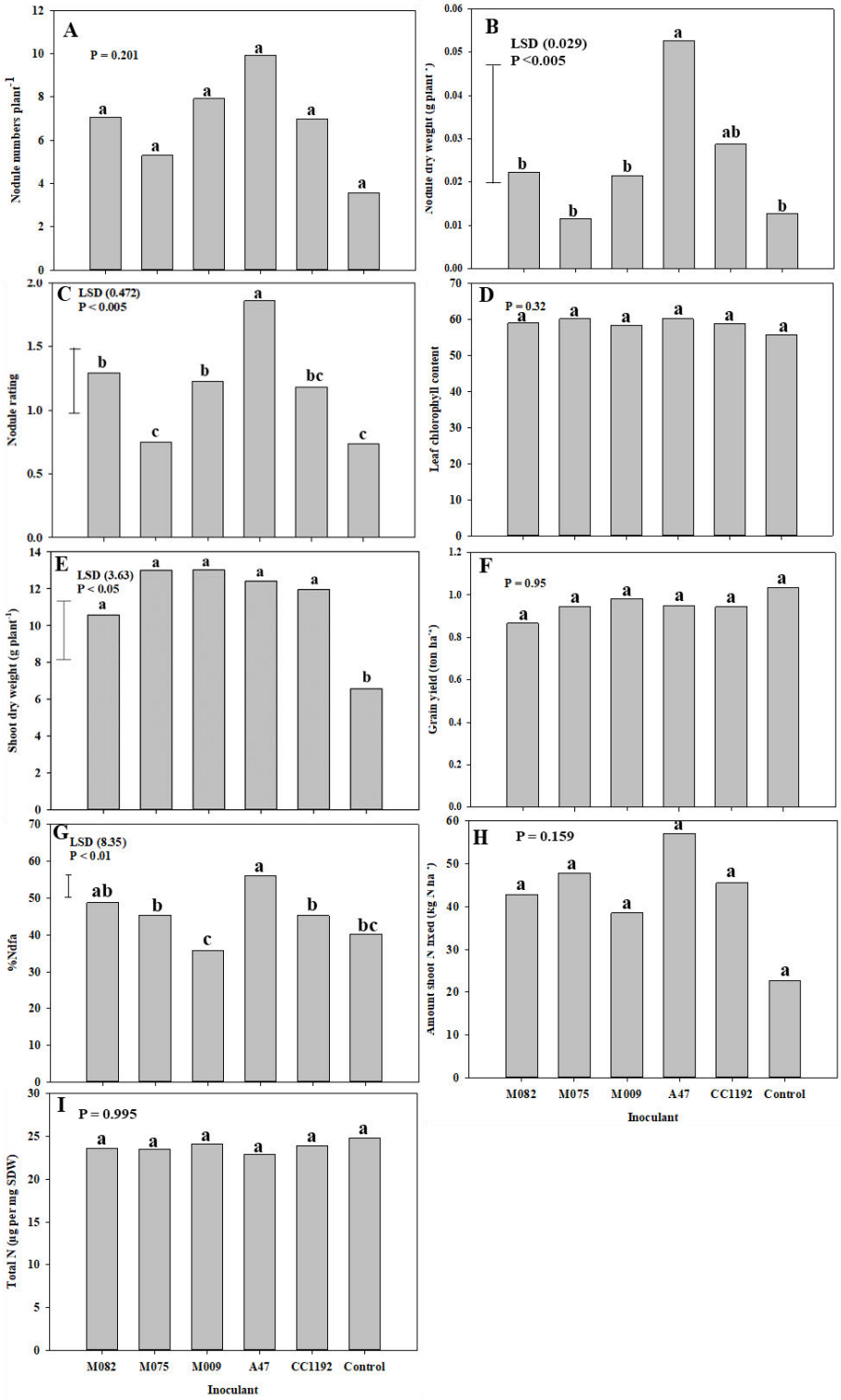

Figure 4 Effect of rhizobial inoculation on (A) nodule numbers, (B) nodule dry weight, (C) nodule rating, (D) shoot dry weight, (E) leaf chlorophyll content, (F) grain yield, (G) %N derived from atmosphere (Ndfa), (H) amount of shoot N fixed of chickpea (HatTrick variety) and (I) total N in shoot dry weight under field conditions at Angas Valley, South Australia, 2020. Note: % 15N of test and reference plants, total N and B value are presented in table S1.

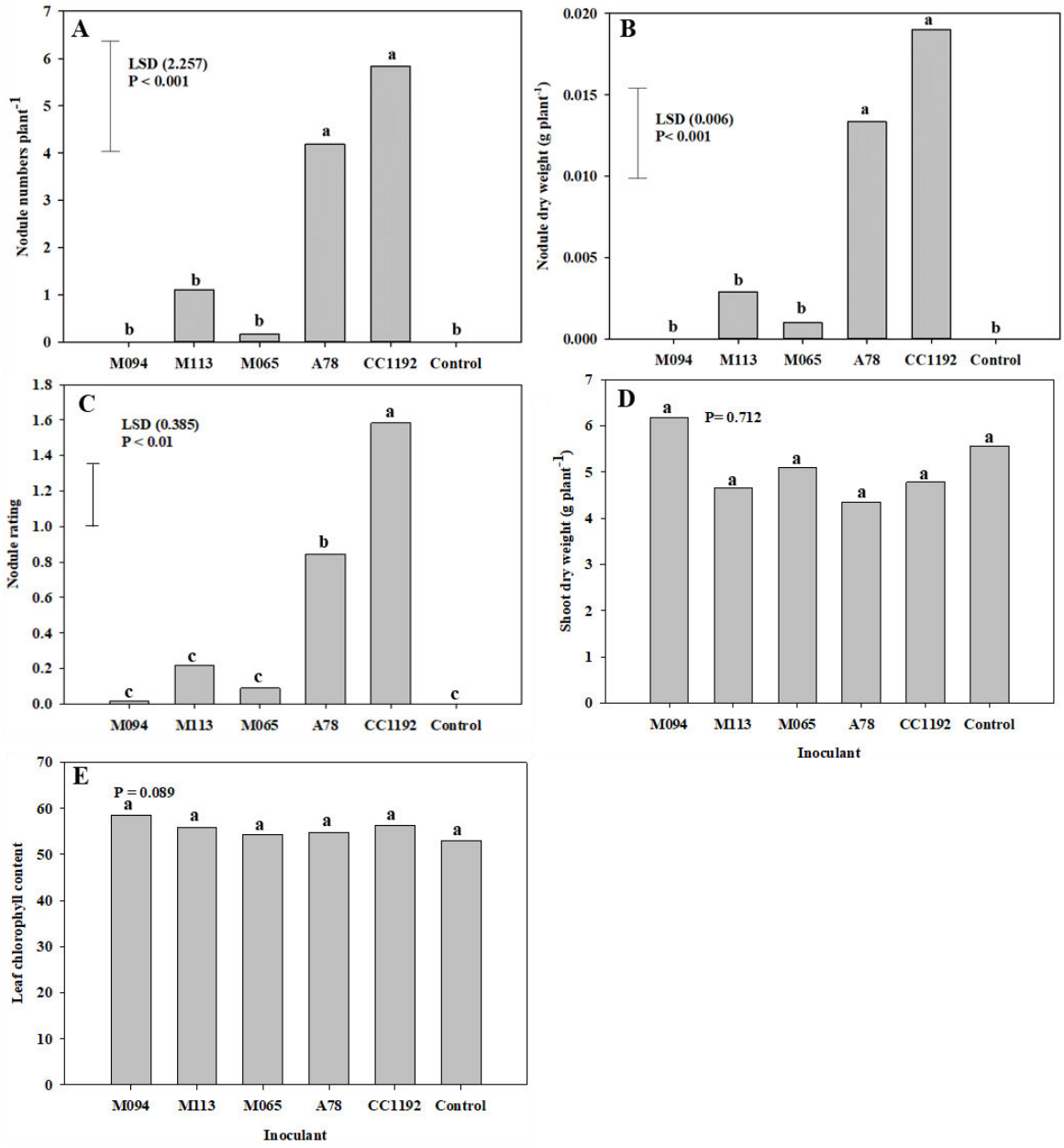

Figure 5 Effect of selected acid tolerant strains on (A) nodule numbers, (B) nodule dry weight and (C) shoot dry weight and (D) leaf chlorophyll content of chickpea (Hattrick variety) in acidic soil at Kapunda, South Australia, 2020.

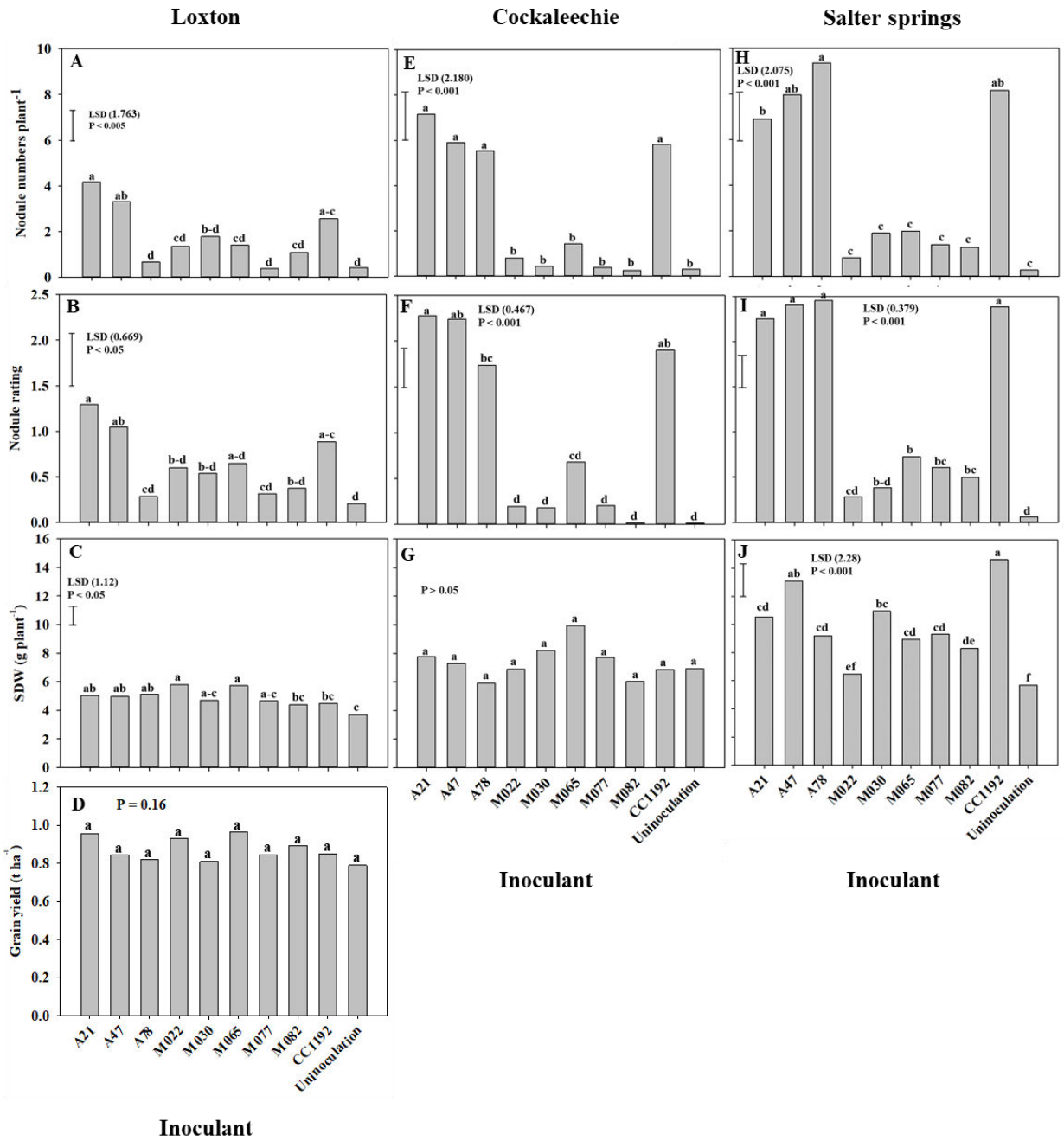

Figure 6 Effect of inoculation with nine selected rhizobial strains on nodule numbers, nodule rating, SDW and grain yield of chickpea (cv. PBA Slasher) in three field experiments at (A-D) Loxton, (E-G) Cockaleechie and (H-J) Salter Springs, 2021.

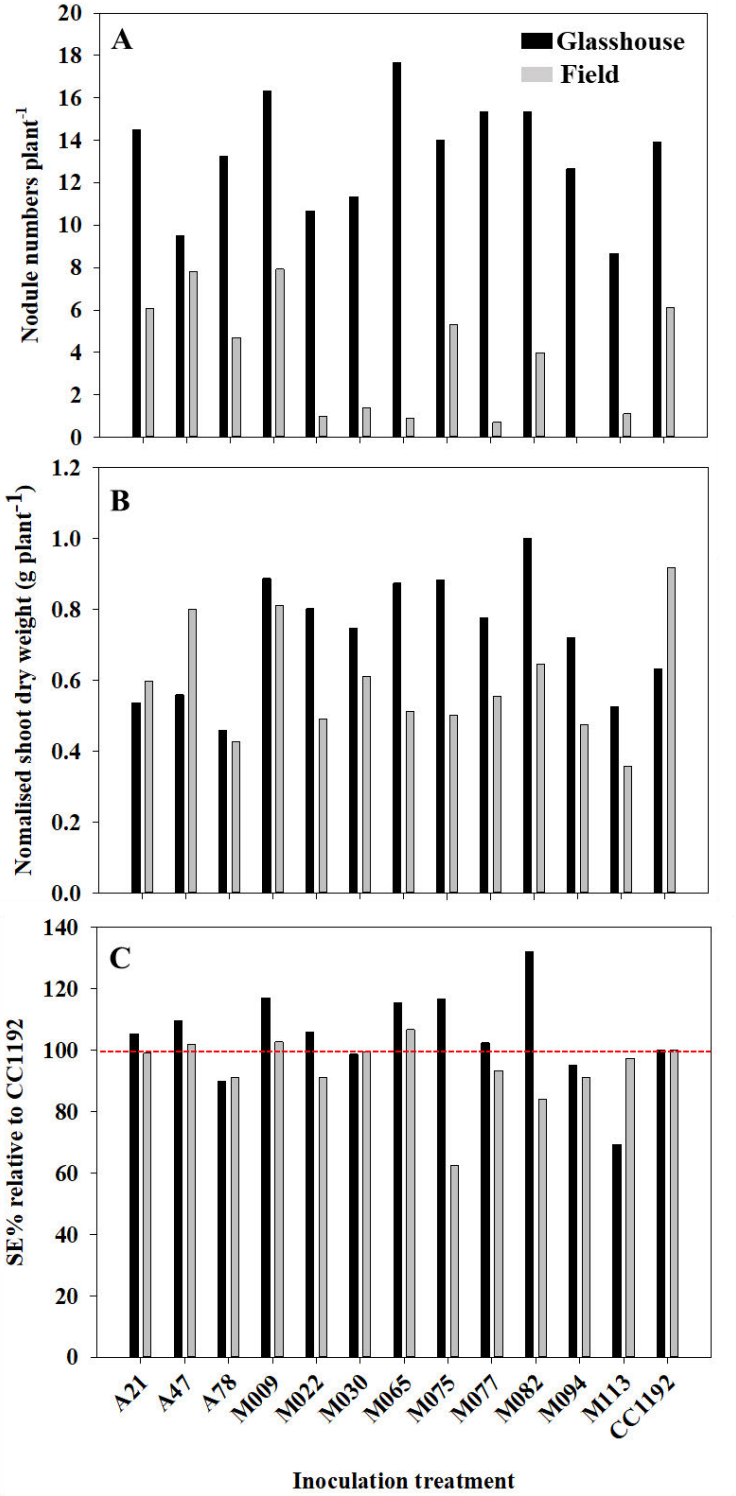

Figure 7 Comparison of strains' performance between glasshouse and field conditions in (A) nodule numbers, (B) shoot dry weight and (C) SE%. The SE% relative to CC1192 (positive check) were calculated independently for each experiment. The red-dotted line indicates the SE% similar with CC1192.

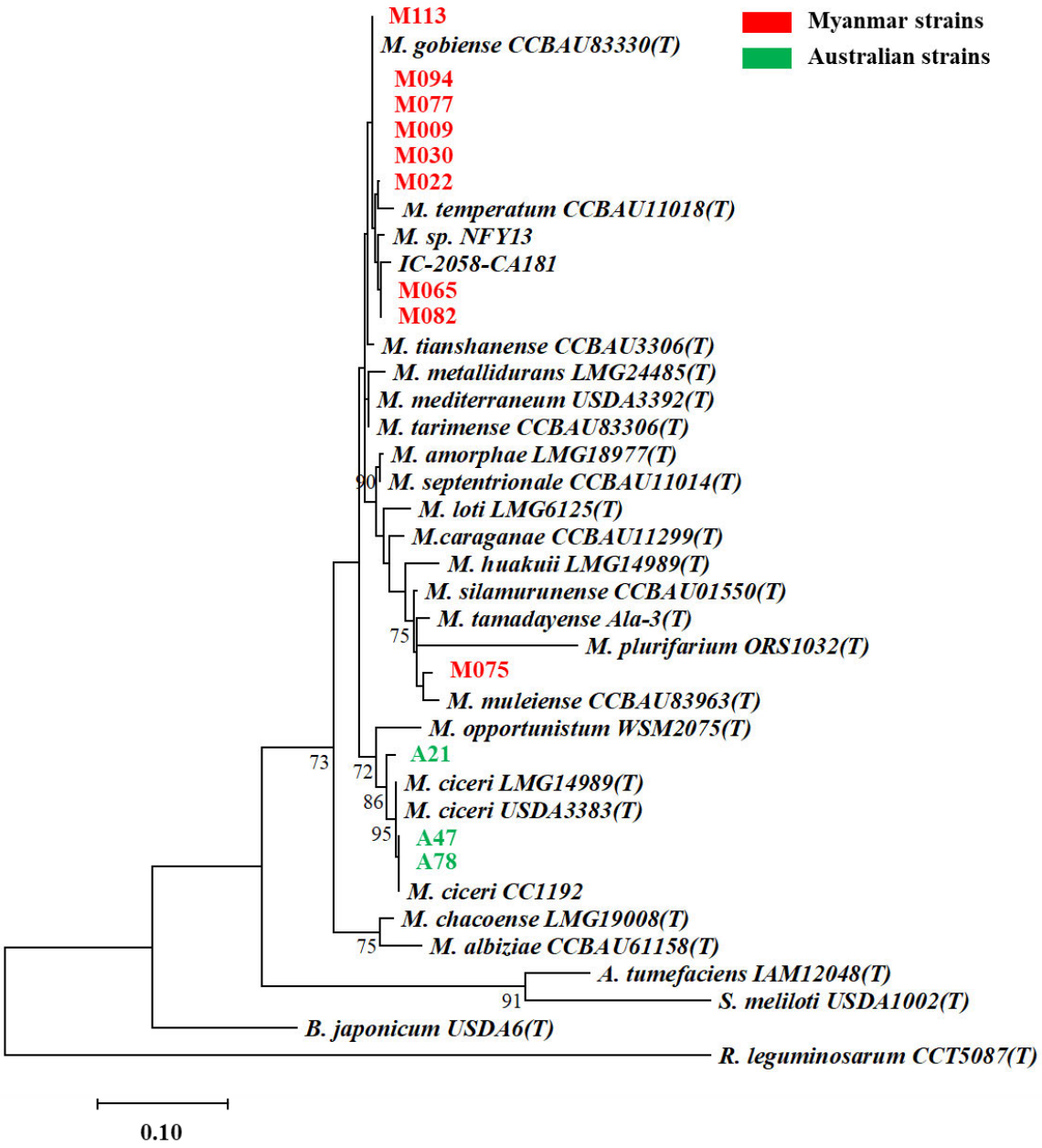

Figure 8. Phylogenetic analysis of 16S-23S rDNA IGS sequences of 12 chickpea rhizobial strains selected for field evaluation, CC1192 and reference strains. Bootstrap values were computed based on 1000 replications. Bootstrap values less than 70% were omitted, the scale bar (0.10) indicates the percentage of nucleotide substitutions per site. *A, Agrobacterium, B, Bradyrhizobium, M, Mesorhizobium, S, Sinorhizobium, R, Rhizobium.*

Table S1 Nitrogen fixation data of inoculated chickpea and reference (canola) plants

Strain	Rep	$o^{15}N$ air %	Total N (ug/mg)	%Ndfa	SDW (g/plant)	Nfixed (ug/mg)	Nfixed (ug/plant)	Nfixed (kg/ha)
M082	1	1.1295283	20.36539444	50.64439	8.34	10.31392968	86018.1735	30.10636
M082	2	1.2948658	25.88218246	47.24253	12.11	12.22739843	148073.7949	51.82583
M082	3	1.243271	24.48937925	48.30411	11.2625	11.82937602	133228.3474	46.62992
M075	1	1.2405368	23.42949483	48.36036	11.96125	11.33058899	135528.0076	47.4348
M075	2	1.6484048	19.37666949	39.96839	15.22875	7.744542087	117939.6953	41.27889
M075	3	1.2620055	27.67704068	47.91864	11.84375	13.26246165	157077.2802	54.97705
M009	1	1.8738215	22.69584706	35.33039	16.27625	8.01853031	130511.604	45.67906
M009	2	2.0977324	26.80339038	30.72337	12.415	8.234904123	102236.3347	35.78272
M009	3	1.5864119	22.76137	41.2439	10.36375	9.387677667	97291.54442	34.05204
A47	1	0.4455524	29.46889231	64.71735	12.59875	19.07148643	240276.8897	84.09691
A47	2	1.2148071	21.86675965	48.88976	12.62375	10.69060599	134955.5374	47.23444
A47	3	0.9357894	17.33553571	54.63061	12.01	9.470509231	113740.8159	39.80929
CC1192	1	1.5996503	23.33905926	40.97152	8.7825	9.562367614	83981.49357	29.39352
CC1192	2	1.5996503	25.66212857	40.97152	14.325	10.51416445	150615.4057	52.71539
CC1192	3	0.9721033	22.70468519	53.88345	12.73625	12.23406656	155816.1302	54.53565
Control	1	1.544695	28.35188621	42.10224	6.09625	11.93677893	72769.58856	25.46936
Control	2	1.6521262	20.94835893	39.89182	7.675	8.356681113	64137.52754	22.44813
control	3	1.7189146	24.99570741	38.51763	5.945	9.627754298	57236.9993	20.03295
Reference	1	4.7095930	35.0300765					
Reference	2	3.3243868	30.074939					
Reference	3	2.7388807	33.3713035					

Chapter 6. **General discussion**

6.1. Discussion of experimental results

The work in this thesis has contributed to a better understanding on the genetic and phenotypic diversity, and physiological attributes of rhizobial strains collected from the nodules of chickpea grown in soils sampled in Australia and Myanmar. An assessment of chickpea isolates was made that is more extensive than previous assessments, covering isolates across the major cropping regions of Australia. In addition, chickpea rhizobial isolates from Myanmar were assessed from the key cropping region of the CDZ, which has not been previously assessed to any great extent. Furthermore, isolates were tested genetically, physiologically and in multiple field environments.

Chickpea was historically considered as a highly specific host that could be nodulated by either *M. ciceri* or *M. mediterraneum* (Broughton and Perret 1999). However, a recent global collection of *Mesorhizobium* revealed 28 species that were classified as chickpea symbionts, including 20 previously undescribed species (Greenlon *et al.* 2019). In the present study, besides the cognate chickpea rhizobia (*M. ciceri*/*M. mediterraneum*), 16S-23S rRNA IGS sequences of isolated strains from Australia were also related to rarely isolated chickpea symbionts such as *M. temperatum*, *M. tianshanense*, *M. opportunistum, M. huakuii*, and *M. plurifarium* (Chapter 3). The mechanism of HGT of nodulation genes from an inoculant strain to other resident *Mesorhizobium* species may have allowed these species to nodulate chickpea in Australian soils (Sullivan *et al.* 1995; Nandasena *et al.* 2007; Andrews *et al.* 2018; Hill *et al.* 2020).

The integration of symbiosis islands from the inoculant strain to resident rhizobia through HGT may result in ineffective strains that are highly competitive for nodule occupancy. This has been observed for *Biserrula pelecinus* in Australia, reducing the efficiency of nodulation and

N fixation by the inoculant strain (Nandasena *et al.* 2007). In the present study, the incongruence between core (16S-23S rRNA IGS, *atpD*, *dnaJ* and *recA*) and symbiosis *(nodC* and *nifH*) gene-based phylogenies were observed in tested mesorhizobial strains (Chapter 3, 4), suggests the potential occurrence of HGT of symbiotic genes from inoculant strains into diverse resident *Mesorhizobium* species that have different genetic backgrounds (Andrews *et al.* 2018). It is also possible that depending on the chromosomal backgrounds of the receiving strains, HGT from the inoculant strain to the resident rhizobia may produce symbiosis of variable effectiveness with chickpea plants (Nandasena *et al.* 2007; Haskett *et al.* 2016). In this study, there was significant variation in SE% between the strains that could be explained, in part, by genetic relatedness and inoculation history.

The diversity and biogeography of the strains may be influenced by soil characteristics (pH), climate (temperature and rainfall), altitude and geology (Bala *et al.* 2003; Bontemps *et al.* 2010; Greenlon *et al.* 2019). In this study, most strains that were more tolerant to high temperatures *in vitro* were isolated from soils from Queensland (Chapter 3), which has warm to hot conditions in summer. More than 50% of Myanmar strains can grow at 40 ºC as they were isolated from hot regions in Myanmar (Chapter 4). Despite this, soil pH and total soil nutrient contents of sampling sites were not associated with the genetic distance of the strains, even though the soil pH of the original site is often related to the pH tolerance of rhizobial strains (Slattery *et al.* 2001; Shamseldin and Werner 2005; Lemaire *et al.* 2015). The poor association found between low pH tolerance and soil origin pH in the present study may have been due to limited variation in pH values (neutral to slightly alkaline) for all sampled soils from both Australia and Myanmar. There was no significant relationship between antibiotic resistance of strains and their geographical origins (Chapter 3), consistent with previous reports for chickpea rhizobia (Alexandre *et al.* 2006; Benjelloun *et al.* 2019).

Symbiotically effective strains may have descended from inoculant strains through a combination of mutation and/or natural selection over time in soil (Brockwell *et al.* 1995). Despite the genetic diversity among strains collected in this study, there was a positive correlation between strains with a similar 16S-23S rRNA IGS sequence to CC1192, and SE. The potential descendant strains mostly had equal or higher SE than the inoculant strain CC1192 (Chapter 3). Other studies have also shown that inoculation with strains distantly related to *M. ciceri* have equal to, or lower, SE with chickpea plants compared with inoculant strains more closely related to *M. ciceri* (Elias and Herridge 2015; Gunnabo *et al.* 2021). These findings suggest that genetic analysis and SE testing of rhizobia closely related to the inoculant strain may be a useful approach for selecting new strains with better adaptation and superior SE than the current commercial inoculant.

Inoculation history was associated with phylogenetic diversity of the re-isolated strains in this study. More than half of the Myanmar strains (68%), based on 16S-23S rRDA IGS, are genetically related to the Indian strain IC-2058 (CA-181), which is also most closely related to *M. gobiense* (Chapter 4). None of Myanmar strains were closely related to the cognate chickpea rhizobial species (*M. ciceri* /*M. mediterraneum*), possibly due to the lack of introduction of chickpea rhizobia through limited inoculation efforts in Myanmar. It is also possible that the Myanmar strains originally came from India with the historic introduction of chickpea or are resident soil strains. The symbiosis genes (*nodC, nifH)* of most Myanmar strains were in a slightly divergent group (*M. muleiense* and IC-2058-CA181) from Australian strains which are most related to *M. ciceri*. It is possible that the symbiosis genes found in Myanmar strains are slightly divergent from those of strains found in Australia where there has frequent inoculation, and the descendants of the inoculant strain were commonly found among re-isolated strains (Zaw *et al.* 2021). This discrepancy was also identified in a study on symbiosis genes of Ethiopian strains that although most strains were closely related to *M. ciceri*, some strains also

shared similar *nodC* and *nifH* sequences with *M. muleiense* (Muleta *et al.* 2022). In the present study, the divergence of symbiosis genes between the Australian and Myanmar strains indicates that the dispersal of symbiosis genes from a common symbiont seems to have been a historical event in Australia, which does not appear to have been repeated recently in Myanmar. It is also possible that symbiosis genes were either dispersed to the resident rhizobial strains via HGT (Sullivan *et al.* 1995; Hill *et al.* 2021) following original historic introduction of chickpea into Myanmar or dispersed more recently through introduction on the seed from neighbouring countries as found in recent phylogenetic diversity of peanut *Bradyrhizobium* species (Bouznif *et al.* 2019).

It is likely that diverse *Mesorhizobium* species found in Myanmar compared with Australia is not only due to inoculation history but also crop management, inputs and resident species that affect the soil chemistry (Hirsch 1996). In this study, three of the four main species groups of chickpea-nodulating rhizobia, *M. gobiense*, *M. huakuii* and *M. muleiense,* were distributed throughout Myanmar while *M. temperatum* was abundant elsewhere but absent in the southwestern area of Magway (Chapter 4). These four species were originally isolated from alkaline sandy desert soils in China (Chen *et al.* 1991; Gao *et al.* 2004; Han *et al.* 2008; Zhang *et al.* 2012). Soils in Magway are mostly sandy uplands, while Vertisols are most common with chickpea grown in rotation with rice in Sagaing and Mandalay. In Magway, the farming systems are mostly rainfed and do not include rice rotations, so they differ from ricelands in the irrigated lowland central regions of the CDZ. It is possible that the *Mesorhizobium* species found in Magway require specific adaptive traits like those of species found in Northern China, while those in heavy Vertisols mostly found in Mandalay and Sagaing have different and less stressful conditions, which support better survival and persistence of *Mesorhizobium* species (Greenlon *et al.* 2019). A study in China showed that the population composition of *M. muleiense* shifted with changing soil nutrient and pH conditions over time (Zhang *et al.* 2018).

Mutation (substitution) or deletion of critical amino acids in the symbiosis genes may result the significant changes in the nodulation phenotypes and the loss of nodulation ability (Göttfert *et al.* 1989; Krause *et al.* 2002; Arashida *et al.* 2021; Ratu *et al.* 2021). The *nod* gene mutant strain of *Rhizobium* produced lower nodule numbers than non-mutant strains, and these nodules failed to fix nitrogen (Burn 1987; Burn *et al.* 1989). In this study, there was no significant effect of amino acid substitutions in *nodC* sequences of Myanmar strains on either symbiotic effectiveness or nodule number per plant, even with a major amino acid change of phenylalanine to valine compared to the *nodC* protein of *M. ciceri* CC1192 (Chapter 4). It is possible that the heavy selection pressure based on annual plantings of chickpea in Myanmar would facilitate the maintenance of symbiotic integrity of nodulation genes even in the absence of HGT from a regularly applied commercial inoculant. Even though the transfer of symbiosis genes to native Myanmar strains was most likely historic and rare, there has been very little divergence relative to the symbiosis genes of inoculant species such as *M. ciceri*, and there is high nodulation of chickpea crops in Myanmar soil, despite the lack of widespread inoculation.

In this study, the physiological attributes of rhizobial strains were more likely associated with local soil environments than their genetic relatedness (Chapter 4). *M. gobiense* and *M. tianshanense* were previously described as acid sensitive strains, and about 50% of the *Mesorhizobium* type strains including *M. ciceri* were tolerant to acidic pH (Laranjo and Oliveira 2011), but the physiological attributes of rhizobial strains in our study did not cluster according to the classification of the type strains. Acid tolerance was not a characteristic detected in many of the strains from Myanmar or Australia, possibly because all strains were isolated from soils with neutral to high pH.

Based on the above experiments, 12 elite strains that were symbiotically effective for chickpea in pot experiments were selected. These 12 strains have the potential to improve chickpea

productivity, particularly for low pH soils. These strains were evaluated in five separate field experiments in the 2020 and 2021 growing seasons (Chapter 5). We hypothesised that the re-isolated strains may have greater symbiotic effectiveness and better adaptation to low pH soils in Australia than the current inoculant strain CC1192. Under field conditions, Australian strains were vastly superior in nodulation efficiency compared with Myanmar strains, however in most cases they did not perform better than the commercial inoculant. Strain A47 improved symbiotic N_2 fixation and strain A78 is adapted to strongly acidic conditions, and these strains should be evaluated in additional environments to test these traits further.

The selection of symbiotically effective strains with higher survival in desiccating environments is important for efficient nodulation and N_2 fixation under field conditions (Fletcher *et al.* 2015; Fletcher *et al.* 2016; Porqueddu *et al.* 2016; Hunt *et al.* 2019). In the field, the delay between inoculation and sowing may cause severe loss of viable rhizobia before seedling germination (O'Hara *et al.* 2002). In this study, a strong positive association was found between survival of rhizobia on seed and nodulation (Chapter 5). Generally, Australian strains, including CC1192, showed higher survival on seed within 3 to 24 hours after inoculation better nodulation in the fields compared with Myanmar strains, indicating that Myanmar strains had low competency for nodulation in Australian soils through a lack of adaptation to Australian conditions. Besides abiotic factors, such as desiccation, temperature and humidity, the inoculated strains may also have to deal with biotic factors such as competitive and antagonistic soil microbes (Bottomley 1992; Jack *et al.* 2019). The critical step in successful nodulation under stress is most likely to be survival of strains as free-living bacteria in the rhizosphere. The communication between rhizobia and legumes in the rhizosphere is crucial to this process (Munns 1965; Robson and Loneragan 1970), which is required for the successful entry of rhizobia into root (Raaijmakers *et al.* 2009; Saeki 2011).

In this study, two Australian strains (A21 and A47) and CC1192 showed consistency in effective nodulation and SDW accumulation across multiple field sites. The poor field performance of the Myanmar strains was in direct contrast with the SE under controlled conditions. However, two strains (M022 and M065) provided significantly higher SDW than CC1192 on dry and high pH sandy soil at the Loxton site, suggesting these two Myanmar strains may have adaptation to high temperatures and drought in soils. Importantly, improved chickpea nodulation and yield requires that competitive strains can tolerate local soil conditions and environmental stresses (Romdhane *et al.* 2008).

In acidic soils, root and shoot growth may be inhibited by the high Al concentrations (Kochian *et al.* 2005; Ryan 2018), which limit the elongation of roots by rapidly damaging the cells in the root cap (Ryan *et al.* 1993). In this study, most strains, except for A78 and CC1192, had compromised nodulation when grown on strongly acid soil (pH 4.18) at Kapunda. This site received poor agronomic management particularly lack of proper weed control and has much higher soil nitrate N concentrations than other sites, which would likely have limited nodulation. Soil nitrate concentrations of 80 mg N kg^{-1}, compared with those of 20 mg N kg^{-1} reduced the nodulation and N fixation in chickpea (Evans 1982). Additionally, nodule initiation of soybean was delayed by soil nitrate concentration of 30 mg N kg^{-1}, with nodule development prevented until 42 days after sowing (Herridge *et al.* 1984). It is also possible that among our potential test strains, only A78 and CC1192 are strongly adapted to acidic conditions and also high soil nitrate and these strains were able to survive and nodulate chickpea under stressful environments. Selected acid tolerant strains were previously shown in field experiments to increase nodulation when used in mildly acid soil, compared with no inoculation and with acid sensitive strains (Denton *et al.* 2007).

In summary, the selection of effective rhizobial strains for chickpea with better adaptive traits to specific regions is crucial to optimise the chickpea productivity. The Australian strain A47 provided greater N fixation and could be considered potential strain to use as alternative inoculant for Australian chickpea production, following further testing. Even with low nodulation, two Myanmar strains (M022 and M065) showed some potential and produced effective SDW on high pH soil at Loxton. Two strains; A21 and A47 performed equivalently with CC1192 in nodulation in both pot and field experiments and could be considered as suitable candidate strains for acidic soils in Australia following additional assessment. Improved inoculants will likely arise from the combined efforts to understand genetic and physiological analysis, with screening in both controlled and field environments.

6.2. References

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Appendices

YMA medium

Chemical	**Quantity (g L^{-1})**
Yeast extract	0.5
Mannitol	2.5
L-glutamic acid Na	0.5
$Na_2HPO_4.2H_2O$	0.36
$MgSO_4.7H_2O$	0.1
$CaCl_2$	0.04
$FeCl_3$	0.004
Agar	12

Congo red: 10 mL (0.0025 g/L)

The pH is adjusted to 7.0 and autoclaved for 20 min at 121 ºC. YMB is prepared using the same ingredients excluding agar.

McKnight's nutrient solution (McKnight et al. 1949)

Chemical	Quantity (per 0.25 L)
$CaSO_4.2H_2O$	6.75 g
$MgSO_4.7H_2O$	1 g
KH_2PO_4	1 g
KCl	1.5 g
A-Z trace elements*	5 mL
D solution**	5 mL
Distilled water	250 mL

*A – Z trace elements (2.86g H_3BO_3, 2.08 g $MnSO_4.7H_2O$, 0.22 g $ZnSO_4.7H_2O$, 0.079 g $CuSO_4.5H_2O$, 0.09 g $H_2MoO_4.H_2O$ L^{-1}).

**D solution is 10 g $FeCl_3$ L^{-1}.