

Variation in morphology, salinity and waterlogging tolerance, and resource allocation in strawberry clover (*Trifolium fragiferum* L.); implications for its use in mildly saline soils in southern Australian farming systems.

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## SUMMARY

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In southern Australian farming systems the replacement of deep-rooted perennial native vegetation with shallow-rooted annual crops and pastures has resulted in rising groundwater tables and an increased incidence of dryland salinity. It has been suggested that to address this issue by restoring hydrological balance, large areas of agricultural land need to be vegetated with perennial plants. One of the most agriculturally productive ways to do this is to introduce perennial pastures, both into upslope groundwater “recharge” areas and low-lying waterlogging and salinity prone “discharge” areas. This thesis examines the perennial legume strawberry clover (*Trifolium fragiferum* L.) in respect to variability of available germplasm, adaptive strategies and growth habits, and tolerance to waterlogging and salinity. The aim of this research was to gain insight into the suitability of strawberry clover for further development for broader use in discharge areas in southern Australian farming systems.

Genetic variation within and between naturalised populations of strawberry clover cv. Palestine collected from five locations in Western Australia was studied in a common garden experiment. Plants from seed collected at each site were grown as spaced plants and 13 morphological traits were measured. Within-population variation was very high presumably due to the outcrossing nature of strawberry clover. In contrast, between-population variation was generally low with most traits showing 8 – 15 % of the total variation. However, all populations were different from each other. It was concluded that although originally sown as the same cultivar, each of the populations had begun to differentiate into ecotypes more suited to the habitats into which they had been sown.

In a similar experiment, the genetic variation within and between eight populations of strawberry clover collected from the Mediterranean Basin and three commercial cultivars was studied. Results were similar to those from the naturalised Western Australian populations. The proportion of between-population variation was a little higher than for the Western Australian populations, ranging from 6 – 40 % of total variation. Variation in the commercial cultivars was similar to variation in the populations from the Mediterranean Basin. From these two studies it was concluded that the high level of variation contained in populations of strawberry clover indicates

ample opportunity to use selective breeding to improve upon current strawberry clover cultivars.

Salinity and waterlogging often occur together in discharge areas in southern Australian farming systems and greatly reduce agricultural production. The response to salinity and waterlogging was compared between four populations of strawberry clover and one population of lucerne (*Medicago sativa* L.) cv. Pioneer L69. There was a significant interaction between population, salinity and waterlogging for root porosity, tap root length, leaf area, root dry weight, shoot dry weight and root-shoot ratio. Waterlogging and salinity alone had little impact on lucerne shoot dry weight but decreased root dry weight. The response of the strawberry clover populations to waterlogging and salinity was variable; waterlogging alone had no impact or increased shoot (up to 125%) and root dry weight in three of the four populations, while salinity alone decreased shoot dry weight by up to 95% in three of the four populations. The combination of salinity and waterlogging magnified the negative effect of salinity alone for lucerne and two populations of strawberry clover while the two populations most negatively affected by salinity alone showed little additional impact from adding waterlogging. Root porosity was lowest in lucerne but the impact of treatment on porosity was extremely variable between strawberry clover populations. Root porosity was not found to be correlated with waterlogging tolerance within species. The differing responses of the strawberry clover populations would appear to indicate ample opportunity to improve tolerance to salinity and waterlogging through targeted selection.

Perennial pasture plants in southern Australian farming systems must possess mechanisms to enable them to survive hot dry summer months, such as extensive and deep rooting systems. Resource allocation patterns were therefore studied in the glasshouse for three species of perennial *Trifolium* and lucerne. Included in the study were two populations of strawberry clover from different environments, two populations of *T. uniflorum* L. from different environments (one from an alpine environment) and one population of a perennial alpine clover, *T. alpestre* L. Lucerne root systems were the deepest and most extensive, followed by the two populations of strawberry clover. The remaining three perennial *Trifolium* species exhibited much slower root growth and less extensive and shallower root systems. None of the populations matched the resource allocation or high productivity of lucerne. Populations from similar environments did not exhibit similar resource allocation

patterns; instead species was the greater determinant of resource allocation. It was concluded that while lucerne was relatively well-adapted to survival in southern Australian farming systems due to its quickly established deep root system, strawberry clover was likely to be less well-adapted but useful in niche areas. The remaining two species of perennial *Trifolium* were probably not adequately deep-rooted to survive summer drought.

Strawberry clover has previously been under-resourced for breeding and development activities. With its tolerance to waterlogging and mildly saline soils, improved cultivars of strawberry clover could prove to be a very useful plant in areas affected by these factors. This thesis has shown there is ample genetic variation in strawberry clover populations to improve agronomic performance as well as salinity and waterlogging tolerance, and its broad adaptability to southern Australian farming systems.

# TABLE OF CONTENTS

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Summary .....	3
Acknowledgements .....	7
Statement of Candidate Contribution .....	8
Thesis Related Publications .....	8
Chapter 1: General Introduction .....	9
Chapter 2: Literature Review .....	12
Chapter 3: Genetic Variation in Five Populations of Strawberry Clover ( <i>Trifolium fragiferum</i> L. cv. Palestine) in Western Australia .....	28
Chapter 4: Genetic Variation in Eight Populations of Strawberry Clover ( <i>Trifolium fragiferum</i> L.) Collected from the Mediterranean Basin Compared to 2 Commercial Cultivars .....	43
Chapter 5: The Response of Four Strawberry Clover ( <i>Trifolium fragiferum</i> L.) Populations and Lucerne cv. Pioneer L69 to Waterlogging and Salinity Under Glasshouse Conditions .....	59
Chapter 6: Resource Allocation and Growth Patterns in Three Species of Perennial <i>Trifolium</i> and Lucerne cv. Pioneer L69 Under Glasshouse Conditions .....	81
Chapter 7: General Discussion .....	104
References .....	115

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## STATEMENT OF CANDIDATE CONTRIBUTION

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I declare that all sources are acknowledged and that this thesis is my own composition and the result of my own research. In all chapters the ideas and concepts were generated by me under supervision.

## THESIS RELATED PUBLICATIONS

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McDonald KS, Cocks PS, Ewing ME (2005) Genetic variation in five populations of strawberry clover (*Trifolium fragiferum* cv. Palestine) in Western Australia. *Australian Journal of Experimental Agriculture* **45**, 1445 – 1451.



# CHAPTER 1

## General Introduction

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Dryland secondary salinity is a major issue affecting Australia's southern agricultural regions. Its expansion has been caused by rising water tables resulting from the inability of the predominantly annual plant-based farming systems to use all the incident rain. The redesign of farming systems around a greater perennial plant component to mimic the water use of the original native perennial vegetation would aid in restoring the water balance and ameliorate the growing salinity problem (Cransberg and Macfarlane 1994). The best way to do this in the extensive and dryland crop-dominated regions is to replace annual plant-based pastures with perennial plant-based pastures in a phase farming system. That is, a system where several years of perennial based pasture are followed by several years of a cropping rotation (Cocks 2001). Perennials that are also legumes would be of most value in these systems due to the nitrogen contribution they provide to following crops. They also provide the opportunity for reduced weed constraints in following crops due to control measures imposed during the pasture phase. There is an opportunity to introduce perennial pastures into areas where cropping is unsuitable, such as areas that are waterlogged and mildly saline. At present there are few perennial options for these areas. Indeed, little is known of the ecology and ecophysiology of the many perennial legumes that may be of use in new farming systems.

The overall aim of the study was to use strawberry clover as a case study to explore the variation, adaptability and growth of perennial legumes. It was hoped to highlight the importance of understanding these factors when selecting target species and source locations for the collection of new genetic material for breeding programs. It was also hoped to highlight the potential of strawberry clover for further cultivar development so as to be able to include it in the array of perennial options that might be available to farmers to increase the productivity of marginal soils and improve the sustainability of southern Australian farming systems.

Strawberry clover is an outcrossing perennial stoloniferous legume that is widely distributed throughout the world and is considered a species with special application to saline and waterlogging prone areas that occur in many parts of Australia, including the

south-west (Craig 1994). A high degree of genetic variation is an important characteristic of pastures targeted for use in climatically variable environments such as those experienced in southern Australia (Cocks 2001). It is also useful as a basis for further selection and breeding of new, better performing cultivars. The presence of long-term established pastures of strawberry clover in a range of environments in Western Australia presents the opportunity to investigate the genetic variation, as inferred from the morphological variation, present in these populations and the adaptability of this species to these environments. This is a major focus of this thesis (Chapter 3). The variation in accessions of strawberry clover collected from its native environment in the Mediterranean Basin is also investigated and compared to that in the Western Australian populations (Chapter 4).

Improvement in plant production on mildly saline and waterlogged agricultural land is not only economically important but will aid in controlling recharge to water tables in these areas. Strawberry clover has consistently demonstrated superior performance over other clovers in saline environments (Craig 1994) and improvements in its tolerance to waterlogging and salinity would greatly increase its value in these areas. In Chapter 5 the variability between populations of strawberry clover from Western Australia and the Mediterranean in their tolerance to waterlogging, salinity and waterlogging and salinity combined is investigated. Demonstration of the presence and extent of variation in these characters would enable selection and breeding of genetic material with improved production and could widen the use of strawberry clover.

Perennial pastures that will grow and survive in southern Australian farming systems must possess adaptations to the abiotic stresses prevalent in these systems. Even in areas suffering waterlogging and mild salinity during winter, a major factor affecting perennial survival is likely to be summer drought, especially in the Mediterranean climate of south-west Western Australia. Among the adaptations for drought survival is the production of roots that can access water deep in the soil profile (Asch *et al.* 2005; Yu *et al.* 2007). In Chapter 6 results are reported from a glasshouse experiment which involved comparing the root growth of 3 species of perennial *Trifolium* with lucerne, the only perennial legume currently widely used in the medium – low rainfall zones of southern Australia. Shoot growth, leaf area, reproductive biomass and root-shoot and reproductive-vegetative ratios were also measured to gain a thorough understanding of the growth of perennial clovers as compared to lucerne. The perennial clovers were

selected from contrasting environments including coastal versus alpine and Mediterranean versus Australian to allow investigation of whether species or environment determines perennial growth patterns. This knowledge could be invaluable when selecting potential germplasm for future cultivar development.

In Chapter 7 the results of all experimental chapters are discussed in context of the potential for further development of strawberry clover for use on mildly saline soils in southern Australian farming systems and the usefulness of increased resources being used for breeding improved cultivars.

## CHAPTER 2

### Literature Review

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This review commences with a brief description of the process of secondary salinisation of a landscape and the impact of rising saline groundwater tables on southern Australian farming systems. The review will highlight the role of perennial pastures in mitigating the productivity and sustainability issues associated with this increasing problem and the current lack of a suite of perennial pasture options for farmers. The growth and adaptations, including resource allocation patterns, necessary for plants to survive and be productive in southern Australian farming systems will be discussed as will the role of genetic variation in conferring success upon a species. Finally, the perennial pasture legume strawberry clover (*Trifolium fragiferum* L.) will be described and its potential usefulness in southern Australian farming systems will be discussed in the context of these issues.

#### **2.1 Dryland salinity in south-western Australia**

##### *Causes of dryland salinity*

Dryland salinity is regarded as one of the most serious forms of land degradation affecting Australia's agricultural regions. It is also one of the most expensive forms of land degradation to treat (Woods 1984). It is widely acknowledged that the cause of dryland salinity is the removal of the native perennial vegetation and its replacement with the autumn/winter-active annual crops and pastures that predominate in the current farming systems in southern Australia (Hatton *et al.* 2003; Williams 1991; Woods 1984). Annual plants are shallower-rooted and have a shorter growing season than perennial plants. This results in reduced evapotranspiration and, consequently, increased deep drainage and runoff (Hatton *et al.* 2003; Williams 1991), which lead to erosion and rising groundwater tables. As the groundwater tables rise, they mobilise the salts stored naturally in the deeper clays that, when brought close to the surface, are the cause of dryland salinity. Dryland salinity results in a loss of agricultural productivity, soil erosion and increased stream salinity (Woods 1984).

Areas where mobilised salts and groundwater tables are close to or at the soil surface are known as “discharge areas” or zones. These areas are typically located low in the landscape on valley floors, but can also be found in isolated areas upslope, such as hillside seeps, where barriers or changes to the slope of the landscape force the groundwater to the surface. Areas where water can enter the groundwater systems are known as “recharge areas” – these are commonly located on the ridges and slopes of the landscape (Hatton *et al.* 2003). However, all parts of the landscape can contribute to recharge, including areas that are generally considered discharge areas.

### *Scope of the problem*

It has been estimated that over 4.7 million hectares of land in Australia are currently affected by dryland salinity (Dolling *et al.* 2001). Some 15 million hectares of the southern Australian wheatbelt has been estimated as being at risk of salinity, mainly in the Murray-Darling Basin and south-western Australia (PMSEIC 1999). In Western Australia, 1.1 million hectares of agricultural and public lands are severely affected by dryland salinity (McFarlane *et al.* 2004) and as much as 30% of the total area of agricultural land in the state could eventually be similarly affected. This will lead to a major decrease in agricultural production in these areas and a significant effect on the state’s economy.

There are also wider-ranging impacts of dryland salinity on the economy apart from the decrease in agricultural production. In 2001 the National Land and Water Resources Audit (Dolling *et al.* 2001) reported that there would be significant costs to the nation from damage to building, rail and road infrastructure, water quality issues, decreases in biodiversity and increased flooding risk caused by rising groundwater tables. As well as agricultural, environmental and infrastructure costs arising from dryland salinity there are also significant social costs to rural communities already struggling to maintain people and services (Pannell 2001). With such wide-ranging impacts, implementation of strategies to contain the spread of dryland salinity is clearly an urgent issue.

## **2.2 Perennial pastures as an option for the management of dryland salinity**

With the increased recognition that dryland salinity poses a major threat to the southern agricultural regions of Australia, the need to increase the use of perennial species in these farming systems to mimic the more stable natural ecosystems that were present on the land prior to European settlement has been highlighted (Cocks 2003; Cransberg and Macfarlane 1994; Dear *et al.* 2003). Perennial pastures such as lucerne (*Medicago sativa* L.) use more water than annual crops and pastures and can reduce recharge of the groundwater table and help prevent rising saline groundwater tables (Latta *et al.* 2001). Lucerne is widely used in south-eastern Australia but is only a relatively recent inclusion in dryland farming in Western Australia. Adoption of lucerne is restricted by its poor adaptation to low pH and aluminium toxic soils and poor tolerance to waterlogging and inundation (Humphries and Auricht 2001). It is also unwise to rely on one species and an increase in the diversity of perennial pasture species will reduce the susceptibility of farming systems to devastation from pests and disease and the vagaries of a variable climate (Cocks 2001; Dear *et al.* 2003). Indeed, the Australian lucerne industry suffered just such devastation in the 1970's when much of the area sown to lucerne was devastated due to lucerne-eating aphids (Cocks 2001).

Perennial legumes offer additional benefits to farmers than perennial herbs and grasses through their ability to fix nitrogen, which is then available to crops grown in rotation with, or as companions to, perennial pastures. However, there are few perennial legume pasture options available to southern Australian wheatbelt farmers and research into the growth and ecology of potential perennial pasture species has been neglected in favour of research into the more traditional annual agricultural plants. Therefore we have little understanding of how perennial pasture legumes may grow and survive in southern Australian farming systems. One perennial legume genus that has had some research undertaken into its ecology and agronomy is *Dorycnium* (Bell *et al.* 2008). Bell *et al.* (2008) highlighted that while *D. hirsutum* L. did show some desirable traits, notably, good drought tolerance, forage quality and establishment reliability were major limitations to its use. They concluded that further research and development would be required to develop *Dorycnium* species as forage plants for agriculture. A series of trials investigating the adaptability of a number of perennial legumes, herbs and grasses across southern Australia have recently concluded (Nichols *et al.* 2008; Nie *et al.* 2008;

Reed *et al.* 2008) and so far few legumes in these trials have come close to matching the persistence and biomass production of lucerne (Dear *et al.* 2008). Better performing species have been targeted from these results for further selection and development.

Research into the development of new perennial legumes in Australia is divided between two groups of species; species targeted for recharge areas and species targeted for discharge areas. Currently, species used commercially for discharge areas consist of annual legumes such as balansa clover (*Trifolium michelianum* L.), perennial grasses such as puccinellia (*Puccinellia ciliata* Bor.) and halophytes such as saltbushes (*Atriplex* spp.) (Barrett-Lennard 2003a). There are very few current commercial perennial legume options for discharge areas: strawberry clover is one.

#### *Attributes of perennial legumes required for survival in southern Australian farming systems*

The survival of perennial legumes in southern Australian farming systems is dependant on their ability to resist drought (Cocks 2001). This is particularly important in the large areas of the agricultural zones that receive minimal summer rainfall and can regularly experience up to 6 months of summer drought conditions, i.e. those areas with a Mediterranean climate. Plants can escape the effects of drought by either avoiding it or being tolerant of it. A deep root system has been suggested as important for survival of a plant in drought conditions (Cocks 2001; Dear *et al.* 2003; Humphries and Auricht 2001). Deep-rooted species have the ability to access water from deeper in the soil profile than shallower rooted species. Allocation of root biomass to deep horizons and, in particular, to the tap root, had been suggested as the reason some common bean genotypes are better adapted to drought environments (Ho *et al.* 2004; Ho *et al.* 2005). Thus the ability to quickly form an extensive, deep rooting system is likely to be an important adaptation for survival of perennials in southern Australian farming systems. Indeed, in a study of root morphologies of two Australian native perennial legumes (*Kennedia prorepens* F.Muell. and *Lotus australis* Andrews.), Denton *et al.* (2006) found that while the native legumes had a lower root mass ratio than lucerne cv. Hunterfield, they had a more even distribution of roots to 1 m depth and therefore a greater proportion of root biomass at depth. Denton *et al.* (2006) speculate that the extensive deep root systems of the native species may confer a greater ability to extract nutrients and water from the soil at depth and thus could be an important adaptation for

the survival of perennial legumes in the summer drought conditions often experienced in southern Australian farming systems.

Perennial legumes may also resist drought by becoming dormant or may reduce their use of water by closing stomata and/or dropping leaves (Cocks 2001). For example, lucerne, in the absence of access to subterranean water tables, responds to water stress by reducing stomatal conductance to less than 70% (Irigoyen *et al.* 1992) and in extreme conditions will drop its leaves. *Dorycnium hirsutum* L. will also drop leaves when exposed to water limiting conditions at similar levels of water stress (Bell *et al.* 2007). Leaf hairs may reduce water loss and increase reflection of radiation and so decrease leaf temperature (Karabourniotis *et al.* 1995). *D. hirsutum* has hairy leaves and this may aid its survival in drought susceptible environments (Bell *et al.* 2007).

To survive and persist in southern Australian farming systems, perennial legumes also need to be tolerant of grazing. Perennials in these farming systems are likely to be subject to a range of grazing management regimes and stock classes, and those plants, such as lucerne, that require specialised grazing management such as strict rotational grazing, are unlikely to be as widely adopted by wheatbelt farmers as a more tolerant plant (Cocks 2001). Other factors such as climate and edaphic adaptation, biomass production and forage quality, particularly during the summer/autumn feed gap, ease of establishment and a low cost seed production system, winter activity and weed control options are all likely to be important in determining whether new perennial legumes will be adopted on a broad scale in southern Australian farming systems.

#### *Attributes of perennial pastures for the management of groundwater recharge*

One of the main inducements for farmers to adopt perennial pastures in southern Australia is their ability to contribute towards control of groundwater recharge. As recharge is generally a catchment-wide phenomenon, a high proportion of the farming landscape will need to be under perennial vegetation in order to substantially reduce recharge (Dear 2003). Perennial pastures such as lucerne, among others, can greatly reduce the amount of recharge to groundwater tables (Bell *et al.* 2006; Dolling *et al.* 2005; Fillery and Poulter 2006; Latta *et al.* 2001; Lolicato and Cook 1993; Verburg *et al.* 2007a; Ward and Micin 2006). The deep rooting habit of these plants aids in the extraction of water from soil depths that shallower-rooted annual plants would not be able to access. As most recharge occurs in the winter when rainfall exceeds



evapotranspiration potential, the main opportunity to reduce recharge is to dry the soil profile in late spring and maximise the dry soil “buffer” (depth of dry soil in the profile) before the opening rains in autumn (Dear 2003). The ability of perennial plants, such as lucerne, to utilise out-of-growing-season (i.e. summer) rainfall is also an attribute enabling them to use more water than annual plants, which are present only as a dormant seed bank at this time, and aid in the reduction of groundwater recharge (Verburg *et al.* 2007b).

#### *Perennial pastures on discharge sites*

There are about 4.7 million hectares of agricultural land in Australia currently affected by dryland salinity or at risk from shallow saline groundwater tables; this area is predicted to increase to 17 million hectares within 50 years (Dolling *et al.* 2001). Land affected by dryland salinity is often also affected by waterlogging. The agricultural productivity of these discharge areas is low, due to the combined effects of salinity and waterlogging making them unsuitable for cropping and reducing the productivity of pastures of conventional agricultural species to very low levels (Rogers *et al.* 2005). There is therefore a need to develop salt and waterlogging tolerant pasture species for discharge areas to improve pasture productivity and reduce recharge of the groundwater table and runoff of saline water into waterways from these areas, which are often currently poorly vegetated. Many saltland pastures currently consist of unmanaged unproductive suites of species including weeds, naturalised annual legumes and grasses such as barley grass (*Hordeum leporinum* Link.) on mildly saline areas and graduate to salt-tolerant halophytes and then bare ground as salinity levels increase. Managed saltland pastures, usually on the more mildly saline areas, generally consist of halophytic shrubs (i.e. saltbush), salt-tolerant perennial grasses such as *Puccinellia* species and tall wheat grass (*Thinopyrum ponticum* Podp.) and sometimes mildly salt-tolerant annual legumes such as balansa clover (*Trifolium michelianum* L.) (Barrett-Lennard 2003b). There are few perennial legumes that can be considered suited to growth in saline and waterlogged conditions but there is a range of legumes with some measure of variation in salinity and waterlogging tolerance (Rogers *et al.* 2005). There are several mechanisms of salt and waterlogging tolerance in plants that may be found in agriculturally useful species (Barrett-Lennard 2003a).

### *Plant adaptations to growth in saline and waterlogged conditions*

Plant adaptation to discharge areas is complicated by the need to possess both salt and waterlogging tolerance. For plant growth there may be a strong negative interaction between salinity and waterlogging (Barrett-Lennard 2003a). Research into the causes, consequences and implications of this interaction has recently been extensively reviewed (Barrett-Lennard 2003a) and its causes listed as increased  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations in the shoot and the impairment of root function. Barrett-Lennard (2003a) highlights that while evidence is sketchy, the literature on the topic does allude to several strategies that plants can adopt to improve the regulation of salt transport under saline waterlogged conditions. These include; firstly, the avoidance of hypoxia (waterlogging) in tissue through the formation of aerenchyma; secondly, the maintenance of regulation of ion uptake and transport through the formation of an endodermis; thirdly, the reduction of stomatal conductance and; fourthly, the protection of metabolism through the implementation of salt removal strategies such as salt glands.

Aerenchyma are gas filled spaces formed by the separation of cells (schizogeny) or breakdown of cells (lysigeny) forming a longitudinally interconnected system along the tissue axis, which provides a low resistance pathway for gaseous diffusion (Gibberd 1997). The volume of aerenchyma cells in the root system can be indicated by the root porosity, or proportion of root volume consisting of air spaces. The formation of aerenchyma, and therefore high root porosity, is considered an adaptation to waterlogged conditions as it may aid oxygen diffusion through the root (Armstrong *et al.* 1991; Colmer *et al.* 1998; Justin and Armstrong 1987; Thomson *et al.* 1990). Rogers and West (1993) investigated salinity and flooding interactions for two perennial and two annual species of *Trifolium* and found that  $\text{Na}^+$  and  $\text{Cl}^-$  concentrations were highest in the species whose growth was most depressed in saline, flooded conditions. They also observed that root porosity for each species was different in different treatments; strawberry clover had its highest porosity measured under saline, flooded conditions, as did white clover (*Trifolium repens* L.), while balansa clover had its highest root porosity when subjected to flooding alone and subterranean clover (*T. subterraneum* L.) when exposed to salinity alone. It is unclear whether high root porosity is an adaptation to saline conditions from these results. Extensive discussion on the role of aerenchyma and other possible mechanisms for salinity and waterlogging tolerance can be found in Barrett-Lennard (2003a) and Rogers *et al.* (2005).

Generally, legumes are less tolerant of salinity and waterlogging than grasses, and halophytic shrubs such as saltbush (*Atriplex* spp.) are much more tolerant of salinity than either grasses or legumes (Rogers *et al.* 2005). Variation in tolerance to salinity within legume species has been reported, including strawberry clover (*Trifolium fragiferum* L.) (Rumbaugh *et al.* 1993) and lucerne (Rogers 2001), as has variation in tolerance to waterlogging alone in perennial legumes such as *Lotus* spp. (Real *et al.* 2008; Teakle *et al.* 2006), *Melilotus* spp. (Rogers *et al.* 2008) and lucerne (Smethurst *et al.* 2005). Many of these studies have not investigated the mechanisms involved in these tolerances, only the variation as indicated by shoot and root growth.

Another issue for plants in salt-affected land is their tolerance of salt and waterlogging at germination. Germination is also a convenient time for testing large numbers of genotypes for salt and waterlogging tolerance but there is almost no correlation between genotypic differences in germination and later plant growth tolerances in a range of species (Rogers *et al.* 2005). Rogers *et al.* (2005) goes on to state that there is, in fact, little difference between species in their response to salinity at germination, in contrast to the variation found at later plant stages within and between species. This is because germination involves simple water relations that dominate the process of imbibition and many of the tolerance mechanisms to salinity and waterlogging take time to develop (Rogers *et al.* 2005). It is also likely that while at maturity, plants in discharge areas in southern Australia will be subject to both salinity and waterlogging together, at the time of germination this may not be the case. At many discharge sites high salinity without waterlogging may be common after the first rainfall event in autumn, before enough rainfall is received to result in waterlogging. Moreover, as sites become waterlogged with successive rainfall events, their salinity levels are likely to decrease as the salt is diluted by the increase in water or leached to below the root zone. Unfortunately, there is also little data from the field on this variability (Barrett-Lennard 2003a). However, even at low levels of salinity, as may occur later in the autumn-winter growth season, the combined effect of salt and waterlogging may produce a large decrease in plant growth (Barrett-Lennard 2003a; Barrett-Lennard *et al.* 1999).

### *Perennial pastures and future farming systems*

Perennial pastures will be a key feature of future farming systems in southern Australia which aim to control groundwater recharge and reduce the impact of dryland salinity on agricultural production. Lucerne is currently the most widely used perennial pasture in such systems, but there is a need to increase the species diversity and reduce reliance on lucerne alone and to find better-adapted species. Lucerne is not suited to growth in discharge areas where waterlogging is common, or to the highly acidic soils which predominate in the south-west of Australia. There is therefore a need to introduce new perennial legumes. However, to assess which new species will have the best chance of survival, a thorough understanding of the growth patterns and adaptive strategies of these plants is required.

### **2.3 Plant population survival in a variable environment**

Southern Australian farming systems must contend with a climatically variable environment (Cocks 2001; Dear 2003). The advent of climate change brings an increasing likelihood of a drier climate with greater extremes for the agricultural areas of southern Australia. Any plant introduced into a self-regenerating pasture in these environments will require a suite of adaptive traits, including genetic variation and the ability to utilise this to change and adapt and ensure population survival. A thorough knowledge of the growth and ecology of new perennial legumes will be a necessity in determining their likelihood for success in southern Australian farming systems. While the ecology of some herbaceous perennial legumes, such as the economically important species white clover and lucerne, have been extensively studied (Barrett and Silander 1992; Brougham *et al.* 1978; Fothergill *et al.* 1997; Hall *et al.* 1993; Hanson 1972; Leach 1978), little is known about the ecology of herbaceous perennial legumes in general. This is especially true of species from the Mediterranean basin and contrasts with our knowledge of the ecology and ecological genetics of Mediterranean annual legumes (Bennett 1998; Cocks 1992; 1993; Ehrman and Cocks 1990; Norman *et al.* 1998; Rossiter and Collins 1989a; 1989b). Some limited work has been attempted with other agriculturally important perennial legume species such as red clover (*Trifolium pratense* L.) (Quesenberry 1976; Taylor and Quesenberry 1996), strawberry clover (*Trifolium fragiferum* L.) (Hill and Luck 1991; McDonald *et al.* 2005) and *Lotus* species (Charlton 1989; Fraser *et al.* 1994; Sevilla *et al.* 1996), and most recently with the potentially useful canary clover (*Dorycnium* spp.) (Bell *et al.* 2008). However, apart

from the work described in the review of Bell *et al.* (2008), these studies are mostly specific in nature and do not cover the whole adaptive strategy and life cycle that allows the species to survive in their native habitats.

#### *Genetic change as part of the adaptive process*

Genetic variation in plant populations is very important for the continuing success of a species in a changing environment. Genetic variation enables populations to adapt to their surroundings during colonisation and subsequently when permanent, stable populations are established. It allows for a population to adapt and change with its habitat.

One way in which genetic variation is generated is through outcrossing. Cocks (1996) suggests that the capacity to outcross is important for the widespread establishment of volunteer species, including pasture legumes. Even the low levels of outcrossing present in some Mediterranean annuals, including subterranean clover, supplies sufficient genetic variation to allow populations to successfully adapt to variable environments (Cocks 1992; Nichols 2004). For example, populations where no more than two strains of subterranean clover were sown developed almost infinite genetic variation within 20 years, the original genotypes being replaced at a rate of about 1.5%/year (Cocks 1992; Rossiter and Collins 1989a; 1989b). Similar results for subterranean clover were also obtained by Nichols (2004) when investigating evolution in pastures over 16 years, where a mixture of 40 different strains of subterranean clover were sown in equal proportions at two sites. Most evolution occurred within three years of sowing due to elimination of poorly adapted genotypes. However, divergent strains, different to those originally sown, were also a significant part of the pasture.

White clover is an outcrossing (Thomas 1987) stoloniferous perennial legume, similar to strawberry clover. In a study on the genecology of permanent white clover pastures in New Zealand, differences between populations from 'wet' (> 1400 mm average annual rainfall) and 'dry' (< 760 mm average annual rainfall) farms were recorded in naturalised pastures that had not been oversown with white clover in the previous 10 years (Caradus *et al.* 1990). One of the main findings of the study was that populations from 'dry' farms were more upright and tap-rooted than populations from 'wet' farms. Dependence on tap roots is surely an adaptation to drier areas where plants must access sufficient water for survival. Unfortunately, Caradus *et al.* (1990) did not state the

origins of the germplasm at each site, that is, whether the naturalised populations were from a single introduction or sowing, or if they were genetically different in the first place. Another critical finding was that the study did not support the theory that early flowering is a feature of naturalised white clover populations adapted to dryland areas. However they did conclude that it would still be possible for plants from dryland areas to flower earlier due to a plastic response to moisture deficit, rather than because the plants were inherently earlier-flowering, but that there was still insufficient information to determine whether white clover populations from dry environments had greater plasticity for reproductive strategy than those from less stressful environments.

There are likely to be differences in the rate of genetic change between annual and perennial species, although this does not appear to have been quantified in any published studies. It would be expected that perennial plants would have a slower rate of change due to individual plants of perennial species living longer than those of annual species and perennial populations not being replenished with new genetic material through seedling recruitment to the same extent as for annual pastures. Indeed white clover has been found to have very low levels of seedling recruitment, with an average of 5.5 seedlings appearing per metre squared per year and only 4.4% of these seedlings surviving to form established plants (Chapman 1987). Seedling recruitment in stoloniferous perennials that can form dense “mats” of herbage, such as white clover, generally occurs on the site of small disturbances of ground in the pasture, from grazing or treading damage by stock, mole hills etc (Chapman 1987; Fothergill *et al.* 1997). However, even with such low levels of seedling recruitment, both Chapman (1987) and Fothergill *et al.* (1997) conclude that this process is likely to play an important ecological role in regeneration of the sward and in maintaining diversity within the pasture to allow adaptation to particular environments.

There has been little work with perennial legumes, apart from white clover, on the evolution of sown pastures, and whether there is sufficient genetic change to cause ecotypic differentiation in populations that started out genetically identical. In Western Australia, strawberry clover (*Trifolium fragiferum* cv. Palestine) has been sown in niche areas in the West Midlands, Great Southern and South Western regions. Strawberry clover is a predominantly outcrossing (Morley 1963; Wright 1964), stoloniferous perennial legume and these previously established pastures present the opportunity to

study the potential adaptation and genetic differentiation from a single genotype in an outcrossing, stoloniferous perennial legume.

### *Adaptive strategies*

Adaptation is defined by Harper (1977) as the features of organisms that improved the fitness of their ancestors and so were passed on to their descendants. Adaptive strategies are those characteristics that confer fitness in the present habitat of the organism. These strategies are reflected in the life histories of species. The life histories are the patterns of growth, reproduction and longevity that are related to specific demands for survival in a particular place at a particular time (Barbour *et al.* 1987). These patterns determine at what place and time a species successfully carries out the processes of germination, growth and reproduction. However, resource allocation to any of these processes can only occur within the confines of the genotype (Barbour *et al.* 1987).

Many of the existing studies on perennial legumes concentrate on their agronomic characteristics and do not consider perennial legumes in terms of the adaptive features they possess and the implications of these for ecological success in different or variable/changing environments. They often focus on one, or a few, particular traits, rather than putting together the whole life history and adaptation of the species. Studies of this type include those on breeding systems (Kalin 1981; Turkington and Cavers 1978), seed dormancy (Chapman and Anderson 1987; Fairey and Lefkovitch 1991; Hall *et al.* 1993), seedling recruitment (Barrett and Silander 1992; Chapman 1987; Ehrlén and Eriksson 1996; Fraser *et al.* 1994), emergence and persistence (Douglas *et al.* 1996; Forde *et al.* 1988; Fulkerson and Slack 1996; Sevilla *et al.* 1996; Sheaffer 1988), stolon (ramet) development (Clark and McFadden 1997; Hay *et al.* 1987; Jahufer *et al.* 1994), the genetic variation of populations (Caradus 1991; Caradus *et al.* 1990) and physiological ecology (Chapman *et al.* 1992; Taylor and Quesenberry 1996).

Genetic variation in these traits within species often occurs when the species is distributed over different environments and must find a way to survive in each environment. The formation of genetically different populations of the same species that are adapted to different environments is known as ecotypic differentiation, and was first defined by botanist Göte Turesson (Barbour *et al.* 1987). The life history traits that confer this “adaptability” are the “adaptive strategies” that a plant uses to survive in that

particular environment. Little is known about what set of adaptive strategies confer success on the survival of perennial legumes in their habitats of origin, how they interact with each other and the range of variability in these traits between and within species, let alone what set of strategies may confer success on species when sown in a variable discharge environment in a southern Australian agricultural system.

#### *Resource allocation*

There have been few studies on the resource allocation patterns of perennial legumes and these are generally interpreted in reference to agriculturally important trends, rather than the classification of life histories. Chapman *et al.* (1992) looked at physiological integration in white clover and noted that there was transfer of carbohydrates between parent plants and stolon branches. However, Chapman did not go on to highlight the ecological significance of this on the survival of the plant in that habitat. Other studies with white clover examined the allocation of resources to reproductive and vegetative structures (Turkington and Cavers 1978) and the genetic variation and tradeoffs that occur (Cain *et al.* 1995), as well as the effects of manipulating rooting on resource allocation to branches (Lotscher and Hay 1995). There have been few studies of resource allocation patterns in other perennial legumes (Bell 2005; Turkington and Cavers 1978), the variability of these patterns within the confines of different ecotypes, or of the ecological implications of any variability.

## **2.4 Strawberry clover – *Trifolium fragiferum* L.**

### *Taxonomy*

Strawberry clover is credited to the botanist Linnaeus and was included in his 1753 'Species Plantarum'. In the taxonomic revision of the genus *Trifolium*, Zohary and Heller (1984) categorise strawberry clover as follows:

Family:	<i>Fabaceae</i>
Tribe:	<i>Trifolieae</i>
Genus:	<i>Trifolium</i>
Section:	<i>Vesicaria</i> ( <i>T. fragiferum</i> L. is the lectotype for this Section)
Species:	<i>fragiferum</i>



An abbreviated description of strawberry clover giving the most important features from Zohary and Heller (1984) is as follows:

Perennial, branching from neck, with prostrate or creeping stocks, 5 – 50 cm long, rooting from nodes. Leaves with erect, hairy or glabrous petioles; stipules linear to lanceolate-linear, membranous, with acuminate or subulate free portion; leaflets ovate to obovate-elliptical, rarely ovate or suborbicular, cuneate at base, obtuse or retuse, spinulose-toothed, glaucous or glaucescent, glabrous or hairy along nerves and margin. Peduncles axillary, terete, furrowed, villous or glabrescent. Flowering heads hemispherical to ovoid, 10 – 30 flowered, subtended by a 2 – 6 cm long involucre made up of united, entire or toothed bracts of lower flowers and often concealing calyx tubes of latter. Flowers short pedicled, each subtended by entire or bifid, more or less ciliate bract. Calyx tubular-obovate, bilabiate, often reddish, mostly pilose to woolly on upper side, rarely all over; teeth unequal, lanceolate, the upper ones becoming subulate- aristate, erect or recurved, shorter than or almost as long as or longer than tube, the 3 lower ones somewhat broader and shorter than upper ones, often ciliate. Corolla whitish, pink or flesh coloured, considerably longer than calyx, marcescent in fruit, not resupinate (rarely slightly resupinate). Fruiting head ovoid or globular; fruiting calyx deflexed, inflated, reticulately nerved, pilose to hispid above or all over, with marcescent corolla largely or slightly protruding from calyx. Pod ovoid to globular, 1 – 2 seeded. Seeds reniform, brown.  $2n = 16$ .

#### *Centre of Origin*

Strawberry clover is assigned to the Eurosiberian and Mediterranean centres of diversity (Townsend 1985), but has been spread widely by man and has become naturalised in many countries. The general distribution of strawberry clover has been described by Zohary and Heller (1984) as: All European countries (including British Isles), West Asia (including West Siberia), all Mediterranean countries, Anatolia, Iraq, Caucasus, Iran, Afghanistan, Pakistan, Middle Asia, Turkistan; also recorded from Ethiopia and cultivated in some countries, especially in Australia. Its habitat is generally grassy and muddy places, sometimes on saline ground (Zohary and Heller 1984).

### *Breeding system*

Strawberry clover is a predominantly self-incompatible, cross-pollinated, diploid ( $2n = 16$ ) species (Morley 1963; Wright 1964). Ecotypes of Mediterranean origin tend to be self-incompatible whereas those from northern latitudes tend to be more self-compatible (Davies and Young 1966). In Australia strawberry clover is pollinated by honey bees and other species of native bees (Townsend 1985).

### *Strawberry clover in southern Australian farming systems*

Strawberry clover is considered tolerant of waterlogging and mild salinity (McGuinness 1991) and has been utilised widely in irrigated pastures in California and other parts of the United States (Townsend 1985). In dryland farming systems in south-eastern South Australia and south-western Victoria it is mostly utilised for the improvement of production in poorly drained, waterlogged and/or saline areas or on non-saline alkaline soils where the pH is too high (8.8 – 9.0, H<sub>2</sub>O) for white clover (Craig 1994; Gramshaw *et al.* 1988). However, in these farming systems, strawberry clover remains a niche species due to its requirement for some soil moisture throughout the year. In the irrigation districts of Victoria and New South Wales, interest in the species is largely in areas where the salt level of irrigation water is so high that it severely retards the growth of white clover (Craig 1994). Strawberry clover has also been sown in areas susceptible to sandplain seeps in the West Midlands region of Western Australia, and in areas subject to waterlogging and inundation in the high rainfall, sandy coastal plain of the south-west of Western Australia as a companion legume with the perennial grass kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.). Strawberry clover is tolerant of a wide range of soil types, growing on acid, sandy soils of pH 5.5 – 6.5 to peats and black clays with a pH of 7.0 – 9.0, and does well in slightly saline and swampy situations where white clover does not thrive (Oram 1990).

There are three certified cultivars of strawberry clover registered in Australia – Palestine, O'Connors and Shearmans (Oram 1990). Palestine is by far the most common and is used solely in agriculture while O'Connors is largely used as a lawn species (Oram 1990) due to its smaller leaves and more prostrate growth habit. Shearmans is an old cultivar derived from a single, naturally occurring plant observed by Mr J.H. Shearman in New South Wales in 1897 (Oram 1990). There appears to be no seed of this cultivar produced. A fourth cultivar, Prinsep Park, has existed and was derived from a natural selection from cv. Palestine from the Bunbury, Western

Australia, area (McDonald 2006). Certified seed production of strawberry clover was estimated at 70 t/year in 1993, comprising 60 t of cultivar Palestine and 10 t of cultivar O'Connors (Craig 1994). Breeding in New Zealand in the late 1980's has also produced two relatively new cultivars of strawberry clover, Grasslands Onwards and Grasslands Upwards (Craig 1994); these have yet to be extensively tested in Australia.

## **2.5 Conclusions**

There can be little doubt that dryland salinity will continue to have a major impact on agricultural production in southern Australian farming systems for some time. The ability of perennial plants to reduce recharge into rising groundwater systems has been well documented and it now remains for researchers to investigate new agricultural systems that are both environmentally and economically sustainable. To do this, information on the adaptive traits of perennial plants is necessary, together with a need to define those attributes required by perennial plants to not only survive, but be productive, in southern Australian farming systems. Work in this area has already begun (Dear and Ewing 2008) and it is hoped that this thesis can contribute to the bank of knowledge required if perennial plants are to be successfully and broadly adopted into southern Australian farming systems. There are very few perennial species that are commercially available for use in these systems, however there are many potential perennial species that could be introduced after substantial breeding efforts. Lucerne has begun to be widely utilised for the purpose of improved production and recharge control but it is not tolerant of waterlogging and so is not suitable for discharge areas and those areas subject to waterlogging or inundation. Strawberry clover has long been sown as a niche species in many agricultural regions of Australia, yet little breeding effort has gone into improving its production and adaptability to farming systems. For strawberry clover to be more widely sown in agricultural areas, improvement is needed in seedling vigour and drought tolerance, as well as improved tolerance to salinity and the interaction between salinity and waterlogging. This thesis examines strawberry clover in respect to the amount of morphological variation between populations, its adaptive strategies and growth habits and its tolerance to waterlogging and salinity, with the aim of gaining insight into its potential for further development for broader use in discharge areas in southern Australian farming systems.

## CHAPTER 3

# Genetic Variation in Five Populations of Strawberry Clover (*Trifolium fragiferum* L.) in Western Australia.<sup>1</sup>

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### 3.1 Abstract

Genetic variation within and between populations of an outcrossing stoloniferous perennial legume, strawberry clover (*Trifolium fragiferum* L.), was studied using seed collected from five locations in Western Australia. The sites ranged from Badgingarra in the north of the wheatbelt to Karridale in the southwest of the state. Seed was collected randomly at each site and grown at the University of Western Australia Field Station at Shenton Park, Perth. Thirteen plant morphological traits were measured and analysed. Within-population variation was extremely high. In contrast, between-population variation was generally low with most characters showing only 8–15% of the total variation. Despite this, all but one of the measured plant traits differed between populations. Principal components analysis highlighted the large amount of variation within the populations with the first three principal components accounting for only 59% of the total variation. The populations appear to have begun to differentiate into ecotypes more suited to the habitats into which they had been sown, but retain high within-population variation due to the outcrossing nature of strawberry clover. The high levels of variation within and between the populations indicate ample opportunity to use selective breeding to improve on current strawberry clover cultivars.

### 3.2 Introduction

Genetic variation in plant populations is important for the continuing success of individual species in a changing environment. It enables populations to colonise and establish in diverse habitats and, when established, to form a stable population that is able to persist under variable environmental conditions. Genetic variation in adaptive traits is evident when a species is distributed over different environments. The formation of genetically different populations of the same species that are adapted to different environments is known as ecotypic differentiation, which was first defined by the botanist Göte Turesson in 1922 (Barbour *et al.* 1987).

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<sup>1</sup> The results of this chapter have been reported by McDonald KS, Cocks PS, Ewing MA (2005) Genetic variation in five populations of strawberry clover (*Trifolium fragiferum* cv. Palestine) in Western Australia. *Australian Journal of Experimental Agriculture* **45**, 1445 – 1451.

One way in which genetic variation is generated is through outcrossing. The capacity to outcross is important for the widespread establishment of volunteer species, including pasture legumes (Cocks 1992). Population genetic theory leads to the expectation that outcrossing populations should reach Hardy–Weinberg equilibrium (heterozygosity) in a single generation and that such populations are expected to remain heterozygous for many generations (Allard 1988). Cocks (1996) stated that even the low levels of outcrossing found in some predominantly inbreeding Mediterranean annuals, including subterranean clover (*Trifolium subterraneum* L.), provide sufficient genetic variation to allow populations to successfully adapt to variable environments. For example, populations where no more than 2 strains of subterranean clover were sown developed almost infinite genetic variation within 20 years, the original genotypes being replaced at a rate of about 1.5% per year (Cocks 1992; Rossiter and Collins 1989a; 1989b).

A series of papers on long-term experiments with the perennial grass *Anthoxanthum odoratum* L. (Davies and Snaydon 1976a; Davies and Snaydon 1976b; Snaydon 1970; Snaydon and Davies 1972) found that populations of *A. odoratum* differed significantly in a number of morphological characteristics when compared with the genetic variation within the populations. More than 90% of the variation for many of the characters could be accounted for by differences in environmental factors associated with the source of the population. These studies also illustrate the ability of outcrossing perennial species to also form distinct populations over short geographic distances.

However, in perennial legumes, apart from white clover (*Trifolium repens* L.) (Caradus *et al.* 1990), there has been little research on the evolution of sown populations, and whether there is sufficient genetic change to cause ecotypic differentiation in populations that started out genetically identical. In Western Australia, strawberry clover (*Trifolium fragiferum* L. cv. Palestine) has been sown in niche areas in the West Midlands, Great Southern and South Western regions. The inclusion of more perennial based farming systems has been cited as a means to improve the sustainability of Australian agriculture, particularly where dryland salinity is a threat (Cocks 2001) and thus it is important to increase our knowledge of the adaptation of strawberry clover to a range of environments. Strawberry clover belongs to the same family as some widely naturalised annual legumes in Western Australia (e.g. *Trifolium tomentosum* L.) that are adapted to waterlogging (Gibberd and Cocks 1997). Strawberry clover is

predominantly outcrossing (Morley 1963; Wright 1964), so existing pastures present the opportunity to study genetic differentiation and potential for adaptation in an outcrossing, stoloniferous perennial legume.

The aim of this chapter is to examine the level of genetic variation in established populations of strawberry clover cultivar Palestine in Western Australia and to determine whether any genetic differentiation has occurred between populations. It was hypothesised that there would be evidence of genetic differentiation between the populations, and that this differentiation would be correlated with environmental factors from each of the collection sites.

### **3.3 Materials and methods**

Seed of five strawberry clover populations, cultivar Palestine, was collected at four locations in February–March 2000; Badgingarra, Brookton, Myalup and Karridale (Table 3.1). Seed from two populations was collected from the Karridale site, one from a natural pasture (Karridale rain) and the other from an irrigated vineyard (Karridale irrigated). The Karridale rain population was a 10-year-old mixed pasture for occasional grazing by cattle and the Karridale irrigated population was sown at the same time and was collected from between rows of an irrigated vineyard on the same property. The Badgingarra site was a sandy valley floor with a 15-year-old mixed pasture of kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.) and strawberry clover that had been intensively managed with fertiliser, rotationally grazed by sheep and regularly cut for hay. The Brookton population was sown approximately 20 years ago and subject to occasional set stocking with sheep in periods of abundant feed. This site was also mildly saline and suffered from summer droughts and winter waterlogging. The Myalup population was an established pasture of kikuyu and strawberry clover, set stocked with cattle for varying periods of time, and sown 30 years previous as a seed production pasture.

Table 3.1. Collection site details for the 5 populations of strawberry clover examined.

Site location	Latitude	Longitude	Average annual rainfall (mm)	Australian Soil Classification (Isbell 2002)	Grazing management
Badgingarra	115°48'E	31°40'S	650	Grey-Orthic Tenosol	Rotationally grazed by sheep and regularly cut for hay
Brookton	117°12'E	32°18'S	460	Yellow Chromosol	Occasional set-stocking by sheep
Myalup	116°E	33°18'S	850	Bleached-Orthic Tenosol	Set-stocked by cattle
Karridale Rain	115°31'E	34°20'S	1200	Brown Chromosol	Occasionally grazed by cattle
Karridale Irrigated	115°31'E	34°20'S	1200	Brown Chromosol	Irrigated and mown, not grazed.

Seed was collected by walking random transects over each paddock with five seed heads picked every 20 paces. Transects at the vineyard were in a straight line between randomly selected rows of vines. Seed heads from each paddock were bulked into one sample for each site. Seed was then separated from the seed heads by rubbing between rubber matting and was scarified and planted into 100 jiffy pots per population in a glasshouse on May 24 and 25, 2000. Three seeds were placed in each pot and were watered with a complete liquid fertiliser and Group B commercial *Rhizobium* inoculum. After germination, pots were thinned to one plant per pot.

Plants were transplanted to the University of Western Australia Field Station at Shenton Park, Perth on June 28 and 29, 2000. The trial was sown as a randomised complete block spaced plant design with two replicates (blocks). Fifty plants from each of the five populations were grown in each block. Each plant was one metre away from others in all directions. The site was fertilised with superphosphate and muriate of potash (100 kg/ha bulk fertiliser) prior to planting. Plants were irrigated for the duration of the experiment. Weeding was conducted by hand and by spraying with glyphosate when required.

Thirteen morphological and flowering characters were measured, of which 12 were quantitative and one was qualitative (leaf mark). The characters measured are listed in Table 3.2. All characters, apart from the seed characteristics (seed head weight, number of seeds per head and 1000 seed weight), were measured at the time of appearance of the first flower, while seed characteristics were measured at the time of seed harvest (March 2001).

*Table 3.2.* Phenological and morphological characters measured on strawberry clover plants, with abbreviations, method of measurement and timing of measurements

Timing; 1, scored on day of first flower; 2, scored at harvest in March 2001

Character	Abbreviation	Method of measurement	Timing
Flowering time	flwrtime	When first flower head fully opened (days from sowing)	1
Node of 1st flower	flwrnode	On main stem, counted from crown	1
Leaflet length	lfltlgth	Length of leaflet (mm), taken on middle leaflet of leaf on the first flowering node	1
Leaf marker	lfmk	Presence of anthocyanin and white patterns	1
Petiole length	pet	Leaf stalk measured in mm at first flowering node	1
Peduncle length	ped	Measured in mm for stalk of first flower	1
Plant height	plthgt	Measured in mm from the crown to the highest leaf tip	1
Plant width	pltwth	Measured in cm across the greatest width of the plant	1
Stolon width	stolon	Stem thickness measured in mm where first flower subtends	1
Head weight	hdwgt	Weight (g) of 20 mature flower heads	2
No. seeds per head	seedhd	Average number of seeds per head, measured from 20 flower heads	2
1000 seed weight	sdwgt	Weight (g) of 1000 seeds	2

### *Data analysis*

Data were analysed using Genstat for Windows Version 6 (VSN International Ltd, Hertz, UK). Pearson's correlations were calculated on the averages of all quantitative variables and on five environmental characters collected from Australian Bureau of Meteorology data for each site (average daily minimum and maximum temperature, annual rainfall, latitude and growing season length). The correlation results do not include measurements from the Karridale irrigated population. These were omitted because irrigation of the population was considered to produce a confounding effect on the environmental factors, as the water management imposed obscured the environmental conditions. Quantitative variables only were subjected to analysis of variance (ANOVA). Tests of normality and homogeneity of variance were made prior to analysis to determine if data required transformation. To determine if variation existed between sites for any of the measured traits, one-way ANOVA was performed on each variable. Variation was then partitioned into between and within site components for each quantitative character measured. Principal components analysis (PCA) was used to examine the spread of variation in the populations, including the Karridale irrigated population. This was conducted on those quantitative plant characters that showed significant differences between populations in the ANOVA, using a correlation matrix to reduce the variables to a common scale.



### 3.4 Results

#### *Correlation between plant traits and environmental variables*

Pearson's correlations (Table 3.3) showed that the plant characters were mostly negatively correlated with the average daily maximum temperature, and there were very few characters correlated to the average daily minimum temperature. Most traits showed a positive relationship with annual rainfall, except seeds/head and head weight, which were negatively correlated. Only a few characters were correlated with length of growing season. Head weight and seeds/head were negatively correlated with growing season, while stolon width and flowering time were positively correlated. Most plant characters were positively correlated with latitude.

Table 3.3. Pearson's correlations between plant characters and environmental variables for strawberry clover populations at 5 sites

Abbreviations for plant characters as in Table 3.2.

\*\*\*,  $P < 0.01$ ; \*\*,  $P < 0.05$ ; \*,  $P < 0.1$

Plant character	Average daily maximum temperature	Average daily minimum temperature	Average annual rainfall	Growing season length	Latitude
flwrtime	-0.50	<b>0.86*</b>	0.66	<b>0.83*</b>	0.36
flwrnode	-0.54	-0.30	0.13	-0.08	0.70
lfltlgth	<b>-0.98***</b>	-0.10	<b>0.89**</b>	0.67	<b>0.95***</b>
pet	<b>-0.95***</b>	0.26	<b>0.87*</b>	0.76	<b>0.91**</b>
ped	<b>-0.82*</b>	-0.19	0.49	0.26	<b>0.92**</b>
plthgt	-0.29	0.78	0.32	0.49	0.24
pltwth	<b>-0.97***</b>	-0.23	0.78	0.51	<b>0.99***</b>
stolon	<b>-0.86*</b>	0.15	<b>0.97***</b>	<b>0.86*</b>	0.74
hdwgt	0.66	-0.76	<b>-0.83*</b>	<b>-0.94***</b>	-0.50
seedhd	0.72	-0.71	<b>-0.84*</b>	<b>-0.92**</b>	-0.58
sdwgt	<b>-1.00***</b>	-0.01	<b>0.86*</b>	0.66	<b>0.99***</b>

#### *Qualitative character – leaf marks*

Between the five field sites there were five distinct leaf marks recorded, each with distinctive patterns of anthocyanin (red), and green and white colouring (Table 3.4). Mark categories 1 and 2 were the most commonly exhibited by all populations, with category 5 only being exhibited by Badgingarra and Karridale rain and then, at very low frequency (Fig. 3.1). The population from Brookton exhibited relatively even numbers of categories 1, 2 and 3 with few plants exhibiting category 4. The remaining populations exhibited similar numbers of plants with categories 3 and 4. The Karridale irrigated population exhibited mostly plants with category 2, while the Badgingarra, Myalup and Karridale rain populations exhibited a majority of plants with category 1.

Table 3.4. Description of leaf mark categories found in 5 populations of strawberry clover

Leaf marker	Description
1	No mark or diffuse red flecking and marking on leaf margins
2	Pale green/white 'v' mark, sometimes with red flecking or edging above 'v'
3	Pale green/white 'v' with red dot, sometime extending to a red 'v' underneath, occasionally with red flecking or edging above 'v'
4	Whole leaf red tinged with green 'v' mark
5	Pale green 'v' with red 'v' underneath and red coloured leaf tip of leaf above the 'v'

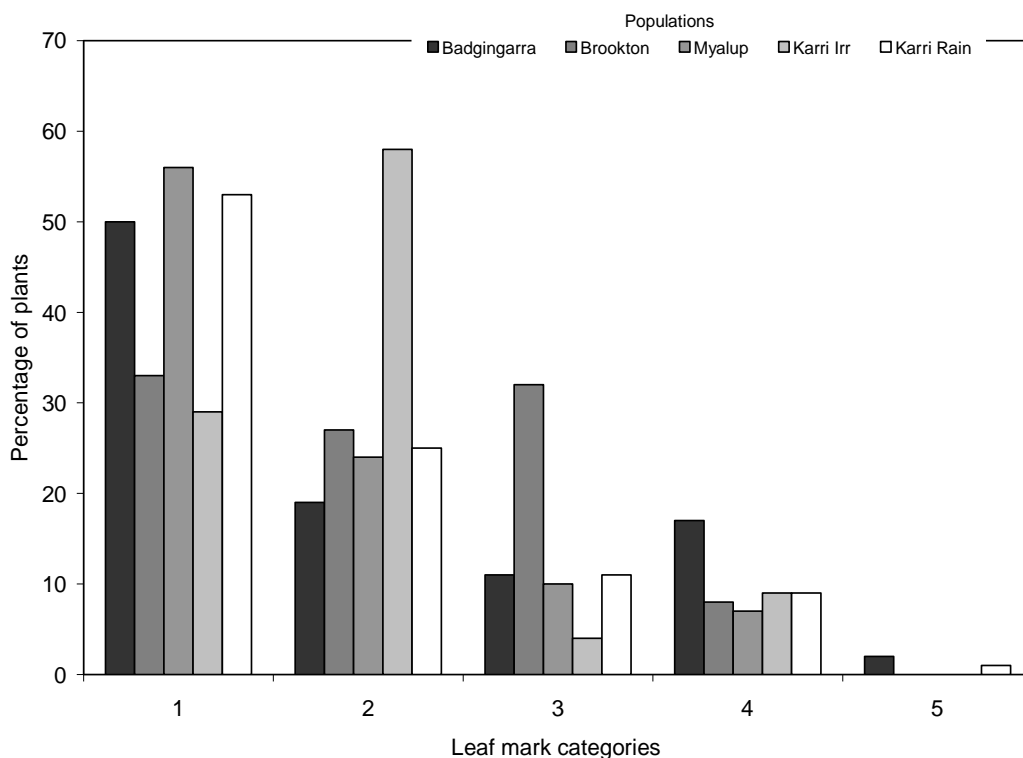


Figure 3.1. Percentage of plants of strawberry clover from 5 populations (Badgingarra, Brookton, Karridale irrigated, Karridale rain, Myalup) scored for the 5 leaf mark categories described in Table 3.4.

### Quantitative characters

The means, standard errors, and percentage within-population variation of the 12 quantitative plant characters are shown in Table 3.5. The Brookton population had the earliest flowering maturity at 168 days post-sowing. Myalup recorded the latest flowering maturity at 184 days. Generally those populations with a heavier seed weight had less seeds per head and a lower head weight. The Karridale irrigated population had the heaviest seed weight, followed by the Myalup population, then Brookton and Karridale rain, with the Badgingarra population having the smallest seed weight and one of the larger number of seeds per head. On average, plants from the Karridale rain

population were larger, had larger leaves, thicker stolons, and longer petioles and peduncles, and were wider and taller than plants from any of the other populations. Plants from the Badgingarra population were, on average, smaller than plants from any of the other populations. There was no difference between any of the populations for node of first flower.

While there were significant ( $P < 0.001$ ) differences for all but one of the plant characters measured (Table 3.5), only a small proportion of the total variation was between-population variation. Stolon thickness showed the greatest variation between populations (~ 19%), with node of first flower showing the least (~ 1.5%). Most characters showed between 8 and 15% variation between populations.

Table 3.5. Means ( $\pm$  standard errors) and percentage among-population variation calculated from ANOVA for 12 plant characters measured on 5 populations of strawberry clover. Abbreviations for plant characters as in Table 3.2. \*\*\*,  $P < 0.001$ ; n.s., not significant ( $P > 0.05$ )

Plant trait	Badgingarra	Brookton	Myalup	Karridale irrigated	Karridale rain	Variation among populations (%)	Significance
flwrtime	176 $\pm$ 2	168 $\pm$ 2	184 $\pm$ 2	178 $\pm$ 2	169 $\pm$ 1	10.9	***
flwrnode	9.4 $\pm$ 0.2	10.0 $\pm$ 0.3	10.1 $\pm$ 0.2	9.8 $\pm$ 0.3	9.4 $\pm$ 0.3	1.5	n.s.
lfltlgth	8.9 $\pm$ 0.2	9.2 $\pm$ 0.2	9.4 $\pm$ 0.1	9.9 $\pm$ 0.2	11.1 $\pm$ 0.3	14.8	***
pet	20 $\pm$ 1	20 $\pm$ 1	23 $\pm$ 1	23 $\pm$ 1	30 $\pm$ 1	12.9	***
ped	41 $\pm$ 2	48 $\pm$ 2	51 $\pm$ 2	50 $\pm$ 2	62 $\pm$ 2	13.3	***
plthgt	20 $\pm$ 1	19 $\pm$ 1	22 $\pm$ 1	20 $\pm$ 1	26 $\pm$ 1	8.4	***
pltwth	43 $\pm$ 1	48 $\pm$ 1	49 $\pm$ 1	52 $\pm$ 1	55 $\pm$ 2	10.6	***
stolon	2.9 $\pm$ 0.05	2.9 $\pm$ 0.05	2.9 $\pm$ 0.05	3.1 $\pm$ 0.05	3.5 $\pm$ 0.06	18.8	***
hdwgt	2.5 $\pm$ 0.05	2.8 $\pm$ 0.04	2.2 $\pm$ 0.08	2.3 $\pm$ 0.04	2.5 $\pm$ 0.05	15.0	***
seedhd	51 $\pm$ 2	55 $\pm$ 1	45 $\pm$ 2	47 $\pm$ 1	52 $\pm$ 1	9.3	***
sdwgt	1.06 $\pm$ 0.01	1.09 $\pm$ 0.01	1.12 $\pm$ 0.02	1.14 $\pm$ 0.01	1.09 $\pm$ 0.01	5.4	***

The percentage variation and character loadings from the principal components analysis are shown in Table 3.6. Principal components analysis explained only 59% of the variation in the first three components with principal component 1 (PC1) and principal component 2 (PC2) explaining less than 50% of the total variation. The leaf characters (leaflet length, petiole length), peduncle length and to a lesser extent plant width, plant length and stolon width had the strongest loading on PC1. Characters with a strong loading on PC2 were head weight and seeds per head and, to a lesser extent, seed weight. Seed weight had the strongest loading on principal component 3 (PC3).

*Table 3.6.* Principal component scores 1, 2, and 3 (PC1, PC2, PC3, respectively) percentage variation and component loadings on plant characters for strawberry clover populations

Abbreviations for plant characters as in Table 3.2

	PC1	PC2	PC3
Proportion of variation	0.324	0.158	0.109
Cumulative variation	0.324	0.482	0.591
Component loadings			
flwrtime	0.210	0.075	0.363
hdwgt	-0.082	0.648	0.222
lfltlgth	-0.460	-0.083	-0.014
ped	-0.411	-0.055	-0.073
pet	-0.454	-0.087	-0.152
plthgt	-0.304	-0.054	-0.169
pltwth	-0.349	-0.049	0.322
seedhd	-0.120	0.674	0.064
sdwgt	0.010	-0.310	0.760
stolon	-0.366	0.032	0.279

The scores of PC1 and PC2 are plotted in Fig. 3.2 and are overlaid with a biplot of the component scores. The biplot shows that the plant morphological traits are all highly correlated with each other, and negatively correlated with time to first flower. There is no correlation between the seed traits and flowering time, and seed weight is negatively correlated with number of seeds per flower head and head weight.

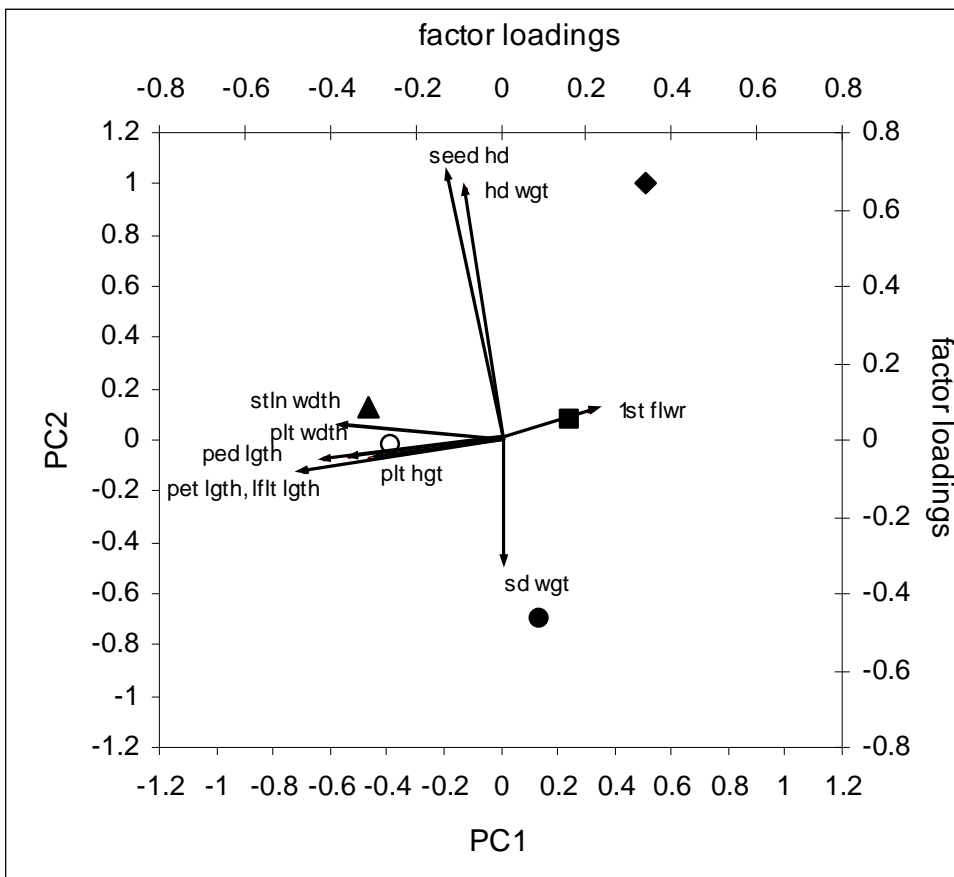


Figure 3.2. Principal components analysis of strawberry clover populations (◆, Badgingarra; ●, Brookton; ▲, Karridale irrigated; ■, Karridale rain; ○, Myalup) showing the averages for each population. An overlay of the biplot represents the component loadings for principal components 1 and 2 for each of the quantitative characters used in the multivariate analysis.

Figure 3.2 shows the Myalup and Karridale irrigated populations close to each other. Both are characterised, compared to the other three populations, by larger plants with large leaves and longer, thicker stems and higher average seed weight, seeds per head and head weight. The remaining populations were characterised by smaller plants with smaller leaves and shorter stems, and by more extreme scores for the reproductive traits. The Brookton population was characterised by high seed weight and low numbers of seeds per head in light seed heads, while the biplot shows the Badgingarra population had low seed weight with high numbers of seeds per head and heavier seed heads. The Karridale rain, Badgingarra and Brookton populations were characterised by earlier flowering than the Karridale irrigated and Myalup populations.

### **3.5 Discussion**

Low levels of variation between populations were found for all plant traits. Despite this, there were significant differences between populations for all measured traits, apart from node of first flower, indicating that the populations did differ from each other. Many of the plant traits had a high correlation with environmental factors, such as latitude, maximum temperature and rainfall, indicating the populations may have developed into more locally adapted ecotypes. Less than 50% of the variation was explained by the first two principal components in the principal components analysis. Only 59% of total variation was explained by the first three principal components, highlighting the variability within the populations of strawberry clover.

The low levels of between-population variation for each of the plant characters can be explained by a consideration of the breeding system of the species and the fact that each population was originally established as a sown pasture of the same cultivar, Palestine. Strawberry clover is an outcrossing species (Morley 1963). Outcrossing promotes high levels of variation within populations through crossing and recombination. Due to the different year of strawberry clover establishment at each site, it is likely that seed originally sown came from different sources and for this reason the initial populations would have varied from each other, perhaps considerably. As most of the collected populations were widely separated from each other it is unlikely that gene flow occurred among them and, therefore, it is reasonable to expect distinct populations to evolve. Many other outcrossing plants have exhibited similar high levels of within population variation, including the annual legumes *Trifolium michelianum* L. and *T. resupinatum* L. (Bennett 1998) and the perennial legumes birdsfoot trefoil (*Lotus corniculatus* L.) cultivar Grassland Goldie (Kelman and Ayres 2004) and white clover (Jahufer and Gawler 2000). A previous study with inbreeding and outcrossing clovers (Bulinska-Radomska 1996) also showed that most of the genetic variation of the strawberry clover populations studied was within-population variation, in contrast to the inbreeding clovers studied. The outcrossing clover species, including strawberry clover, were also found to contain greater total variation (Bulinska-Radomska 1996).

Characters with the most variation between populations are likely to be those that improve the adaptability of a population to its environment, as selection pressure for these characters would be expected to be strongest. In a study on the outcrossing annual

weed capeweed (*Arctotheca calendula* L.) in the south-west of Western Australia, Dunbabin (2001) found the highest percentage of between-population variation occurred with flowering time. He concluded that as flowering time was crucial for the survival of annual plants, extreme selection pressure for this trait was imposed by the environment resulting in large differences among environments. In the present study, the characters showing the most between-population variation were those related to plant habit, not flowering time. As each population came from pastures experiencing strongly contrasting grazing regimes (rotational, intermittent, set stocking) with different classes of stock (sheep, cattle) it may be that certain plant types were selected for, and came to dominate, these pastures. For example, a study undertaken in Norway suggested that grasses growing under intense grazing are likely to evolve low growth activity and prostrate habit (Myserud and Myserud 2000).

In strawberry clover, many of the plant habit traits (e.g. petiole length, stolon width and plant height) were positively correlated with annual rainfall and growing season length, whereas in capeweed the opposite was true (Dunbabin 2001). The reason suggested for the negative correlation between morphological traits and rainfall in the capeweed study was adaptation to the more intense grazing pressure likely to be experienced under high rainfall conditions where grazing animals will have selected smaller, shorter plants. In this study, the most intensive grazing of strawberry clover was likely to have occurred at the drier sites. The biplot (see Fig. 3.2) confirms the notion that plant habit is being selected for through grazing pressure. The Karridale irrigated population is characterised by large plants with long stems and large leaves. This population was located between rows of vines at a vineyard and did not experience any grazing. Those populations that had the most constant grazing (Badgingarra in a constant rotational system and Brookton with a periodic set stocked system) were also those characterised by the smallest plants, indicating that intensified grazing pressure had selected for small plants with shorter stems. Natural selection in white clover evolving in an environment of close continuous grazing was also found to favour small-leaf genotypes (Williams 1987).

The ANOVA showed the five populations to be significantly different from each other for all measured plant traits except node of first flower. This indicates that the different populations had formed genetically distinct ecotypes. In a study on the inbreeding annual species *T. glomeratum* L. (Bennett 1997), the formation of two distinct



populations with different population structures was the result of more extreme climatic influences on one of the populations. Ecotype formation has also been described in several other naturalised annual species including capeweed (Dunbabin 2001), wild radish (*Raphanus raphanistrum* L.) (Bhatti 2004) and annual ryegrass (*Lolium* sp.) (D. Ferris, pers. comm.). Subterranean clover is known to have produced distinctly different ecotypes over very short distances as a result of position on a slope (Cocks 1992), which is likely to be related to the time of onset of summer drought. A study of the genecology of white clover also showed differences between white clover populations from dry and wet farms in New Zealand (Caradus *et al.* 1990), although it is not stated if these populations were originally introduced from the same source. Significant genetic differentiation in white clover populations collected from old pasture sites in northern New South Wales has also been recorded (Lane *et al.* 2000). The results from the present study illustrate the high variability in strawberry clover populations derived from the cultivar Palestine and that each population changed slightly to be better suited to its new environment. Leaf mark, a trait that would be unaffected by environmental conditions and not expected to change, varied widely among populations (see Fig. 3.1) providing further evidence of genetic differentiation among the studied populations.

Genetic variation in pastures is important to maintain the pasture throughout variable seasonal conditions. Cocks (1994) suggested that a monoculture is at high risk of climatic, biotic and edaphic stresses and also may confer an inability to colonise the range of habitats that may be present. The same can be inferred for a single species where presumably the greater the diversity or genetic variation a population can maintain, the greater the number of micro-habitats that population could colonise and the higher the chance of it persisting in a variable environment, such as a wheatbelt pasture in Western Australia (Bennett 1997).

A genetic shift in populations away from the original strain as a result of adaptation to new sites has been reported for other pasture species. Studies on the predominantly inbreeding annual, subterranean clover, have shown that even the small amounts of outcrossing exhibited by this species allows enough genetic variability into the population to change them from the original sown cultivar to a mixed strain pasture (Cocks 1992; Rossiter and Collins 1989a; 1989b). Strawberry clover, as an outcrossing, insect pollinated species, is likely to be particularly prone to adaptive shifts in strain

when sown in new environments. There is a high probability that other agriculturally-important, outcrossing perennial legumes would do the same.

There has been limited resources allocated to research on cultivar improvement of strawberry clover in Australia (Craig 1994), but given its tolerance of waterlogged and mildly saline conditions (Hoveland and Mikkelsen 1967; Rumbaugh *et al.* 1993), strawberry clover could have an important role to play in improving production on these marginal soils. In a review of the efficiency of breeding strategies for white clover in Australia, Jahufer *et al.* (2002) highlighted the amount of genetic variation in existing cultivars and populations of white clover. They concluded that the characterisation of germplasm in Australia and overseas indicated that there was extensive genetic potential available for use in breeding programs. With the high levels of within population variation shown in strawberry clover in this study it is likely that gains in production, seedling vigour, and waterlogging and salinity tolerance could be made with existing, as well as introduced, genetic material through appropriate screening and breeding programs. Results from the current study also highlight issues with the integrity of cultivars of outcrossing perennial species like strawberry clover. These are likely to have important implications for plant breeders and the seed industry and highlight the role of seed certification to maintain the genetic integrity of proprietary lines so that farmers can have confidence that the seed they purchase is true to type.

## CHAPTER 4

# Genetic Variation in Eight Populations of Strawberry Clover (*Trifolium fragiferum* L.) Collected from the Mediterranean Basin Compared to Two Commercial Cultivars.

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### 4.1 Abstract

Genetic variation within and between populations of the outcrossing stoloniferous perennial legume, strawberry clover (*Trifolium fragiferum* L.) was studied using seed of eight populations collected from the Mediterranean Basin and two commercial cultivars (one of which was represented twice using seed from two sources). Seed was sourced from genetic resource centres and available passport data for each population was also collected. The seed was grown at the University of Western Australia Field Station at Shenton Park, Perth. Thirteen morphological plant traits were measured and analysed. The proportion of between-population variation was at low-medium levels with most characters showing 6 – 40% of their total variation occurring as between-population variation. Thus most variation was within-population variation. Despite this, all of the measured plant traits differed between the populations. Principal components analysis highlighted the large amount of variation within the populations, with the first three principal components accounting for only 57% of the total variation. Variation in the commercial cultivars was similar to variation in the populations from the Mediterranean Basin. The amount of genetic variation within and between all populations indicates ample opportunity to use selective breeding to improve upon current strawberry clover cultivars.

### 4.2 Introduction

Strawberry clover (*Trifolium fragiferum* L.) is one of seven species in section Vesicaria of the *Trifolium* genus. Its centre of origin is the Mediterranean Basin (Zohary and Heller 1984), although it is widely naturalised throughout the world. Strawberry clover is a stoloniferous perennial legume that is predominantly outcrossing (Morley 1963; Wright 1964). In Australia strawberry clover is used in medium to high rainfall zones as a niche species for poorly drained, waterlogged and/or saline areas or on non-saline

alkaline soils where the pH is too high for white clover (*Trifolium repens* L.) (Craig 1994).

Historically, strawberry clover has not had an important role in Australian agriculture. The current commercial cultivar, Palestine, was first certified by the South Australian Department of Agriculture in 1950-51 (Oram 1990) and subsequently there have been no new cultivars released. With the salinisation of agricultural lands now requiring urgent attention there may now be impetus for development of improved cultivars of strawberry clover.

Primitive landraces, wild crop relatives and other wild species from the Mediterranean Basin remain a valued source of genetic material for improving agricultural production in the temperate world (Tan 1996). This is particularly the case for the genus *Trifolium* with 110 species from seven of the eight sections originating in the Mediterranean Basin (Zohary and Heller 1984). A high degree of genetic variation in pastures is important to allow productivity to be maintained throughout a variety of seasonal conditions; a circumstance where a monoculture is at high risk of climatic, edaphic and biotic stresses (Cocks 1994). The same can be inferred for a single species i.e. the greater the diversity or genetic variation a population can maintain, the higher the chance of that population persisting in a variable environment (Allard 1988; 1996; Woodward and Morley 1974). As the Mediterranean Basin is the centre of diversity for strawberry clover, it would be likely that individual populations from there should contain greater genetic variation than commercially bred cultivars.

This chapter reports on the investigation of the extent of genetic variation occurring in populations of strawberry clover collected from the Mediterranean basin in comparison to two commercially available cultivars. It is a sister study to the study reported in Chapter 3 which investigated the variation in five naturalised populations of strawberry clover in Western Australia. All populations in Chapter 3 were originally sown as pastures of the commercial cultivar, Palestine. Variation within the populations was found to be much higher than variation between the populations. Despite this, each population was different and these differences were attributed to adaptation to different habitats, and hence were due to ecotype formation. Based on these findings, three hypotheses were tested in Chapter 4;

- (1) Variation will be greater in populations of strawberry clover from the Mediterranean Basin than commercial varieties;
- (2) There will be a greater proportion of within-population variation than between-population variation for all populations of strawberry clover; and
- (3) Genetic variation will be greater in populations from the Mediterranean than naturalised populations from Western Australia.

### **4.3 Materials and methods**

Seed of eight populations of strawberry clover originating in the Mediterranean, as well as two commercial cultivars, were obtained from the genetic resource centres at the South Australian Research and Development Institute, the Margot Forde Centre, New Zealand and the Department of Agriculture and Food, Western Australia. The commercial cultivars were O’Connors, a lawn type variety developed in South Australia, and Palestine, a cultivar developed from an ecotype originating near the Dead Sea in Israel and introduced into Australia in 1929 (Oram 1990). There were two sources of seed for Palestine, one from the Trifolium Genetic Resource Centre at the Department of Agriculture and Food, Western Australia and the other from the Margot Forde Centre in New Zealand. Available passport data on each of the Mediterranean populations is presented in Table 4.1.

*Table 4.1.* Populations of strawberry clover examined and available passport data for collections from the Mediterranean basin

Population	Country of Origin	Population label	Altitude (m)	Latitude	Longitude	Average annual rainfall (mm)
Palestine SA	Australia	Pal (Au)				
Palestine NZ	New Zealand	Pal (NZ)				
O’Connors	Australia	O’Con				
SA14345	Syria	Syria	350	35°9’N	36°44’E	325
SA14911	Turkey	Turkey	950	36°55’N	31°44’E	1050
SA14958	Spain	Spain1	800	40°N	5°W	350
SA14959	Spain	Spain2	400	41°N	4°10’W	370
SA16600	Iran	Iran1	1280	36°57’N	46°6’E	250
SA16596	Iran	Iran2	15	36°44’N	53°38’E	600
SA19684	Tunisia	Tunisia1				
SA19773	Tunisia	Tunisia2				

Seed was scarified and planted into 40 jiffy pots per population in a glasshouse on May 24 and 25, 2000. Three seeds were placed in each pot, and then watered with a liquid fertilizer and Group B commercial *Rhizobium* inoculum. After germination, plants were thinned to one per pot.

Plants were transplanted to the University of Western Australia Field Station at Shenton Park, Perth on June 28 and 29, 2000. The trial was sown as a randomised block spaced plant design with two replicates (blocks). Twenty plants from each of the 11 populations were grown in each block. Each plant was one metre away from other plants in all directions. The site was fertilised with super phosphate and muriate of potash (100 kg/ha bulk fertiliser of each) just prior to planting. Plants were irrigated for the duration of the experiment. Weeding was conducted by hand and by spraying with glyphosate.

Thirteen morphological and flowering characteristics were measured, of which 12 were quantitative and one (leaf mark) was qualitative (Table 4.2). All characters, apart from the seed characteristics (seed head weight, number of seeds per head and 1000 seed weight), were measured at the time of appearance of the first flower, while seed characteristics were measured at the time of seed harvest (March 2001).

Table 4.2. Phenological and morphological characters measured on strawberry clover plants, with abbreviations, method of measurement and timing of measurements

Timing; 1, scored on day of first flower; 2, scored at harvest in March 2001

Character	Abbreviation	Method of measurement	Timing
Flowering time	flwrtime	When first flower head fully opened, as days from sowing	1
Node of 1 <sup>st</sup> flower	flwrnode	On main stem, counted from crown	1
Leaflet length	lfltlgth	Length of leaflet (mm), taken on middle leaflet of leaf on the first flowering node	1
Leaflet width	lfltwth	Width of leaflet (mm), taken on the widest part of the middle leaflet of leaf on first flowering node	1
Leaf marker	lfmk	Presence of anthocyanin and white patterns	1
Petiole length	pet	Leaf stalk measured in mm at first flowering node	1
Peduncle length	ped	Measured in mm for stalk of first flower	1
Plant height	plthgt	Measured in mm from the crown to the highest leaf tip	1
Plant width	pltwth	Measured in cm across the greatest width of the plant	1
Stolon width	stolon	Stem thickness measured in mm where first flower subtends	1
Head weight	hdwgt	Weight (g) of 20 mature flower heads	2
No. seeds per head	seedhd	Average number of seeds per head, measured from 20 flower heads	2
1000 seed weight	sdwgt	Weight (g) of 1000 seeds	2

#### *Data analysis*

Data were analysed using Genstat for Windows Version 9 (VSN International Ltd, Hertz, UK). Pearson's correlations were calculated on four environmental characters collated from each collection's passport data (altitude, latitude, longitude and annual rainfall). The correlation results do not include measurements from the commercial cultivars, O'Connors or Palestine (both sources), or from the two populations from Tunisia, as there was no passport data available. Quantitative variables only were subjected to analysis of variance (ANOVA). Tests of normality and homogeneity of variance were made prior to analysis to determine if data required transformation. To determine if variation existed between populations for any of the measured traits, one-way ANOVA was performed on each variable. Variation was then partitioned into between and within population components for each quantitative character measured. Principal components analysis (PCA) was used to examine the spread of variation in the populations, including the commercial cultivars and populations from Tunisia. The PCA was conducted on those quantitative plant characters that showed significant differences among populations in the ANOVA, using a correlation matrix to reduce the variables to a common scale.

## 4.4 Results

### *Correlations between plant traits and environmental variables*

Pearson's correlations showed that six of the plant characters were negatively correlated with latitude and five were positively correlated with longitude (Table 4.3). Only plant width had a significant correlation (negative) with altitude, while none of the characters measured were correlated with average annual rainfall at the population collection site. None of the reproductive characters were correlated with any of the environmental indices.

*Table 4.3.* Pearson's correlations between plant characters and environmental indices for strawberry clover populations from the Mediterranean Basin

Abbreviations for plant characters as in Table 4.2. \*\*,  $P < 0.01$ ; \*,  $P < 0.05$

Plant characters	Altitude	Latitude	Longitude	Average annual rainfall
Flwrtime	0.25	-0.49	0.67	0.59
Flwrnode	-0.22	0.70	-0.63	-0.18
Lfltlgth	-0.12	<b>-0.87*</b>	<b>0.91*</b>	-0.11
Lfltwth	-0.11	<b>-0.88*</b>	<b>0.90*</b>	-0.06
Pet	-0.14	<b>-0.86*</b>	0.73	-0.16
Ped	-0.21	<b>-0.89*</b>	<b>0.96**</b>	-0.01
Plthgt	-0.08	<b>-0.96**</b>	<b>0.89*</b>	0.49
Pltwth	<b>-0.92**</b>	-0.27	0.26	-0.02
Stolon	-0.16	<b>-0.98**</b>	<b>0.89*</b>	0.22
Hdwgt	-0.04	-0.22	0.22	0.52
Seedhd	-0.50	-0.38	0.20	0.06
Sdwgt	0.59	0.72	-0.61	-0.34

### *Qualitative character – leaf marks*

There were five distinct leaf mark categories recorded for the populations, each characterised by different patterns of anthocyanin (red), green and white patterns (Table 4.4). Either leaf mark categories 1 or 2 were the most commonly recorded categories for all populations, with category 5 only recorded for Pal (Au) and Tunisia1, or then in very low numbers (Fig. 4.1).

*Table 4.4.* Description of leaf mark categories found in 11 populations of strawberry clover.

Leaf mark	Description
1	No mark or diffuse red flecking and marking on leaf margins
2	Pale green/white 'v' mark, sometimes with red flecking or edging above 'v'
3	Pale green/white 'v' with red dot, sometime extending to a red 'v' underneath, occasionally with red flecking or edging above 'v'
4	Whole leaf red tinged with green 'v' mark
5	Pale green 'v' with red 'v' underneath and red coloured leaf tip of leaf above the 'v'



In the three commercial cultivars, leaf mark category 1 was most common, making up about 60 – 70 % of the populations. Both Pal (Au) and Pal (NZ) had more variety of leaf marks than O’Con which only showed three of the five leaf mark categories. Unlike Pal (Au), Pal (NZ) did not have leaf mark 5, which was the rarest category in all populations.

In the eight Mediterranean populations, leaf mark 1 was generally the most common mark. Turkey had similar percentages of leaf mark categories 1 and 2, and both Iran1 and Tunisia1 had high percentages of leaf mark category 2. Both populations from Spain had only leaf marks 1 and 2, with the populations from Syria and Turkey having the most variety in their leaf marks. Apart from these 2 populations, the Mediterranean populations of strawberry clover generally exhibited less variety of leaf marks than either source of the cultivar Palestine.

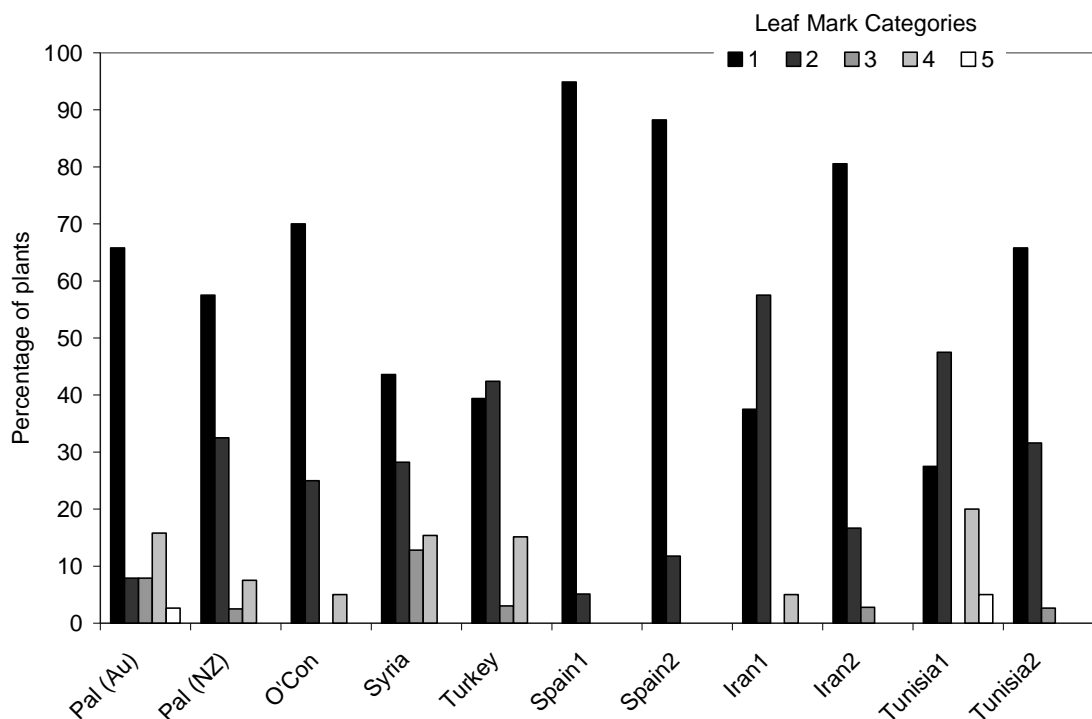


Figure 4.1. Percentage of plants of strawberry clover from populations collected in the Mediterranean Basin and 2 commercial cultivars (2 sources of cultivar Palestine), scored for the 5 leaf mark categories described in Table 4.4.

#### Quantitative characters

The means, standard errors, and percentage between-population variation of the 12 quantitative plant characters are shown in Table 4.5. While there were significant ( $P \leq$

0.05) differences for all quantitative plant characters measured, only a medium - small proportion of the total variation was between-population variation. Stolon thickness and plant height showed the greatest degree of variation between populations (about 40%), with node of first flower showing the least (about 7%). Most other plant traits showed between 20 and 30% of variation as between-population variation.

Generally populations from the same country recorded similar measurements. Tunisia<sup>2</sup> had the earliest flowering maturity, at 165 days post-sowing. This was closely followed by Tunisia<sup>1</sup> and Pal (Au) - Australian-sourced seed of the cultivar Palestine. Iran<sup>1</sup> had the latest flowering maturity at 198 days, along with the population from Turkey and Iran<sup>2</sup> at 198 and 196 days respectively. Pal (Au) was earlier flowering than Pal (NZ). Generally those populations with a high number of seeds per flowering head also recorded a higher head weight. The Tunisian populations had the largest number of seeds per head, followed by the cultivar Palestine from both Australian and New Zealand sources and the Syrian population. The populations with the lowest number of seeds per head were Iran<sup>2</sup> and Spain<sup>2</sup>. On average, plants from Pal (NZ) had bigger leaves, larger and thicker stems and were overall larger plants than those from the Pal (Au) population, and larger also than the other populations studied. The two populations from Spain and the population from Turkey had the smallest plants in all aspects.

Variances for all characters measured on each population are presented in Table 4.6. Within-population variance differed for each character. Pal (Au) showed higher variances for most characters, higher than the other commercial cultivars and most of the populations from the Mediterranean. Variances were also generally higher for the Syria population and, for some characters (i.e. flwrtime, seedhd), the Turkey population. Both Spanish populations had lower variances in comparison to the other populations for most plant characters.

Table 4.5. Means ( $\pm$  standard errors) and percentage between-population variation calculated from ANOVA for 12 plant characters measured on 11 populations of strawberry clover. Commercial cultivars are in bold. Abbreviations for plant characters as in Table 4.2. \*\*\*,  $P < 0.001$ ; \*\*,  $P = 0.005$

Plant trait	<b>Pal (Au)</b>	<b>Pal (NZ)</b>	<b>O'Con</b>	Syria	Turkey	Spain1	Spain2	Iran1	Iran2	Tunisia1	Tunisia2	Variation between populations (%)	Significance
flwrtime	<b>168 <math>\pm</math> 3</b>	<b>181 <math>\pm</math> 3</b>	<b>177 <math>\pm</math> 3</b>	178 $\pm$ 4	198 $\pm$ 4	183 $\pm$ 2	181 $\pm$ 4	198 $\pm$ 3	196 $\pm$ 4	169 $\pm$ 2	165 $\pm$ 2	28.7	***
flwrnode	<b>8.7 <math>\pm</math> 0.4</b>	<b>9.1 <math>\pm</math> 0.3</b>	<b>9.2 <math>\pm</math> 0.4</b>	8.2 $\pm$ 0.4	8.2 $\pm$ 0.3	10.3 $\pm$ 0.4	8.9 $\pm$ 0.5	8.9 $\pm$ 0.4	7.9 $\pm$ 0.5	8.5 $\pm$ 0.5	8.7 $\pm$ 0.4	6.7	**
lfltlgth	<b>9.7 <math>\pm</math> 0.3</b>	<b>10.2 <math>\pm</math> 0.4</b>	<b>8.8 <math>\pm</math> 0.3</b>	8.9 $\pm$ 0.3	7.4 $\pm$ 0.2	7.1 $\pm$ 0.3	6.1 $\pm$ 0.3	9.0 $\pm$ 0.2	8.7 $\pm$ 0.3	12.9 $\pm$ 2.8	10.8 $\pm$ 0.3	33.6	***
lfltwth	<b>6.5 <math>\pm</math> 0.2</b>	<b>7.4 <math>\pm</math> 0.2</b>	<b>6.6 <math>\pm</math> 0.2</b>	6.7 $\pm$ 0.2	5.7 $\pm$ 0.1	5.5 $\pm$ 0.2	4.5 $\pm$ 0.2	6.8 $\pm$ 0.2	6.5 $\pm$ 0.3	7.4 $\pm$ 0.3	7.5 $\pm$ 0.2	25.7	***
pet	<b>23 <math>\pm</math> 2</b>	<b>27 <math>\pm</math> 2</b>	<b>31 <math>\pm</math> 2</b>	21 $\pm$ 2	15 $\pm$ 1	14 $\pm$ 1	12 $\pm$ 1	17 $\pm$ 1	17 $\pm$ 2	23 $\pm$ 2	30 $\pm$ 2	21.6	***
ped	<b>47 <math>\pm</math> 4</b>	<b>51 <math>\pm</math> 3</b>	<b>51 <math>\pm</math> 3</b>	44 $\pm$ 4	37 $\pm$ 2	32 $\pm$ 3	31 $\pm$ 3	46 $\pm$ 3	43 $\pm$ 3	43 $\pm$ 3	56 $\pm$ 2	14.4	***
plthgt	<b>19 <math>\pm</math> 1</b>	<b>22 <math>\pm</math> 1</b>	<b>17 <math>\pm</math> 2</b>	13 $\pm$ 1	14 $\pm$ 1	7.1 $\pm$ 0.7	6.2 $\pm$ 0.8	14 $\pm$ 1	11 $\pm$ 1	25 $\pm$ 2	27 $\pm$ 2	39.8	***
pltwth	<b>44 <math>\pm</math> 2</b>	<b>50 <math>\pm</math> 2</b>	<b>47 <math>\pm</math> 2</b>	45 $\pm$ 2	34 $\pm$ 2	36 $\pm$ 1	37 $\pm$ 2	45 $\pm$ 2	30 $\pm$ 2	43 $\pm$ 2	45 $\pm$ 1	24.2	***
stolon	<b>2.6 <math>\pm</math> 0.09</b>	<b>3.1 <math>\pm</math> 0.07</b>	<b>2.4 <math>\pm</math> 0.08</b>	3.0 $\pm$ 0.09	2.6 $\pm$ 0.09	2.2 $\pm$ 0.07	2.2 $\pm$ 0.09	2.7 $\pm$ 0.09	2.6 $\pm$ 0.10	3.3 $\pm$ 0.10	3.5 $\pm$ 0.10	40.5	***
hdwgt	<b>2.1 <math>\pm</math> 0.09</b>	<b>2.1 <math>\pm</math> 0.08</b>	<b>1.9 <math>\pm</math> 0.07</b>	1.9 $\pm$ 0.07	2.0 $\pm$ 0.9	2.0 $\pm$ 0.13	1.7 $\pm$ 0.09	2.2 $\pm$ 0.10	1.6 $\pm$ 0.14	2.7 $\pm$ 0.10	2.7 $\pm$ 0.09	25.5	***
seedhd	<b>44 <math>\pm</math> 2</b>	<b>47 <math>\pm</math> 1</b>	<b>39 <math>\pm</math> 1</b>	44 $\pm$ 2	34 $\pm$ 2	40 $\pm$ 3	28 $\pm$ 2	40 $\pm$ 2	28 $\pm$ 2	51 $\pm$ 1	47 $\pm$ 2	27.6	***
sdwgt	<b>1.07 <math>\pm</math> 0.006</b>	<b>1.10 <math>\pm</math> 0.001</b>	<b>1.05 <math>\pm</math> 0.002</b>	0.95 $\pm$ 0.002	1.0 $\pm$ 0.002	1.09 $\pm$ 0.004	1.05 $\pm$ 0.002	0.97 $\pm$ 0.002	1.06 $\pm$ 0.004	1.07 $\pm$ 0.002	1.30 $\pm$ 0.005	18.7	***



PCA explained only 57% of the variation in the first three components with principal component 1 (PC1) and principal component 2 (PC2) explaining < 50% of the total variation (Table 4.7). Leaflet width, petiole and peduncle length, plant height and width, and stolon thickness all had the strongest loading on PC1. Characters with a strong loading on PC2 were head weight and number of seeds per head and to a lesser extent, node of first flower. Flowering time had the strongest loading on principal component 3 (PC3).

Table 4.7. Principal component scores 1, 2, and 3 (PC1, PC2, PC3, respectively), percentage variation and component loadings on plant characters for 11 *T. fragiferum* populations  
Abbreviations for plant characters as in Table 4.2.

	PC1	PC2	PC3
Proportion of variation	0.357	0.118	0.094
Cumulative variation	0.357	0.475	0.569
Component loadings			
flwrtime	0.248	-0.050	-0.565
flwrnode	0.052	-0.448	0.379
lfltlgth	-0.223	-0.097	-0.251
lfltwth	-0.357	0.183	-0.271
pet	-0.392	0.325	0.024
ped	-0.343	0.313	0.168
plthgt	-0.376	-0.015	-0.169
pltwth	-0.322	-0.000	0.457
stolon	-0.309	-0.130	-0.158
hdwgt	-0.242	-0.506	-0.156
seedhd	-0.253	-0.514	0.050
sdwgt	-0.154	-0.104	-0.286

The scores of PC1 and PC2 are plotted in Fig. 4.2 and are overlaid with a biplot of the component scores. The biplot shows that the plant morphological traits were generally negatively correlated with flowering time. Peduncle and petiole lengths were highly correlated with each other, as were stolon thickness and leaflet length, plant width and height, and head weight and seeds per head. There was no correlation between the seed traits (head weight, seed weight and number of seeds per head) and flowering time.

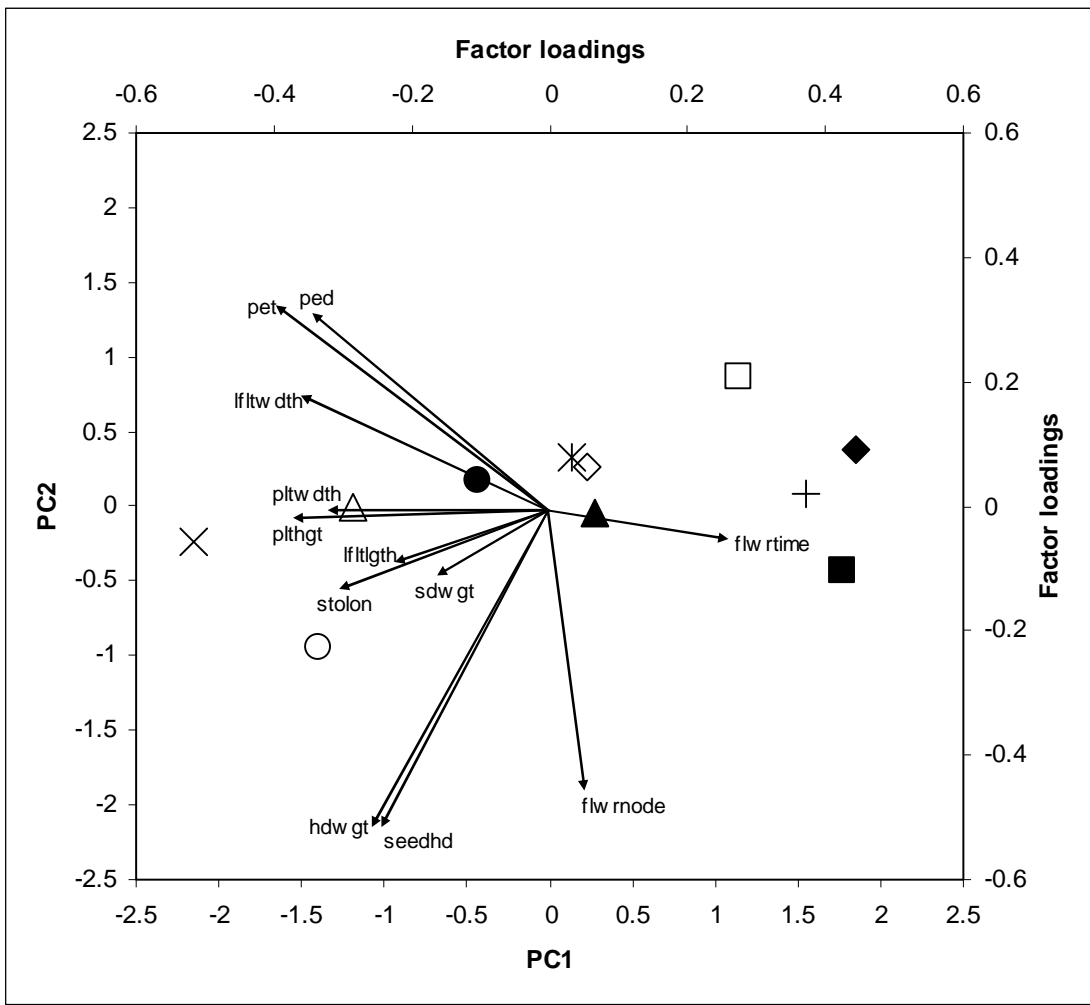


Figure 4.2. Principal components analysis of 11 *T. fragiferum* populations (\*, O'Con; Δ, Pal (NZ); ●, Pal (SA); ◇, Syria; +, Turkey; ■, Spain1; ◆, Spain2; ▲, Iran1; □, Iran2; ○, Tunisia1; ×, Tunisia2) showing the averages for each population. An overlay of the biplot represents the component loadings for principal components 1 and 2 for each of the quantitative characters used in the multivariate analysis.

Figure 4.2 shows the commercial cultivars in relative proximity to each other compared to the wide spread of the Mediterranean populations, indicating there was less variation between them than between populations collected from the Mediterranean. The biplot also shows the populations collected within the same country in relatively close proximity to each other indicating their relative similarity. Tunisia2 had the shortest time to flowering, and Turkey, Spain1, Spain2 and Iran2 were all characterised by longer flowering times. Pal (NZ) and the two Tunisian populations were all characterised by larger plants, with thicker stolons and bigger leaves, while the population from Turkey, both populations from Spain and Iran2 were characterised by generally smaller plants. The biplot shows the remaining 4 populations in the study were characterised by relatively average values for plant traits, including 2 of the 3 commercial cultivars. Head weight, number of seeds per head, and node of first flower

all had only a small impact in differentiating the populations compared to flowering time, plant width, plant height, leaflet length, stolon thickness and seed weight.

#### **4.5 Discussion**

The degree of within-population variation for strawberry clover was similar for both Mediterranean populations and the commercial cultivars, disproving the first hypothesis. There was a greater proportion of within-population than between-population variation for all measured plant characters for all populations of strawberry clover in this study, with the proportion of between-population variation ranging from 7 – 40 %. Despite this, all populations were different from each other. Variation in the Mediterranean populations was generally comparable with the variation found within and between naturalised populations of strawberry clover from Western Australia (Chapter 3). The proportion of between-population variation for the Western Australia strawberry clover populations ranged from 8 – 15 %, somewhat lower than for the Mediterranean populations, and the total variation explained in the first three principal components was 59 % (Chapter 3; McDonald *et al.* 2005). For the Mediterranean populations and commercial cultivars examined in this chapter, total variation explained in the first three principal components was 57 %.

##### *Variation within populations*

As strawberry clover is an outcrossing species (Morley 1963), high levels of variation within populations due to crossing and recombination were expected. It was also expected that the commercial cultivars would exhibit less within-population variation than the Mediterranean populations due to being the product of breeding programs concentrating on specific traits and plant types. In fact, the commercial cultivars had similar amounts of within-population variation as the populations collected in the Mediterranean. It is likely that the outcrossing nature of strawberry clover has resulted in significant genetic drift away from the original parent material of the cultivars, and large amounts of variation are now inherent in these cultivars. Many other outcrossing legumes also have high levels of within-population variation in both cultivars and wild populations, including the annuals balansa clover (*Trifolium michelianum* L.) and Persian clover (*T. resupinatum* L.) (Bennett 1998) and the perennials birdsfoot trefoil (*Lotus corniculatus* L.) (Kelman and Ayres 2004), greater lotus (*L. uliginosus* Schkuhr.) (Ayres *et al.* 2007) and white clover (*Trifolium repens* L.) (Jahufer and Gawler 2000).

A previous study of outcrossing and inbreeding clover species (Bulinska-Radomska 1996) also showed that most of the variation expressed in strawberry clover populations was within-population variation, in contrast to the inbreeding populations.

#### *Variation between populations*

There was generally a medium - low proportion of variation apportioned to between populations compared to within populations for all plant traits. Despite this, there were significant differences between the populations for all measured plant traits, indicating that the populations did differ from each other. The widely varying distribution of the different leaf mark categories also suggests a high degree of genetic variation between the populations. Many of the measured plant traits had a high correlation with some of the environmental factors, mainly latitude and longitude, indicating that differences between the populations may reflect adaptation to the site of collection.

Populations collected from the same country were more similar to each other than those collected from further afield. The biplot shows those populations in close proximity to each other, indicating their similarity, in comparison to populations collected from other countries. Loi *et al.* (1999) also found that differences between populations of two annual legumes (*Biserrula pelecinus* L. and *Ornithopus compressus* L.) were greater where the distance between the collection sites was greatest. Gene flow between populations is more likely as populations become closer.

Those characters associated with plant size (plant height, leaflet length and stolon thickness) exhibited the most variation between the populations. Naturalised strawberry clover in Western Australia (Chapter 3; McDonald *et al.* 2005) showed a similar trend and it was postulated that this reflected ecotypic differentiation due to differences in grazing pressures and regimes. Due to the poor detail of passport data available on the collections of strawberry clover from the Mediterranean Basin it is difficult to draw any conclusions on the reason for the variation in the plant size characters. However as most of the morphological characters measured were strongly negatively correlated with latitude and positively with longitude, and plant width was strongly negatively correlated with altitude it may be inferred that some of the variation among the populations was due to ecotypic differentiation. Latitude, longitude and altitude have been used previously to indicate climatic trends in plant morphological data in the absence of reliable climatic figures (Bennett 2000).



Large differences between the populations in vegetative characters and only a narrow diversity in the reproductive characteristics has also been shown for greater lotus in eastern Australia (Ayres *et al.* 2007). Significant variation between populations in stolon thickness and leaf size has also been shown for white clover (Lane *et al.* 2000). It has previously been concluded that when white clover populations evolve under close continuous grazing, natural selection favours small-leaf genotypes (Williams 1987) and a similar hypothesis has been presented for strawberry clover populations in Western Australia (Chapter 3; McDonald *et al.* 2005). The results in this chapter support the formation of ecotypes through grazing pressure and climate as, from the limited collection site data available, populations from the drier sites in the Mediterranean were generally smaller with smaller leaves and stems than populations from sites with a higher average annual rainfall

#### *Variation in Mediterranean compared to naturalised Western Australian populations*

The proportion of variation attributed to within-population ranged from 7 to 40 %. In a study of the genetic variation between populations of strawberry clover pastures in Western Australia (Chapter 3), McDonald *et al.* (2005) attributed lower proportions of variation to between populations. In that study, variation for the different plant traits measured ranged from 1.5% to 19%. As the populations from the present study were collected from the centre of diversity for strawberry clover (the Mediterranean Basin), it is not surprising that there was more variation between populations than found for Western Australian populations that all originated from the same cultivar.

Principal components analysis highlighted the variability inherent in the strawberry clover populations. This result was very similar to that reported for the naturalised Western Australian pastures of strawberry clover (Chapter 3; McDonald *et al.* 2005).

#### *Genetic variation as a tool for cultivar development*

The high levels of variation found within and between the populations of strawberry clover in this study suggest there is ample scope for judicious breeding programs to target desirable characteristics in the breeding of improved cultivars for use in southern Australia. Lane *et al.* (2000) reached the same conclusion while studying the morphological characteristics of white clover populations collected in New South Wales, Australia. In a study on the genetic variation of seasonal herbage yield, growth habit and condensed tannins in *Lotus pedunculatus* Cav. and *L. corniculatus*, strong

genetic correlations for associations between characters were described for accessions grouped by geographic origin (Kelman *et al.* 1997). It was concluded that selection within, and hybridisation between, the populations in the different geographic groups should be effective in producing cultivars with favourable combinations of the agronomically desirable characteristics. Continued study into the variation for agronomically important traits such as biomass production and seedling vigour could also see improvement in these characters for strawberry clover.

In a review of breeding strategies for white clover in Australia, Jahufer *et al.* (2002) highlight the considerable amount of genetic variation present in existing cultivars and populations of white clover and conclude that extensive genetic potential is available for use in breeding programs. It appears that the same can be concluded for strawberry clover. Together with the high levels of within population variation found in naturalised populations of strawberry clover in Western Australia (Chapter 3; McDonald *et al.* 2005), the considerable variation shown in the Mediterranean Basin populations means that there are ample genetic resources to breed varieties of strawberry clover with improved agronomic characteristics, such as higher seedling vigour, drought tolerance and biomass production. Previous research has shown that strawberry clover is more tolerant of saline conditions than subterranean clover and white clover (Craig 1994). However the value of strawberry clover for improving production on these marginal soils will be increased if better varieties, more suited to southern Australian conditions, can be bred. The targeting of genetic material from populations collected in regions with similar conditions could reveal the characters necessary for these improvements (Bennett 1999; Kelman *et al.* 1997; Lane *et al.* 2000) and thereby speed cultivar development.

## CHAPTER 5

# The Response of Four Strawberry Clover (*Trifolium fragiferum* L.) Populations and Lucerne cv. Pioneer L69 to Waterlogging and Salinity Under Glasshouse Conditions.

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### 5.1 Abstract

Salinity and waterlogging often occur together in southern Australian farming systems and greatly reduce agricultural production. In this chapter the response to salinity and waterlogging is compared between four populations of strawberry clover (*Trifolium fragiferum* L.) and one population of lucerne (*Medicago sativa* L.) cv. Pioneer L69. Plants were grown in the glasshouse for eight weeks and then subjected to four treatments for 30 days – no salinity or waterlogging (control), salinity (120 mM) with no waterlogging, waterlogging with no salinity, and waterlogging + salinity (120 mM). Root porosity, tap root length, leaf area, root dry weight, shoot dry weight and root-shoot ratio were measured. For each of these parameters there was a significant interaction between population, salinity and waterlogging. Waterlogging and salinity alone had little impact on lucerne shoot dry weights but decreased lucerne root dry weights. Waterlogging alone had no impact or increased shoot (up to 125%) and root dry weight in three of the four strawberry clover populations and decreased shoot (40%) and root dry weight in one population. Salinity alone decreased shoot dry weight (8-95%) in three of the four populations and had no impact in one population. Root dry weight was decreased in two populations of strawberry clover and remained unaffected in the remaining two populations by salinity alone. The combination of waterlogging and salinity magnified the negative effect of salinity alone on shoot and root dry weight for lucerne and two populations of strawberry clover; but for the two populations of strawberry clover that were most negatively affected by salinity alone, there was little additional impact from adding waterlogging. Root porosity was lowest in lucerne. The impact of treatment on root porosity was extremely variable. For strawberry clover, maximum porosity (~ 12-16%) occurred in a different treatment for each population and did not correspond with waterlogging tolerance. Root-shoot ratio was highest in lucerne in the control (1.8) and was reduced to ~0.7 by the three treatments. Root-shoot ratio varied from 0.6-1.2 in the strawberry clover populations and the impact of treatment

varied greatly between populations. This study highlights the generally very good tolerance of strawberry clover to waterlogging and the extremely large degree of variation that exists between the populations of strawberry clover in their response to salinity and waterlogging. The study indicates that it should be possible to select breeding lines with an increased tolerance to these two stresses and thereby enhance their adaptation to areas affected by dryland salinity.

## **5.2 Introduction**

Large areas of agricultural land in Australia are under threat from secondary salinity. The acknowledged cause of the spread of secondary salinity is rising groundwater tables (Williamson 1986) due to the replacement of native deep-rooted perennial vegetation with annual, shallow-rooting agricultural crops and pastures. Secondary salinity in Australia is also widely associated with waterlogging in the landscape (Barrett-Lennard *et al.* 1999; Qureshi and Barrett-Lennard 1998). Thus in areas where groundwater tables reach close to the surface, plants often have to cope with both salinity and waterlogging, at least at some times during the year. The largest impact of waterlogging on plant growth occurs due to decrease in the availability of oxygen in the soil. In saline conditions, this then inhibits the ability of plant roots to screen out salt at the root surface (Qureshi and Barrett-Lennard 1998), leading to a large increase in shoot sodium and chloride concentrations that have adverse effects on plant growth and survival (Barrett-Lennard 2003).

To slow the rise of ground water tables it is necessary to reduce recharge to groundwater systems. To achieve this, new farming systems incorporating perennial vegetation need to be developed (Cocks 2001). Perennial pastures can provide an economic and environmentally sustainable option to increase the percentage of agricultural land under perennial vegetation (Ewing and Moore 1999). Perennial pastures use more water than annual crops and pastures (Latta *et al.* 2001) and therefore can reduce groundwater recharge. To manage groundwater recharge on a landscape scale it will be necessary to grow perennial pastures on the valley floor discharge zones, where groundwater comes to the surface, as well as on the mid-slopes. To successfully incorporate perennial pastures into farming systems, a sound understanding of their growth and ecology is necessary. Unfortunately until recent times, there has been relatively little investigation of the ecology and growth of perennial pastures in

Australia (Cocks 2001). Lucerne (*Medicago sativa* L.) has received much recent attention as a commercially viable perennial pasture suited to broadacre agriculture (Dolling *et al.* 2007; Verburg *et al.* 2007a; Verburg *et al.* 2007b; Ward and Micin 2006). However, lucerne is considered to lack tolerance to waterlogging (Humphries and Auricht 2001; Leach 1978) and so is unsuitable for the increasing area of soils in discharge zones.

Strawberry clover (*Trifolium fragiferum* L.) has been classified as moderately tolerant to waterlogging (Hoveland and Mikkelsen 1967) and it is regarded as one of the more important pasture legumes for saline and sodic soils in the United States of America (Townsend 1985). It is one of seven species in the section Vesicaria of the *Trifolium* genus and has its centre of origin in the Mediterranean Basin (Zohary and Heller 1984), although it is widely naturalised throughout the world. Plants from section Vesicaria are considered to be physiologically adapted to soil waterlogging and indeed some grow better under waterlogged conditions than under free drainage (Gibberd and Cocks 1997). In Australia, strawberry clover has predominantly been used in medium – high rainfall zones as a niche species for waterlogged and/or mildly saline soils, or soils that are too alkaline for white clover (*Trifolium repens* L.) (Craig 1994).

A study by Rumbaugh *et al.* (1993) investigated the variation in salinity tolerance of strawberry clover cultivars and wild introductions, but did not include waterlogging as a treatment. They found ample genetic diversity present within the species to permit the development of cultivars with superior tolerance to salinity during seed germination. Conversely a study on lucerne investigating the nutritional status and photosynthetic response of different genotypes to waterlogging found no significant genotypic difference between the four cultivars included in the study, all were adversely affected by waterlogging (Smethurst *et al.* 2005). Other studies have investigated the response of different *Trifolium* species, including strawberry clover, to waterlogged and saline conditions and have found that those species better adapted to growth in these conditions have the ability to form gas spaces, or aerenchyma, in their roots; that is, they have an increased root porosity (Gibberd and Cocks 1997; Gibberd *et al.* 1999; Rogers and West 1993).

Root porosity is the proportion of root volume consisting of air spaces and is indicative of the amount of aerenchyma cells in the root. Aerenchyma are gas filled spaces formed

by the separation of cells (schizogeny) or the breakdown of cells (lysigeny) forming a longitudinally interconnected system along the tissue axis, which provides a low resistance internal pathway for gaseous diffusion (Gibberd 1997). These cells are believed to aid oxygen diffusion through the root and are hence considered an adaptation of plants to waterlogged conditions (Colmer *et al.* 1998; Justin and Armstrong 1987; Thomson *et al.* 1990). High root porosity is therefore considered to be an adaptation for waterlogging tolerance in plants (Armstrong *et al.* 1991). A study of six species of *Trifolium* showed that roots produced by waterlogged strawberry and white clover plants had a higher porosity than those grown in drained conditions (Rogers and West 1993). The same result was reported by Gibberd *et al.* (1999) who investigated root porosity and oxygen movement in waterlogging-tolerant *T. tomentosum* L. and waterlogging-intolerant *T. glomeratum* L. Root porosities for both species were increased under waterlogged conditions compared to drained conditions (from 6.7% to 11.2% in *T. tomentosum* and from 3.9% to 6.1% in *T. glomeratum*), although waterlogging-tolerant *T. tomentosum* had the higher root porosity for both treatments. Other recent studies investigating the effects of waterlogging on various perennial legumes with potential for development for Australian farming systems have focused on lucerne (Smethurst *et al.* 2005), *Lotus corniculatus* L. and *L. tenuis* Wadst. & Kit. (Teakle *et al.* 2007; Teakle *et al.* 2006), a range of *Lotus* species (Real *et al.* 2008) and *Melilotus* species (Rogers *et al.* 2008).

In this chapter, the variation in root and shoot responses, including root porosity, to salinity and waterlogging of four populations of strawberry clover from widely differing origins is compared with the response of a commercial cultivar of lucerne. The inclusion of two strawberry clover populations from long-term established pastures from Western Australia, that were originally sown with the same cultivar, allowed investigation into changes in population response to waterlogging and salinity due to adaptation to different environments. The inclusion of two populations of strawberry clover collected in the Mediterranean Basin allowed comparison of the variation in plant response to waterlogging and salinity in wild populations from the centre of origin of strawberry clover. Lucerne was included as a salinity and waterlogging-sensitive control (Humphries and Auricht 2001). It was hypothesised that: (a) all populations of strawberry clover would show better root and shoot growth than lucerne when grown in waterlogged and saline conditions; (b) the strawberry clover populations would have an increased root porosity compared to lucerne when grown in waterlogged conditions; and

(c) the effect of the treatments on root and shoot growth, and root porosity, of the strawberry clover populations would differ due to their evolution in different environments.

### **5.3 Materials and methods**

Four populations of strawberry clover were included in this experiment. Two populations were from the Mediterranean Basin (Spain and Tunisia – see Chapter 4 for site details) and sourced from the Trifolium Genetic Resource Centre at the Department of Agriculture and Food, Western Australia. The other two populations were collected from naturalised Western Australian populations of cultivar Palestine (Badgingarra and Karridale Irrigated – see Chapter 3 for site details). The commercial lucerne cultivar Pioneer L69 was also included in the experiment as a waterlogging-sensitive control.

In September 2002, six seeds of each population were sown directly into 12 pots each of 15 cm diameter by 15 cm depth filled with washed coarse river sand. Each pot was one experimental unit. The appropriate rhizobia (commercial rhizobia Group B) were watered on at the time of sowing. At first trifoliate leaf emergence, seedlings were thinned to three per pot. Pots were arranged in a completely randomised design with 4 replicates of each treatment. They were placed in a naturally lit glasshouse and were watered with tap water and fertilised with Thrive, a commercial liquid fertiliser (N:P:K 77:5.5:9 plus trace elements) at sowing and then weekly until treatments were imposed.

Treatments were imposed after eight weeks of growth, with most plants having around three trifoliate leaves emerged. Pots were in a closed system so that no water or nutrients were lost. To reduce the effects of osmotic shock on the plants, the NaCl treatments (0 and 120 mM NaCl) were applied in increments of 20 mM per day, watered on, until the desired level of 120 mM of NaCl was reached. Waterlogging treatments (0 and 30 days of soil saturation) were imposed once the target NaCl level had been reached by simply flooding the pots with tap water. Waterlogging was maintained by keeping the pots inundated with water at 1 cm above the soil surface; it was assumed that this flooding would reduce oxygen levels in the pots to sufficiently low levels so as to affect plant growth. Watering of non-waterlogged treatments was twice per week to prevent wilting of plants due to moisture stress. Harvests were taken on the day treatments were imposed (day 0) and at the end of the experimental period

(day 30). At each harvest, shoots and roots were removed from the pots, separated and weighed. Leaf area was measured with an electronic planimeter. Roots were washed from the soil and tap root length measured. Root porosity measurements were taken from the root samples in the manner described below. Roots and shoots were then dried for 48 hours at 70 °C and dry weights recorded.

Root porosity was measured by the method described by Raskin (1983) and was calculated from equations developed by Raskin (1983), and modified in Thomson *et al.* (1990). Root systems of all plants in each pot were cut into 50 mm sections, gently blotted to remove surface water, and weighed to determine fresh weight in air ( $W_a$ ). The roots were then held in a large paper clip and weighed submerged in water from the pan hook of a four point balance to determine their weight submerged ( $W_s$ ). Roots were then infiltrated with water by three cycles of five minutes of vacuum while submerged. Roots were again weighed while submerged in water to determine the submerged weight of infiltrated roots ( $W_{si}$ ). Root porosity was then calculated using the following equations:

$$\text{Volume of roots} = \frac{(W_a - W_s)}{\text{Density of water}^*}$$

$$\text{Root porosity (\%)} = \frac{(W_{si} - W_s)}{\text{Volume of roots}} \times 100$$

\*weights were measured in milligrams as the density of water at room temperature can be approximated as 1 mg mm<sup>-3</sup> effectively making Volume of roots =  $W_a - W_s$ .

Plant health and leaf colour were recorded at the final harvest as a visual assessment on all plants. Details were recorded as to whether plants were dead, dying (no hope of recovery), in poor health (possibility of recovery) or healthy. Leaf colour (green, yellowing or yellow (including dead leaves)) was assessed as the colour of the majority of leaves on each plant.



### *Statistical analysis*

Data for shoot dry weight, root dry weight, root-shoot ratio, tap root length, root porosity and leaf area were analysed with analysis of variance (ANOVA) with a general factorial treatment structure of population  $\times$  +/- waterlogging  $\times$  +/- salinity (Genstat 7, VSN International Ltd, Hertz, UK). Tests for normality and homogeneity of variance were conducted and data transformed prior to analysis if required. Where transformation was required, untransformed means are also presented.

## **5.4 Results**

### *Initial harvest*

There were no differences between populations in root weight, root porosity, leaf area or root-shoot ratio at the initial harvest. Lucerne had a longer tap root length and greater shoot weight than any of the strawberry clover populations, which did not differ from each other (Table 5.1). Root porosity and root-shoot ratio results exhibited particularly high coefficients of variation.

*Table 5.1.* Root length, root porosity, leaf area, root weight, shoot weight and root-shoot ratio means for lucerne cv. Pioneer L69 and 4 populations of strawberry clover at the initial harvest after 8 weeks of growth

ns – not significant, CV% = co-efficient of variation

Species/Population	Tap root length (cm)	Porosity (%)	Leaf area* (sqrt cm <sup>2</sup> )	(cm <sup>2</sup> )	Root weight (mg)	Shoot weight (mg)	Root:shoot
Lucerne	30.5	2.5	17.8	(3.21)	145	173	0.8
Badgingarra	21.2	3.6	11.3	(1.34)	70	78	1.1
Karridale Irrigated	16.5	8.5	10.6	(1.33)	68	38	2.2
Spain	14.0	5.3	8.3	(0.72)	68	45	3.3
Tunisia	16.5	8.8	15.0	(3.17)	110	70	1.4
p	0.009	ns	ns		ns	0.009	ns
lsd (p = 0.05)	8.8					70.1	
CV %	29.7	77.8	46.5		58.6	56.6	79.6

\* Means presented are from square root transformed data, with actual means in parentheses

### *Final harvest*

Results for the final harvest are contained in Table 5.2 and Figures 5.1 – 5.6. There was a significant 3-way interaction between population, salinity and waterlogging for all variables.

Table 5.2. ANOVAs of measures of lucerne and strawberry clover growth: shoot dry weight (mg/plant) (CV% = 5.4, lsd = 0.2), root dry weight (mg/plant) (CV% = 6.1, lsd = 0.2), root-shoot ratio (CV% = 16, lsd = 0.2), tap root length (cm) (CV% = 15.3, lsd = 3.5), leaf area (cm<sup>2</sup>) (CV% = 5.1, lsd = 0.5) and root porosity (%) (CV% = 30.8, lsd = 3.6). Parameters included are population (lucerne, Badgingarra, Karridale, Spain, Tunisia), water (+/- waterlogging) and salt (+/- salinity). All interaction terms are included. n = 80. ns – not significant

Dependant variable	Predictor variable	F-ratio	Prob.
log <sub>10</sub> Shoot dry weight	population	15.5	< 0.001
	water	6.1	0.016
	salt	495.0	< 0.001
	population × water	2.6	0.043
	population × salt	38.4	< 0.001
	water × salt	28.9	< 0.001
	population × water × salt	7.0	< 0.001
log <sub>10</sub> Root dry weight	population	12.5	< 0.001
	water	79.7	< 0.001
	salt	545.0	< 0.001
	population × water	7.2	< 0.001
	population × salt	20.7	< 0.001
	water × salt	34.6	< 0.001
	population × water × salt	17.6	< 0.001
Root-shoot ratio	population	27.3	< 0.001
	water	208.3	< 0.001
	salt	50.2	< 0.001
	population × water	15.3	< 0.001
	population × salt	15.1	< 0.001
	water × salt	0.1	ns
	population × water × salt	22.5	< 0.001
Root length	population	11.8	> 0.001
	water	28.4	> 0.001
	salt	1119.7	> 0.001
	population × water	6.2	> 0.001
	population × salt	19.5	> 0.001
	water × salt	20.5	> 0.001
	population × water × salt	5.1	0.001
log <sub>e</sub> Leaf area	population	22.8	< 0.001
	water	0.8	ns
	salt	1691.0	< 0.001
	population × water	2.1	ns
	population × salt	8.0	< 0.001
	water × salt	8.5	0.005
	population × water × salt	2.7	0.039
Porosity	population	17.6	< 0.001
	water	6.7	0.012
	salt	20.9	< 0.001
	population × water	4.5	0.003
	population × salt	11.4	< 0.001
	water × salt	0.3	ns
	population × water × salt	10.2	< 0.001

### Shoot dry weight

Lucerne shoot dry weight was generally less affected by all treatments than strawberry clover (Fig 5.1). Relative to the controls, salinity alone had no impact on shoot dry weight of lucerne and Badgingarra strawberry clover, but decreased shoot dry weight in the other strawberry clover populations, from 50% - 95%. Waterlogging alone had no impact on shoot dry weight of lucerne and Badgingarra and Tunisia strawberry clovers, decreased shoot dry weight in Karridale strawberry clover by 40% and increased shoot dry weight of Spain strawberry clover by 125%. Salinity + waterlogging substantially decreased shoot dry weight in lucerne by 39% and the strawberry clover populations by 77% - 95%. This treatment always resulted in a lower shoot dry weight than waterlogging alone, but did not differ from salinity alone for Karridale and Tunisia strawberry clovers, the populations that suffered the greatest negative impact from salinity alone.

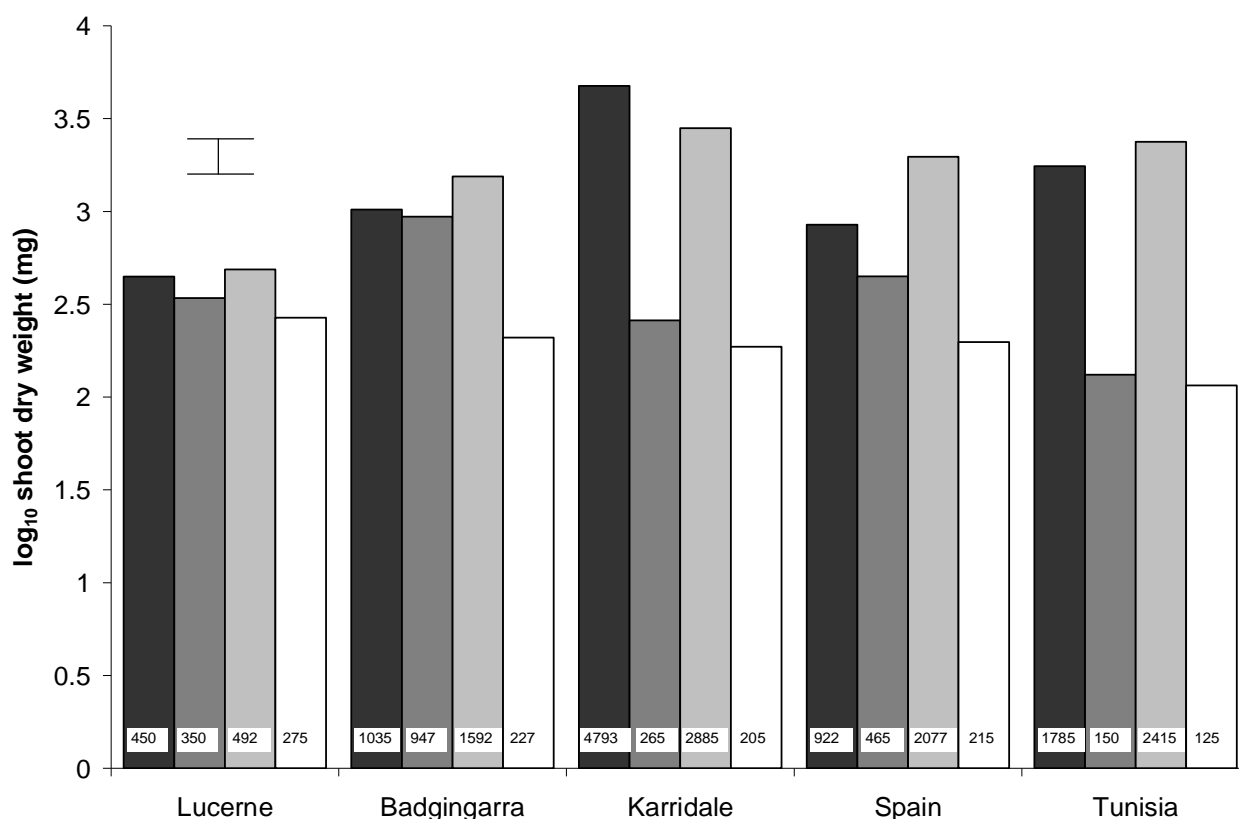


Figure 5.1. Estimated means and LSD at  $p=0.05$  for shoot dry weight measured for a commercial cultivar of lucerne and 4 populations of strawberry clover subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity. Data for shoot dry weight was  $\log_{10}$  transformed and untransformed means in mg are given at the base of each column.

### Root dry weight

Root dry weight was quite similarly affected by all treatments for lucerne and strawberry clover (Fig 5.2). Salinity alone decreased root dry weight for lucerne (67%) and Karridale (96%) and Tunisia (89%) strawberry clovers, and had no effect on Badgingarra and Spain strawberry clovers. Waterlogging alone increased root dry weight in Badgingarra (51%) and Spain (88%) strawberry clovers, decreased root dry weight in lucerne (54%) and Karridale strawberry clover (81%) and had no effect on Tunisia strawberry clover. Salinity + waterlogging substantially decreased root dry weight in all populations (78% - 99%). As with shoot dry weight, those populations which were most affected by salinity alone, Karridale and Tunisia strawberry clovers, were then the least affected by the addition of waterlogging to salinity.

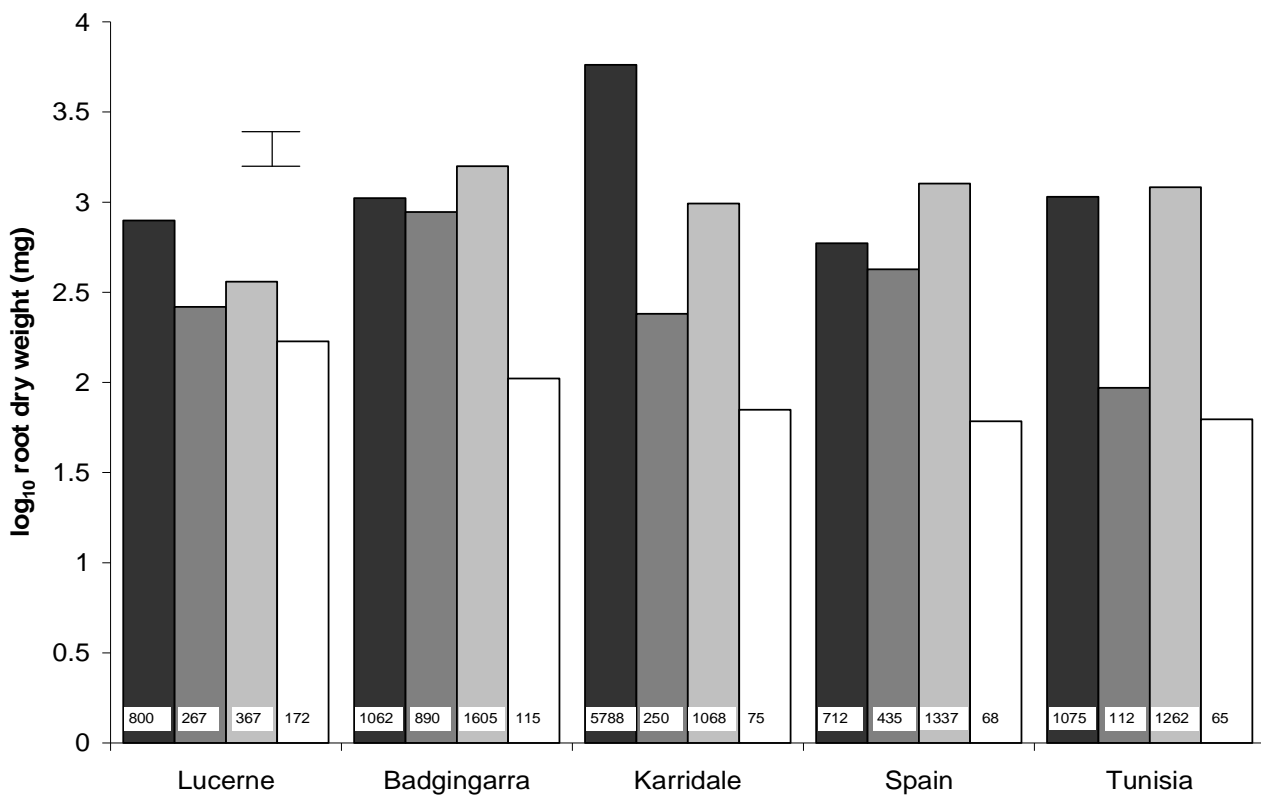


Figure 5.2. Estimated means and LSD at  $p=0.05$  for root dry weight measured for a commercial cultivar of lucerne and 4 populations of strawberry clover subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity. Data for root dry weight was  $\log_{10}$  transformed and untransformed means in mg are given at the base of each column.

### Root-shoot ratio

The root-shoot ratio was higher for lucerne than strawberry clover and was reduced to a greater degree by all treatments for lucerne than strawberry clover (Fig 5.3). Salinity alone decreased the root-shoot ratio in lucerne and Karridale strawberry clover, increased the root-shoot ratio in Spain strawberry clover and had no effect on root-shoot ratio in Badgingarra and Tunisia strawberry clovers. Waterlogging alone decreased the root-shoot ratio in lucerne and Karridale strawberry clover and had no effect on Badgingarra, Spain and Tunisia strawberry clovers. Salinity + waterlogging decreased root-shoot ratios in all populations apart from Tunisia strawberry clover, which was unaffected by all treatments.

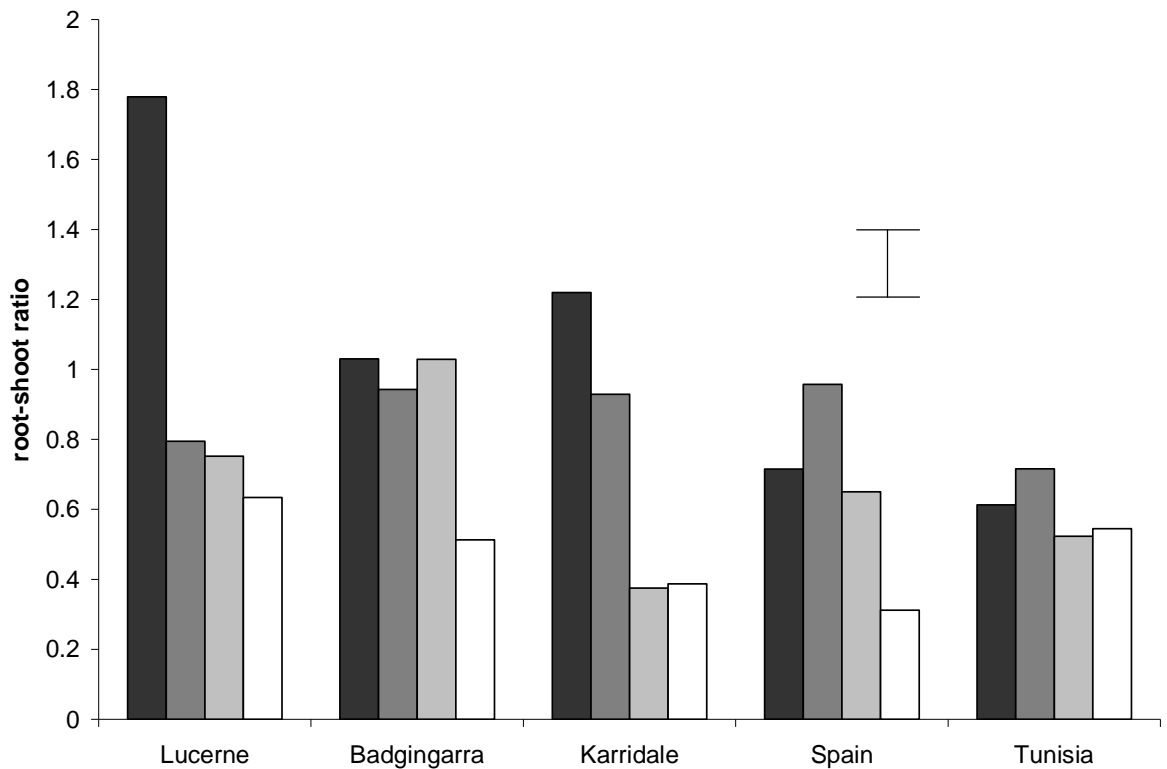


Figure 5.3. Estimated means and LSD at  $p=0.05$  for the root-shoot ratio measured for 4 populations of strawberry clover and one population of lucerne subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity.

### Tap root length

Tap root length was similar for lucerne and strawberry clover and was, in general, similarly affected by the treatments (Fig 5.4). Salinity alone substantially decreased tap root length in all populations by 65% - 83%. Waterlogging alone decreased tap root length in lucerne (47%) and Badgingarra strawberry clover (31%), and had no effect for Karridale, Spain and Tunisia strawberry clovers. Salinity + waterlogging substantially decreased tap root length in all populations, but only to the same degree as salinity alone.

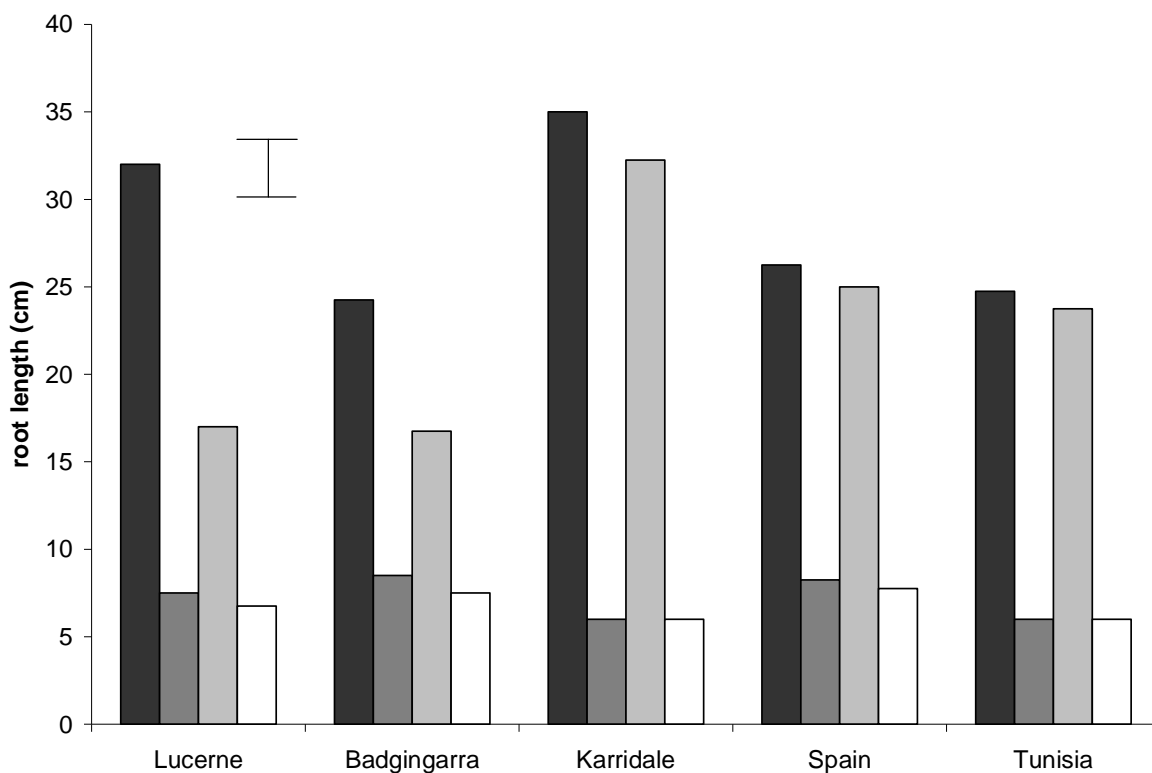


Figure 5.4. Means and LSD at  $p=0.05$  for tap root length measured for a commercial cultivar of lucerne and 4 populations of strawberry clover subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity.

### Leaf area

Leaf area was similarly affected by the treatments for lucerne and strawberry clover (Fig. 5.5). Salinity alone decreased leaf area by 92% for lucerne and 89% - 97% for strawberry clover. Waterlogging alone decreased leaf area in Karridale strawberry clover by 44%, increased leaf area in Spain strawberry clover by 135% and had no effect on lucerne and Badgingarra and Tunisia strawberry clovers. Salinity + waterlogging decreased leaf area in all populations to a similar extent as salinity alone. The populations with the highest leaf area in the control, Badgingarra and Karridale strawberry clovers, experienced the greatest reduction in leaf area when exposed to salinity, with or without waterlogging.

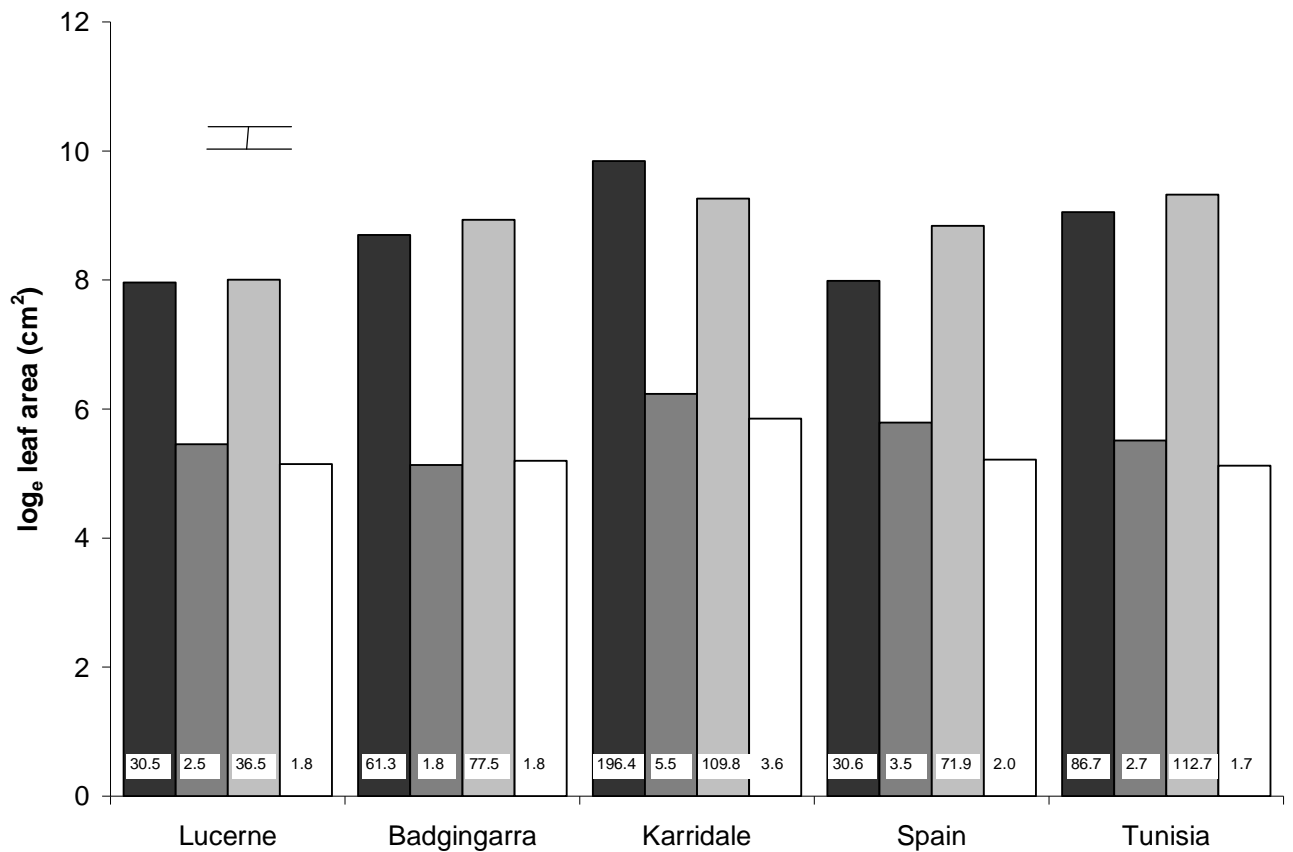


Figure 5.5. Estimated means and LSD at  $p=0.05$  for leaf area measured for a commercial cultivar of lucerne and 4 populations of strawberry clover subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity. Data for leaf area was  $\log_e$  transformed and untransformed means in  $\text{cm}^2$  are given at the base of each column.

### Root porosity

Root porosity was generally lower for lucerne than strawberry clover and was less affected by the treatments for lucerne than strawberry clover (Fig. 5.6). There was a high degree of variation in root porosity between the strawberry clover populations. For instance, maximum porosity (~ 12-16%) occurred in a different treatment for each population. Salinity alone decreased root porosity in Spain strawberry clover, increased root porosity in Karridale and Badgingarra strawberry clovers and had no effect on root porosity for lucerne and Tunisia strawberry clover. Waterlogging alone increased root porosity in Tunisia strawberry clover and had no effect on root porosity in lucerne and Badgingarra, Karridale and Spain strawberry clovers. Salinity + waterlogging increased root porosity for lucerne and Badgingarra strawberry clover and had no effect for Karridale, Spain and Tunisia strawberry clovers.

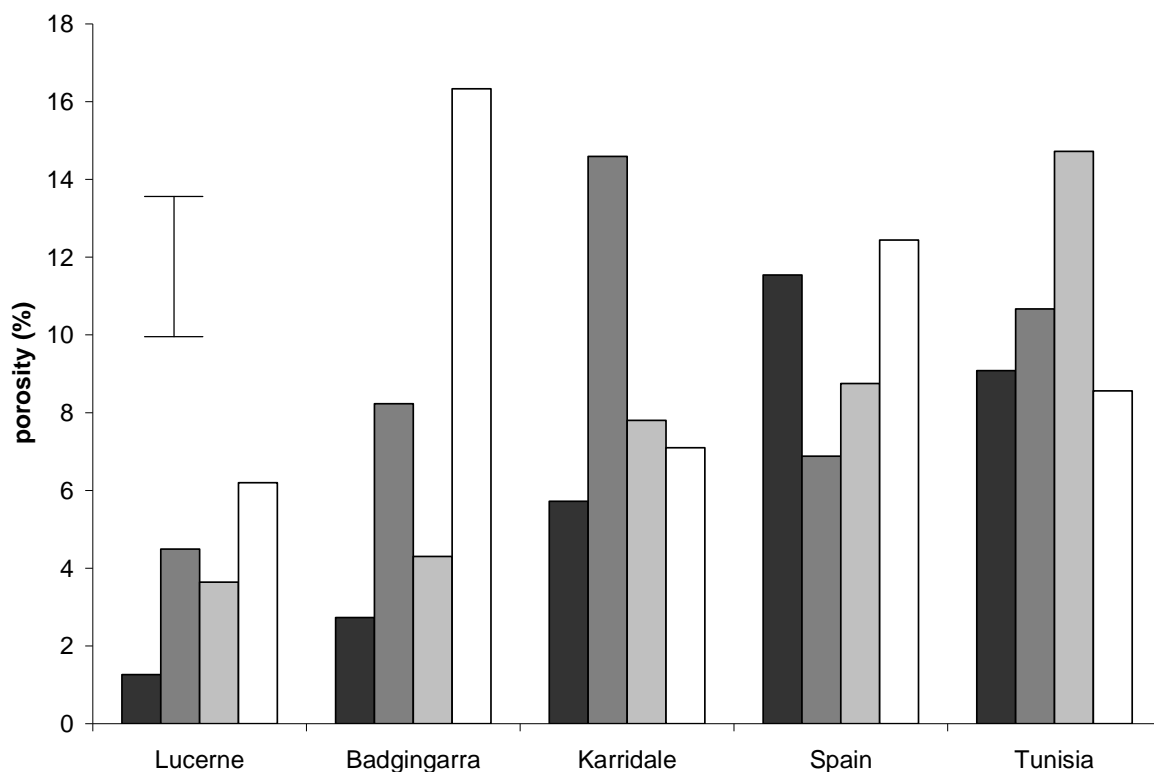


Figure 5.6. Means and LSD at  $p=0.05$  for root porosity measured for a commercial cultivar of lucerne and 4 populations of strawberry clover and one population of subjected to 4 waterlogging and salinity treatments; (a) ■ - waterlogging - salinity (b) ■ - waterlogging + salinity (c) ■ + waterlogging - salinity (d) □ + waterlogging + salinity.



### *Plant health*

Plant health varied considerably between populations and treatments (Table 5.3). Despite having similar or better shoot dry matter weights than the strawberry clover plants in the three treatments, lucerne plants generally appeared in poorer health. When exposed to waterlogging alone all populations of strawberry clover appeared in good health and had green, healthy leaves. When exposed to salinity alone all populations of strawberry clover appeared in poor health. However, apart from Karridale strawberry clover which had yellowing leaves, leaves on these populations were still green. When exposed to the combined stresses of salinity and waterlogging all populations appeared in poor plant health, or were dead. The ability of the four populations to recover from the treatments had the salinity and waterlogging been removed can only be speculated on, based on their apparent plant health. It seems unlikely that lucerne could have recovered from any of the treatments. All populations of strawberry clover probably would have been able to recover from the waterlogging alone treatment, and it is possible that the Badgingarra, Spain and Tunisia populations could also have recovered from the salinity alone treatment. It is very unlikely that any of the strawberry clover populations would have been able to recover from the salinity + waterlogging treatment.

*Table 5.3.* General plant health (good, poor, dying or dead) and leaf colour (green, yellowing or yellow) of lucerne cv. Pioneer L69 and 4 populations of strawberry clover at the time of the final harvest

Plant health	Waterlogging	Salinity	Plant health	Leaf colour
lucerne	-	-	good	green
	-	+	dying	yellow
	+	-	poor	yellowing
	+	+	dead	yellow
Badgingarra	-	-	good	green
	-	+	poor	green
	+	-	good	green
	+	+	dying	yellowing
Karridale Irrigated	-	-	good	green
	-	+	poor	yellowing
	+	-	good	green
	+	+	poor	yellowing
Spain	-	-	good	green
	-	+	poor	green
	+	-	good	green
	+	+	dead	yellow
Tunisia	-	-	good	green
	-	+	poor	green
	+	-	good	green
	+	+	dead	yellow

## **5.5 Discussion**

The major findings of interest in this study were i) the variable, but often positive, response of strawberry clover to waterlogging, and ii) the variable response of strawberry clover to salinity and salinity + waterlogging. These results suggest considerable potential to breed for better salinity and waterlogging tolerance in strawberry clover. These findings, and others of interest, are now discussed in more detail.

### *Shoot and root growth*

Shoot and root growth of lucerne was affected by waterlogging alone in a similar manner to strawberry clover. This was an unexpected result as lucerne is often reported to be relatively intolerant of waterlogging (Humphries and Auricht 2001); although it appears that no previous studies have used the cultivar Pioneer L69. For instance, Rogers *et al.* (2008) observed that when subjected to stagnant (de-oxygenated agar nutrient solution) conditions from four weeks after sowing for a period of four weeks, shoot dry weight for lucerne cv. Sceptre suffered substantial decreases in comparison to strawberry clover which was unaffected. However in the current experiment lucerne plants exposed to waterlogging were not in good health at the end of the treatment period as opposed to the strawberry clover populations (Table 5.3), suggesting that longer exposure to waterlogging may have resulted in further reductions in plant growth, and possibly death. In addition, waterlogging damage for lucerne is increased by factors such as infection by fungal pathogens (Barta and Schmittener 1986) and these presumably were not present in the washed river sand used for the experiment. Indeed, Humphries and Auricht (2001) described the response of lucerne to waterlogging as “wet soil syndrome” and suggested that there was no one single causative factor. Instead, the cause and intensity of the wet soil syndrome depends on the magnitude and balance of all the individual stresses (Alva *et al.* 1985). In this chapter, while Karridale strawberry clover showed a large decrease in shoot and root dry weight in response to waterlogging, the other three populations were unaffected or showed increased growth. This indicates the potential good tolerance of strawberry clover to waterlogging, but that all germplasm can not be assumed to possess this tolerance. Such tolerance of waterlogging has been reported for other species including white clover (Pugh *et al.* 1995).

Salinity alone at 120 mM had a negative impact on shoot dry weight of three strawberry clover populations and no impact on Badgingarra strawberry clover or lucerne.

Although, leaf area and tap root length were dramatically reduced by salinity for Badgingarra strawberry clover and lucerne, and for lucerne only, root dry weight was also reduced. Lucerne is considered one of the more tolerant legumes to salinity as it is able to regulate the uptake and translocation of  $\text{Na}^+$  and  $\text{Cl}^-$  to prevent excessive build up in the shoots (Rogers *et al.* 2005). However, Rogers (2001) found dry matter production was still retarded for six lucerne cultivars tested (which did not include Pioneer L 69, the cultivar used in this study) in saline soil (3.8 dS/m). Small increases in shoot growth for both lucerne cv. Sceptre and strawberry clover cv. Palestine have been reported at 80 mM NaCl, while at 160 mM NaCl small decreases in shoot growth occurred (Rogers *et al.* 2008). The present study used a salt concentration of 120 mM in the salinity and salinity + waterlogging treatments. Even at salt concentrations of 20 mM NaCl, growth of wheat plants was severely affected, beyond recovery, when also exposed to waterlogging (Barrett-Lennard 2003). A better understanding of the response of the strawberry clover populations to salinity may have been achieved with a lower salt concentration which did not affect plant growth so severely.

Shoot dry weight was substantially decreased for all strawberry clover populations when the stresses of salinity and waterlogging were combined. Lucerne shoot growth was also negatively affected, although to a lesser extent. Interestingly, in the two strawberry clover populations where salinity alone had the greatest impact, Karridale and Tunisia, the addition of waterlogging did not further decrease shoot dry weight, although for Karridale, root dry weight was further reduced. Rogers and West (1993) also found that saline (60 mM) waterlogged conditions produced an overall decrease in shoot dry weight in their investigation of a range of *Trifolium* species, but not the large decrease found in this study. They found that increasing external salinity caused increased shoot concentrations of both  $\text{Na}^+$  and  $\text{Cl}^-$  and a decrease in shoot  $\text{K}^+$ , thus inhibiting shoot growth. Increased transportation and accumulation of  $\text{Na}^+$  and  $\text{Cl}^-$  in the shoots was further increased when salinity was combined with waterlogging.

Root dry weight and tap root length were generally less affected by waterlogging for strawberry clover than lucerne. Reduced tap root lengths in response to waterlogging have been reported in a wide range of annual accessions of *Trifolium* (Gibberd *et al.* 1999) and decreased root growth rates have also been observed in *Trifolium glomeratum*

and *T. tomentosum* in waterlogged conditions (Gibberd *et al.* 1999) and in wheat under saline and hypoxic conditions (Barrett-Lennard *et al.* 1999). It is likely that many waterlogged saline discharge areas in southern Australian farming regions become dry in summer and suffer drought conditions. For perennial plants in these areas, a reduction in root dry weight and tap root length could be very detrimental to their survival due to a reduced ability to access soil moisture and nutrients at depth. Thus populations with improved root growth in saline and waterlogged soils may have a better chance at maintaining a productive pasture over variable seasonal conditions.

Previous studies report that *Trifolium* species tend to form new lateral roots in response to waterlogging (Gibberd *et al.* 1999; Gibberd *et al.* 2001; Rogers and West 1993).

While no measurements of lateral root formation were taken in this study, it was noted that lateral roots were more prolific in the waterlogged only treatment for all populations of strawberry clover, and to a lesser extent in lucerne. Gibberd *et al.* (1999) suggested that the lateral roots may become a sink for oxygen from the aerenchyma in the primary root and therefore decrease oxygen supply to the primary root apex and so limit the root length of these species. Thus, it is possible that proliferation of lateral roots was limiting the performance of strawberry clover when exposed to waterlogging, as reduced oxygen supply would adversely affect plant growth.

Root-shoot ratios were generally decreased in all treatments compared to the control. Exceptions to this were a small increase in root-shoot ratio for Spain strawberry clover in salinity alone, and no change in Badgingarra strawberry clover for salinity or waterlogging alone and for Tunisia strawberry clover across all treatments. This is in contrast to an earlier study of annual species of *Trifolium* where the root-shoot ratios of species considered 'tolerant' of waterlogging (*T. resupinatum* L., *T. michelianum* Savi. and *T. tridentatum* L.) all increased significantly under waterlogged relative to drained conditions (Gibberd *et al.* 2001). There does not appear to be an explanation for this, except that in the present study both shoot and root growth were very similarly affected by the salinity and waterlogging treatments, and so root-shoot ratios were not as affected. It may be that lateral root formation (contributing to an increase in root dry weight and hence an increased root-shoot ratio) was not as prolific in the perennial strawberry clover as for its annual counterparts. The large decrease in root-shoot ratio for lucerne is comparable to results for waterlogging susceptible accessions of *T. glomeratum* and *T. subterraneum* L. (Gibberd *et al.* 2001) and reflects a large decrease

in root growth while shoot growth was maintained thus raising the possibility that plants affected in this way would subsequently have reduced drought tolerance.

#### *Root porosity*

Root porosity tended to be higher for strawberry clover populations than for lucerne. However, only one strawberry clover population showed an increase in porosity in response to waterlogging alone (Tunisia). Karridale strawberry clover showed an increase in root porosity in response to salinity alone while Spain showed a decrease in response to this treatment. Badgingarra strawberry clover had increased root porosities in response to salinity alone and salinity + waterlogging, while lucerne showed increased root porosity only when exposed to salinity + waterlogging. Rogers and West (1993) found that root porosity for strawberry clover, *T. michelianum*, *T. subterraneum* and white clover increased under waterlogged + saline conditions compared to the non-saline and non-waterlogged control. Strawberry clover and white clover had about 14% root porosity when grown under saline flooding, which is consistent with this study where porosities of 8 – 16% were measured under the salt + waterlogging treatment. Rogers and West (1993) also showed that while waterlogging produced the highest root porosities for *T. michelianum*, *T. subterraneum* exhibited its highest porosity in saline only conditions, and strawberry clover and white clover had higher porosities under a combination of saline and waterlogged conditions.

A study into root aeration in two annual *Trifolium* species concluded that the better root growth of *T. tomentosum* in low oxygen conditions when compared with *T. glomeratum* seemed associated with greater porosity and enhanced oxygen supply (Gibberd *et al.* 1999). However, in the present study increasing root porosity in waterlogged compared to drained conditions did not seem well correlated with waterlogging tolerance. This result suggests that other factors that differed between populations were responsible for conferring waterlogging tolerance (*e.g.* the formation of aerial or aerotropic roots (Gibberd 1997)). Unfortunately the presence of such structures was not noted in this study and other physiological adaptations to waterlogging were not investigated.

#### *Variation between strawberry clover populations*

Variation in the response to waterlogging and salinity of strawberry clover could be exploited in breeding programs to produce improved cultivars for niche environments with particular growing conditions. The four populations of strawberry clover

responded differently to the waterlogging and salinity treatments (Table 5.2). For example, root dry weight was reduced in all treatments for Karridale strawberry clover, while Badgingarra strawberry clover root dry weight was decreased by salinity + waterlogging but was unaffected by the other treatments. This might be explained by the origin of the Badgingarra population in a sandplain valley floor soil in the West Midlands of Western Australia (see Chapter 3) where waterlogging by fresh water was a frequent occurrence, but there was little salinity. With the limited amount of information available about the environments of origin for the strawberry clover populations, especially those from the Mediterranean Basin, it is difficult to determine if the varied responses to salinity and waterlogging are due to adaptations to their environments of origin. It is unlikely that any of the populations included in this study were exposed to high levels of salinity in their natural environments, although nothing is known of the environment from which Tunisia strawberry clover was collected. It is more likely that the variation shown between the populations is a reflection of the naturally high levels of variation in strawberry clover (Chapters 3 and 4). This does not make this variation any less useful for exploitation in breeding programs to improve production in saline and waterlogged environments, but means presence of tolerance to salinity or waterlogging may be hard to predict without testing of populations.

Variation in plant response to salinity and waterlogging has been observed in other legume species (Gibberd *et al.* 2001; Teakle *et al.* 2006). Schachtman and Kelman (1991) investigated the shoot yield of a number of *Lotus* and *Trifolium* species in saline conditions and found that there were significant differences between populations from the same species, including two cultivars of strawberry clover, Palestine and O'Connors. Variation in salt tolerance of cultivars and selected natural populations of white clover has also been investigated (Rogers *et al.* 1993). They showed that the rate of yield decline in response to increasing salinity levels was greater in those cultivars that had the highest yield under non-saline conditions. The study concluded that genotypes of white clover that have greater salt tolerance or greater yield potential under non-saline conditions could be selected in order to maximise yield under moderately saline conditions. In an investigation into the salinity tolerance of a number of strawberry clover cultivars and introductions to Australia, Rumbaugh *et al.* (1993) found a large variation in shoot dry weight, shoot height and branch number and some populations grew more when exposed to salinity than in the control treatments.

### *Future research*

This study has raised many questions that require further investigation. As the physical environment of saline discharge areas is so highly variable in respect to waterlogging intensity and duration and salinity levels (Barrett-Lennard 2003) further study on the impact of differing levels of the combined stresses could yield interesting results. The use of soil to screen for variability in salinity and waterlogging tolerance can often be confounded by the variability, both temporally and spatially, of these stresses in the field; the addition of a treatment in a controlled environment such as a nutrient solution would have allowed for an interesting comparison of the results. Further investigation with a range of salinity levels and waterlogging intensities may further describe the pattern of variation to these stresses both within and between populations of strawberry clover, and the use of lower levels of salinity than that utilised in the current study (120mM) may also allow further expression of that variation. Investigation into the salinity tolerance at seed germination of different populations of strawberry clover is also warranted due to the likelihood in the Mediterranean climate of Western Australia of higher saline conditions without waterlogging at the time of opening rains, before winter rainfall dilutes the salt content of the soil. Due to the lack of correlation of apparent waterlogging tolerance with root porosity measurements, further investigation into other mechanisms of waterlogging tolerance, such as aerotropic roots and other physiological responses, would also be warranted, as would further study into possible salinity tolerance mechanisms in strawberry clover. This work was beyond the scope of this study due to its focus largely on ecological implications of the variation in these stresses.

### *Conclusions*

The first hypothesis was only partially supported by the results of Chapter 5. Three of the four populations of strawberry clover investigated showed evidence of adaptation to waterlogged conditions as indicated by little effect or improved shoot growth. Root growth of strawberry clover was generally less affected by salinity and waterlogging than for lucerne. While none of the strawberry clover populations showed better growth in saline conditions (with or without waterlogging) than in control conditions, some populations showed more tolerance to salinity than others. The second hypothesis was not supported by the results. Root porosity was higher for strawberry clover than lucerne but was not clearly related to tolerance of waterlogging or salinity. The final hypothesis was supported by the results. There were differences in growth and porosity,

and therefore in tolerance between the strawberry clover populations when subjected to the three treatments. However, whether these differences were due to evolution or random genetic differences is unknown. The variation between the strawberry clover populations in their response to salinity and waterlogging does indicate scope for breeding programs to produce improved cultivars better suited to the conditions in discharge zones during autumn and early winter. Any increase in the productivity of strawberry clover on waterlogged and mildly saline land would be a positive step towards improved sustainability for southern Australian farming systems.



## CHAPTER 6

### Resource Allocation and Growth Patterns Differ Between Three Species of Perennial *Trifolium* and Lucerne (*Medicago sativa* L.) Under Glasshouse Conditions.

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#### 6.1 Abstract

Perennial pasture plants in southern Australia must possess mechanisms to enable them to survive hot dry summer months. For instance, a rapidly formed, extensive and deep root system should enhance access to soil moisture at depth. Resource allocation patterns were therefore investigated for three species of perennial *Trifolium* (*T. fragiferum* L. from Spain and Badgingarra, Western Australia, *T. uniflorum* L. from Turkey and Greece, and *T. alpestre* L. from Switzerland) and lucerne (*Medicago sativa* L.) cv. Pioneer L69. Plants of each of the populations were grown in a glasshouse in one metre deep PVC tubes and four destructive harvests were done at six weekly intervals from sowing with a 5<sup>th</sup> harvest three weeks after the 4<sup>th</sup> harvest. Leaf area, maximum root depth, and dry weight of shoot, reproductive structures and roots were measured.

Lucerne roots reached the bottom of the tubes (100 cm) 18 weeks after sowing, followed by both populations of strawberry clover at 24 weeks. The remaining populations did not reach the bottom of the tubes during the experiment, with *T. alpestre* at 77 cm, and the Greek and Turkish *T. uniflorum* reaching 81 cm and 83 cm, respectively. There were differences between all species in the proportion of resources allocated to roots and shoots. Although by the final harvest the root-shoot ratio of *T. alpestre* (3.37) was close to that of lucerne (3.58), the root and shoot biomass of *T. alpestre* was significantly lower than lucerne and the root-shoot ratios for all but the final two harvests were also lower, showing the *T. alpestre* root system was slower to establish. None of the other populations had a similar pattern of resource allocation to lucerne. Populations from similar environments did not exhibit similar resource allocation and growth patterns; instead species was the greater determinant of resource allocation. Lucerne is relatively well-adapted to survival in southern Australian climatic conditions due to a quickly established deep root system. Strawberry clover is likely to

be less well-adapted but useful in niche areas, while *T. alpestre* may not be adequately deep-rooted to survive summer drought.

## **6.2 Introduction**

With the increased recognition that dryland salinity poses a major threat to the southern agricultural regions of Australia, a need to increase the use of perennial species in farming systems to mimic the water use patterns of more stable natural ecosystems is now widely accepted (Cransberg and Macfarlane 1994; Dear *et al.* 2003). However, there are few perennial legumes available for use in southern Australian farming systems. Lucerne (*Medicago sativa* L.) is widely used in eastern Australia, but is a relatively new introduction to dryland farming systems in Western Australia. Adoption of lucerne in Western Australia may be restricted by poor adaptation to low pH and aluminium toxic soils (Humphries and Auricht 2001). In addition, there exists a need to increase the diversity of pasture legume species to reduce the susceptibility of farming systems to devastation from pests and diseases (Cocks 2001). There is, therefore, a need to examine other species of perennials, particularly legumes, which may show adaptation to southern Australian farming systems. A series of large trials investigating the adaptability of a number of perennial legumes, herbs and grasses across southern Australia has recently concluded (Reed *et al.* 2008; Nie *et al.* 2008; Nichols *et al.* 2008). So far, few legumes in these trials have come close to matching the persistence and biomass production of lucerne (Dear *et al.* 2008).

Adaptation was defined by Harper (1977) as the features of organisms that improve the fitness of their ancestors and so are passed on to their descendants. Adaptive strategies are those characteristics that confer fitness in the present habitat of the organism. These characteristics are reflected in the life histories of species, and these life histories are the patterns of growth, reproduction and longevity that are related to specific demands for survival in a particular place at a particular time (Barbour *et al.* 1987). They determine at what place and time a species successfully carries out the processes of germination, growth and reproduction. The allocation of resources to these processes is somewhat flexible, reflecting the habitat in which the plant presently finds itself, but is only so within the confines of the genotype of that individual. This is known as phenotypic plasticity and has been reported in such species as St John's Wort (*Hypericum*

*perforatum*) (Maron *et al.* 2007) and white clover (*Trifolium repens* L.) (Forde *et al.* 1988).

There have been few studies on the resource allocation patterns of perennial legumes, apart from white clover. Chapman *et al.* (1992) looked at physiological integration in white clover and noted that there was transfer of carbohydrates between parent plants and stolon branches. Other studies with white clover have examined the allocation of resources to reproductive and vegetative structures for the first 18 weeks after germination (Turkington and Cavers 1978) and the genetic variation and tradeoffs that may occur between vegetative growth and reproductive capacity (Cain *et al.* 1995), as well as the effects of root pruning on resource allocation to branches (Lotscher and Hay 1995). The relative growth rates, resource allocation patterns and root morphologies of *Dorycnium* species have also recently been compared to that of lucerne (Bell 2005) and used to draw conclusions about the adaptation of two *Dorycnium* species to southern Australia. However, the characteristics of perennial legumes that may confer adaptability to southern Australian farming systems have otherwise been little explored.

In a climate with hot dry summer months it has been suggested that a deep rooting depth and relatively high resource allocation to root biomass may be important for survival and a high rate of water use by perennial species (Cocks 2001; Humphries and Auricht 2001). Deep-rooted species have the ability to access water from deeper in the soil profile than shallower rooted species. This ability, along with their potential for perennial growth all year, allows these plants to use more water than their annual, shallow-rooted counterparts (Cocks 2001; Cransberg and Macfarlane 1994; Dolling 1999). The ability to quickly produce an extensive deep, root system is likely to be an important adaptation for survival of perennials in southern Australian farming systems as it should confer an ability to extract stored soil moisture from depth over the summer drought period. This water is unavailable to shallower rooted annual species that instead rely on a dormant seed bank to ensure survival of the population during this period. Allocation of root biomass to deep horizons and, in particular, to the tap root, has been suggested as the reason some common annual bean genotypes are better adapted to terminal drought environments (Ho *et al.* 2005).

Evidence on the importance of a high root-shoot ratio for drought avoidance and hence survivability of perennial legumes in southern Australian farming systems is unavailable. Reductions in root-shoot ratio have been associated with reduced competitive ability of perennial ryegrass (Ennik and Hofman 1983) and Smith *et al.* (2002) expressed concerns that breeding for high water soluble carbohydrate concentration in perennial ryegrass may lead to a decreased root mass and less drought tolerance. Denton *et al.* (2006) found that two native perennial legumes, *Kennedia prorepens* F. Muell. and *Lotus australis* Andrews., had a lower root mass ratio than lucerne cv. Hunterfield, but had a more even distribution of roots to one metre depth and hence a greater proportion of root biomass at depth. Overall, it seems reasonable to assume that a greater allocation of resources to roots, especially roots at depth, may indicate a greater ability to extract nutrients and water from the soil and so lead to a greater ability to survive summer drought conditions.

This chapter examines the resource allocation patterns of three species of perennial *Trifolium* with differing life histories and from different environments, over a period of 27 weeks. The species selected for this experiment include paired populations of the same species collected from site of widely varying environments, as well as different species collected from sites of similar environments. A commercial cultivar of lucerne was also included as it is currently the best adapted perennial legume for use in medium-low rainfall southern Australian farming systems (Humphries and Auricht 2001). The aim of this chapter was to compare the resource allocation patterns and root depth in perennial *Trifolium* to that of lucerne and to then draw conclusions as to their suitability for inclusion in southern Australian farming systems. A secondary aim was to examine the resource allocation patterns of these three perennial *Trifolium* to investigate whether the environment of origin affects resource allocation.

### **6.3 Materials and methods**

#### *Species details*

The study consisted of 5 populations from 3 species of perennial *Trifolium*, with lucerne cv. Pioneer L69 included for comparative purposes (Table 6.1). These were:

- Two populations of strawberry clover from different environments; medium rainfall, low altitude (Badgingarra, Western Australia) and low rainfall, higher altitude (Spain).
- Two populations of *Trifolium uniflorum* from different environments; coastal (Turkey) and alpine (Greece).
- One population of *Trifolium alpestre* from an alpine environment.

This selection of species allows a comparison of resource allocation patterns between the two clover populations from alpine environments (*T. uniflorum* Greece and *T. alpestre*), as well as, for strawberry clover and *T. uniflorum*, 2 populations of the same species from different environments. The selection of alpine populations was to investigate resource allocation in widely different environments; both alpine populations were not considered to be adapted to survival in southern Australian conditions.

Strawberry clover is from the section Vesicaria in the genus *Trifolium* and forms stolons that can root at the nodes to form clonal offspring (Zohary and Heller 1984). It is also predominantly out-crossing (Wright 1964) and has been observed to show some tolerance to waterlogging and mild salinity (Taylor 1978). *Trifolium uniflorum* is from the section Lotoidea in the genus *Trifolium* and forms a matted caespitose (Zohary and Heller 1984) with single flowers formed within the centre of the clump and held closely to the plant. *Trifolium alpestre* is from the section Trifolium in the genus *Trifolium*, and is found in alpine regions and spreads by forming a creeping rhizome (Zohary and Heller 1984) as well as producing seed from flowers. The cultivar of lucerne was Pioneer L69, a privately developed winter-active cultivar.

Table 6.1. Site descriptions for the locations where the 5 populations of perennial *Trifolium* were collected.

Species	Country of origin of seed	Location	Habitat	Altitude (m)	Average annual rainfall (mm)
<i>T. fragiferum</i>	Australia	Badgingarra, WA	managed pasture	280	650
<i>T. fragiferum</i>	Spain	40°N 5°W	grassland	800	350
<i>T. uniflorum</i>	Turkey	Cheshme, Erythrea	hillside, near the sea	50	650
<i>T. uniflorum</i>	Greece	Abies forest, west of Athens	alpine forest	1800	400
<i>T. alpestre</i>	Switzerland	Ex Champex, Valais	alpine	1500	700

### *Experimental design*

Populations were grown from seed in one metre deep  $\times$  15 cm diameter PVC seeding tubes in a glasshouse. The design of the experiment was 5 populations  $\times$  5 harvests  $\times$  4 replicates, arranged in a randomised complete block design in the glasshouse. On the 10<sup>th</sup> August, 2001, five seeds were placed in each tube in standard potting mix with slow release general fertiliser and watered. After two weeks, seedlings were thinned to one per tube. Plants were fertilised with Thrive liquid fertilizer (NPK (77:5.5:9) and trace elements) at label recommended rates at sowing and, thereafter, monthly until the end of the experiment. Plants were watered to 80% field capacity by weight on a regular basis. To mimic the seasonal change in water availability under field conditions from autumn to summer, watering was daily for the first two weeks, reduced to three times a week over the winter/spring period from August 25<sup>th</sup> and was further reduced to once a week over the summer period from December 1<sup>st</sup> until the end of the experiment on February 15<sup>th</sup>. Maximum and minimum temperatures were recorded in the glasshouse from the time of harvest 2 (beginning of high summer temperatures) through to the end of the experiment and the maximum and minimum temperatures for each week are presented (Figure 6.1).

### *Harvests*

Five destructive harvests were conducted throughout the experiment; the first four harvests were at six weekly intervals, starting six weeks from sowing, with the fifth and final harvest being three weeks after the fourth. At each harvest whole plants (shoots and roots) were removed from the tubes. Shoots were removed from the tubes by cutting the base from the root system. Leaves were removed from stems for the first four harvests so that leaf area could be measured. Columns of soil were removed from the tubes and the distance from the surface to the deepest penetrating root was recorded as root depth. Roots were then washed out of the soil and all component parts of the plants dried for three days at 80°C and weighed. Reproductive-vegetative ratios (RA/VA) (Hickman 1975) and root-shoot ratios were calculated.

It is important to note that dry weights of plant components were used to determine resource allocation rather than calorimetric measurements for the reasons cited by Turkington and Cavers (1978) and because previous research had concluded that there was no significant difference between resource allocation patterns based on calories or dry weights (Hickman and Pitelka 1975). Also, the reproductive values have been

calculated from weight of seed and flowers only. Vegetative forms of reproduction, such as stolons in the strawberry clover and rhizomes in the *T. alpestre* have only been included as a measure of shoot and root weights, respectively, and not reproduction. If considered as a reproductive structure, then these would have greatly increased the reproductive allocation (RA) of these two species. Unfortunately this study did not include the collection of this data.

#### *Statistical analysis*

Means, standard errors and coefficient of variations are presented for ratio results. Analyses of variance (ANOVA) were computed for root, shoot, reproductive and ratio results for each individual harvest using the GENSTAT statistical package version 7 (VSN International Ltd, Hertz, UK).

### **6.4 Results**

Temperatures recorded in the glasshouse from the time of the 2<sup>nd</sup> harvest (early November) through to the finish of the experiment and ranged from a minimum of 10°C to a maximum of 46°C (Figure 6.1). While minimum temperatures remained fairly consistent over the duration, the maximum temperatures rose around week 14 (mid November), remained steadily high until week 22 (December/January period), then began to fall and were still falling at the conclusion of the experiment in mid February. These temperatures were higher than would be expected in the field.

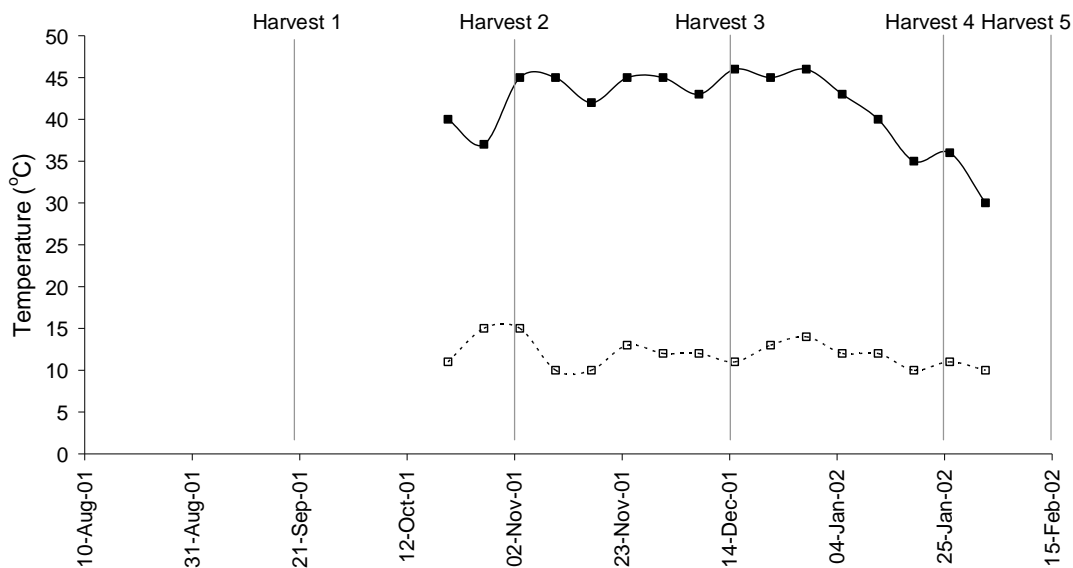


Figure 6.1. Maximum (-■-) and minimum (- □ -) temperatures recorded each week in the glasshouse from the time of the second harvest through to the end of the experiment. Watering was reduced to once per week from 1<sup>st</sup> December.

Lucerne had the highest root dry weight throughout the experiment. This difference was established by the time of the second harvest (Figure 6.2a). Root dry weight for the alpine *T. uniflorum* and *T. alpestre*, also from an alpine environment, was similar at the end of the experiment, but roots of *T. alpestre* were slower to establish, and did not reach the same depth as *T. uniflorum* until the final harvest. Root dry weight for both strawberry clover populations was similar throughout the experiment, while root dry weights for the 2 *T. uniflorum* populations started out similar, but by harvest 4 had decreased for the Greek *T. uniflorum* which only started to catch up by the final harvest.

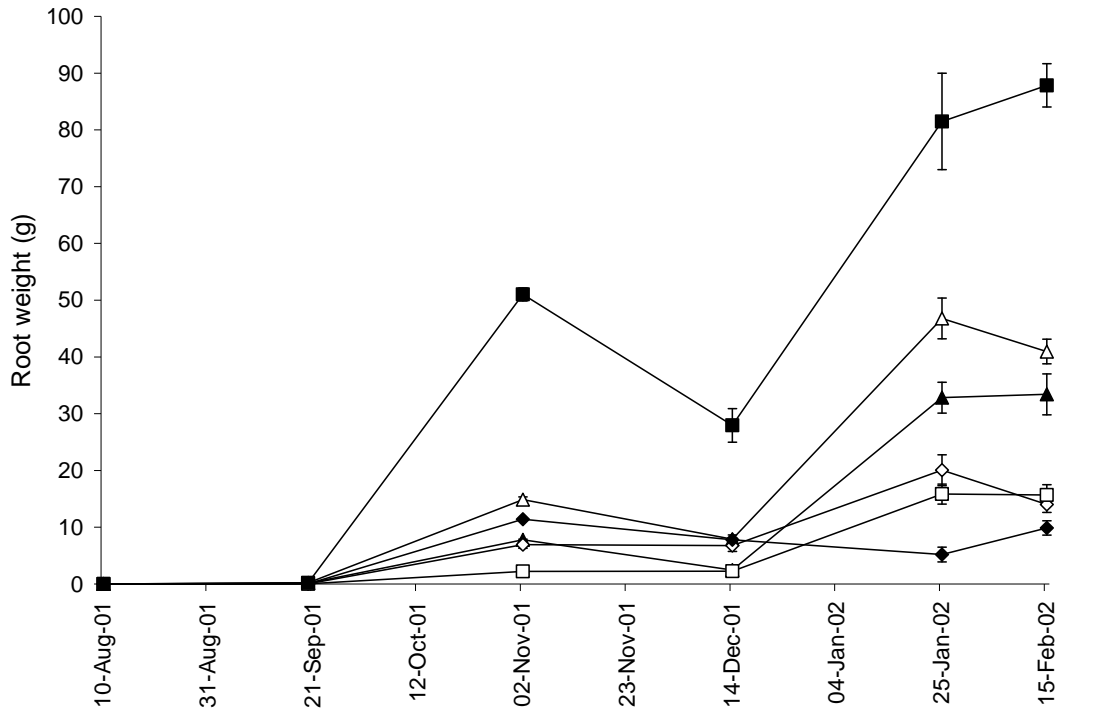
In contrast to results for root dry weight, lucerne did not have the highest shoot dry weight during the experiment (Figure 6.2b). Shoot growth patterns over the duration of the experiment were different for each population. Shoot dry weights for both strawberry clover populations were similar to lucerne for the first 2 harvests but by harvest 3 Badgingarra strawberry clover had the highest shoot dry weight. Between harvests 3 and 4 (14<sup>th</sup> December – 25<sup>th</sup> January) the Spanish strawberry clover population exhibited very rapid shoot growth and ended the experiment with the highest shoot dry weight. Lucerne growth slowed between harvests 3 and 4 but increased again by harvest 5 so that it had the same shoot dry weight as the Badgingarra strawberry clover. Shoot dry weights for the two populations from alpine environments were not similar. *T. alpestre* maintained a slow, even shoot growth throughout the experiment



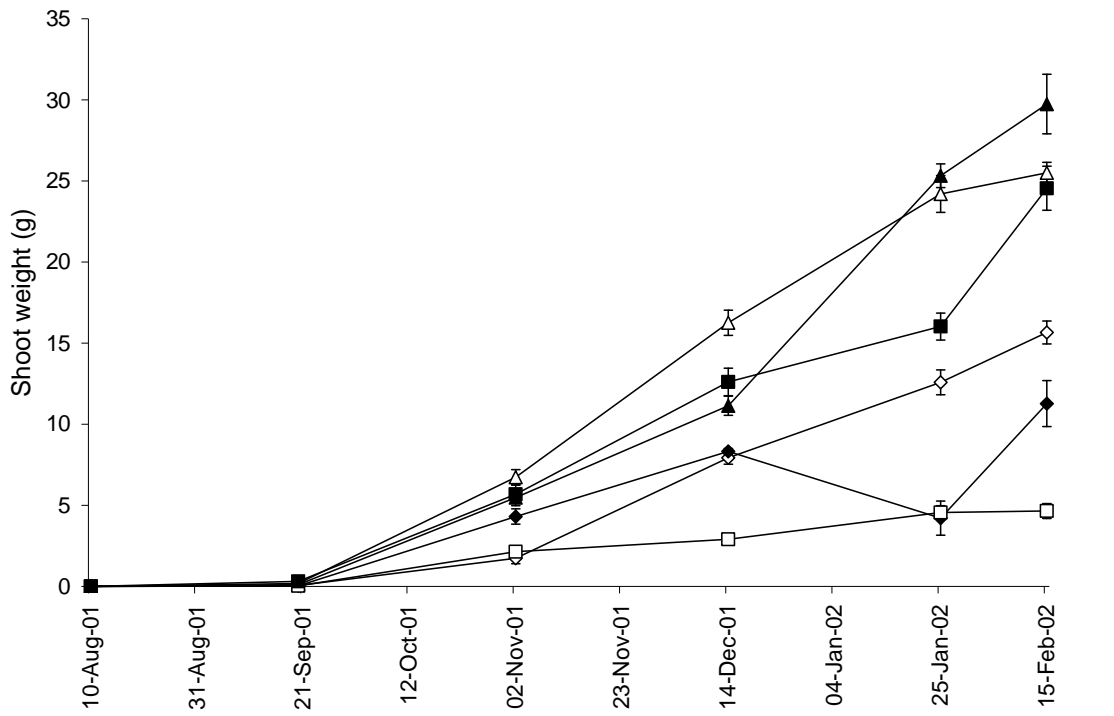
and recorded the lowest shoot dry weight at the completion of the experiment. The two populations of *T. uniflorum* had similar shoot growth patterns until harvest 4, where the Greek population showed a big drop in shoot dry weight before increasing once again by the final harvest.

Reproductive biomass included flowers and seeds. Lucerne had one of the highest reproductive weights throughout the duration of the experiment. The two alpine populations did not match each other with their reproductive growth except at the final harvest. The Greek *T. uniflorum* had one of the highest reproductive structure weights up to harvest 3, but then decreased to one of the lower weights. Both populations of strawberry clover ended with similar reproductive dry weights and were similar to lucerne; however the Spanish strawberry clover was later to begin flowering. Apart from harvests 2 and 3, where the Greek population was higher, both populations of *T. uniflorum* recorded similar reproductive weights.

(a)



(b)



(c)

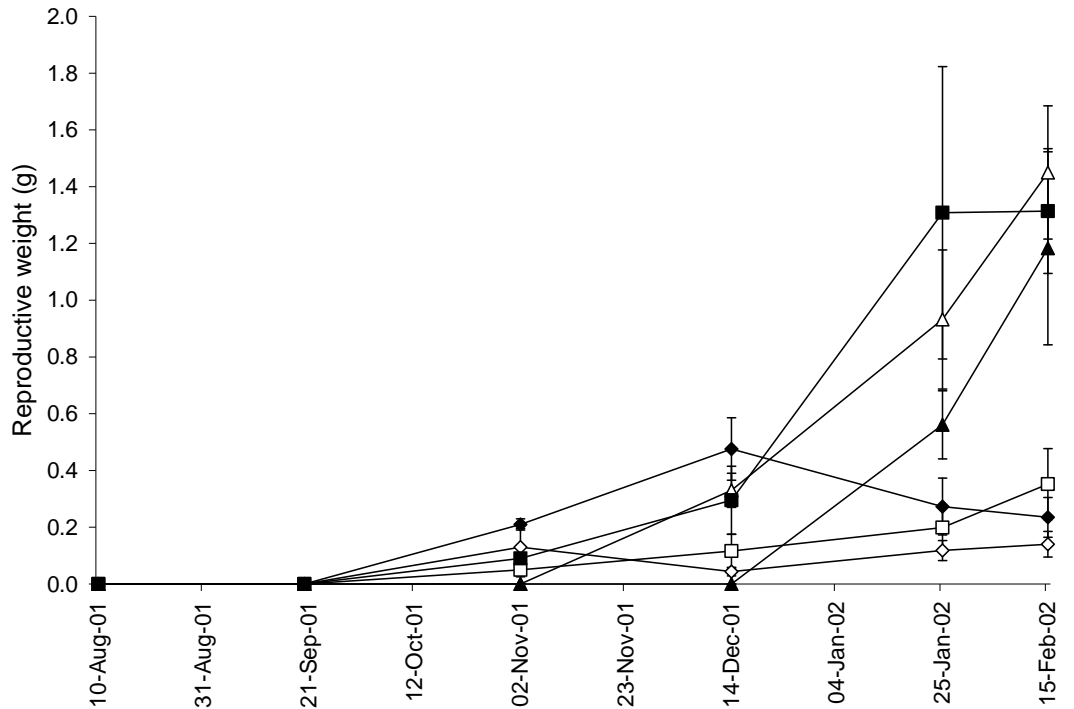


Figure 6.2. Dry weight with standard errors for (a) roots, (b) shoots and (c) reproductive structures for strawberry clover from Western Australia ( $\triangle$ ) and Spain ( $\blacktriangle$ ), *Trifolium uniflorum* from Greece ( $\blacklozenge$ ) and Turkey ( $\diamond$ ), *Trifolium alpestre*, ( $\square$ ), and a commercial cultivar of lucerne ( $\blacksquare$ ), in a glasshouse experiment. Results for root and shoot biomass were significantly different between populations at the  $P < 0.05$  level at each harvest. Results for reproductive biomass were not significantly different between populations. Reproductive biomass includes both flower weight and seed weights where seeds were produced. Watering was reduced to once per week from 1<sup>st</sup> December.

Table 6.2 presents the root-shoot and reproductive allocation-vegetative allocation (RA/VA) ratios for each population from the final harvest, along with standard errors and coefficients of variation. Lucerne had the highest root-shoot ratio at the final harvest. Results for each species were different at  $P < 0.05$ ; however populations from the same species did not differ. There was no difference between the RA/VA for each population. *T. alpestre* showed the most variation for root/shoot ratio (CV 75%), while lucerne showed the least amount of variation (CV 17%). RA/VA ratios showed a lot more variation in each of the populations, with CVs ranging from 59% for Badgingarra strawberry clover to 130% for *T. alpestre*.

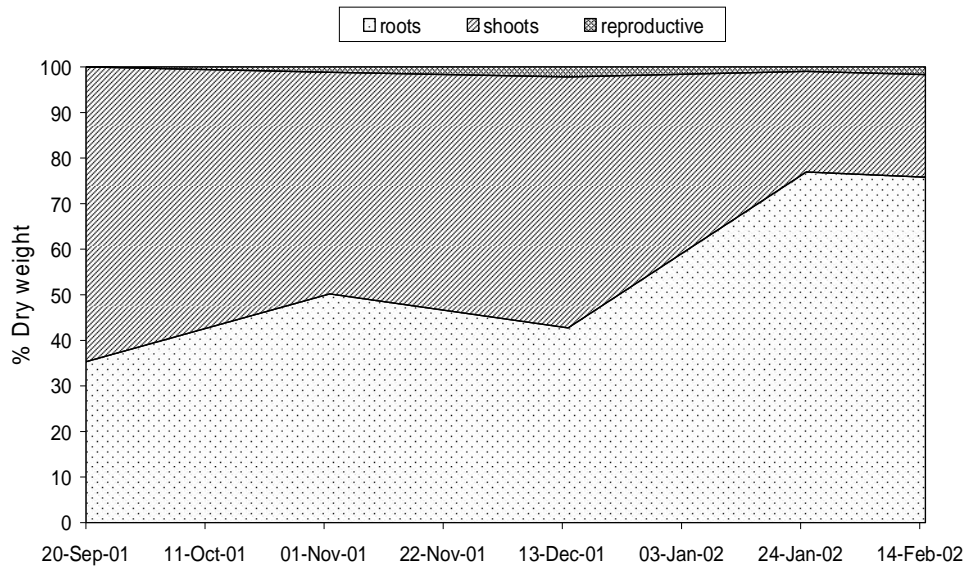
Table 6.2. Average root/shoot and reproductive allocation (RA)/vegetative allocation (VA) ratios with standard errors (se) and coefficients of variation (CV) at the final harvest for 3 species of *Trifolium* and *Medicago sativa*. Root/shoot ratios differed significantly between for each species at  $P < 0.05$  while the RA/VA ratios did not significantly differ between species.

Species	Origin	root/shoot			RA/VA		
		mean	se	CV (%)	mean	se	CV (%)
<i>T. fragiferum</i>	Spanish grassland	1.11	0.16	43	0.024	0.008	102
<i>T. fragiferum</i>	Badgingarra, WA	1.61	0.25	30	0.018	0.006	59
<i>T. uniflorum</i>	Greek mountains	0.87	0.12	27	0.011	0.006	105
<i>T. uniflorum</i>	Turkish coast	0.89	0.14	31	0.005	0.002	97
<i>T. alpestre</i>	Swiss alps	3.37	1.63	75	0.017	0.009	130
<i>M. sativa</i>	Pioneer L69	3.58	0.31	17	0.012	0.004	74

Patterns of resource allocation for each of the populations are displayed in Figures 6.3 - 6.5. None of the populations had a similar pattern of resource allocation to that displayed by lucerne. The distinguishing characteristic of lucerne is the very rapid establishment of its root system by the second harvest and the high proportion of root dry weight maintained throughout the experiment (Figure 6.3b). Resource allocation patterns of the two populations from alpine areas were not similar (Figures 6.3a and 6.4b). However both the populations of *T. uniflorum* (Figure 6.4) and strawberry clover (Figure 6.5) were remarkably similar to each other.

All populations showed a decrease in the proportion of resources allocated to root biomass at week 18, 2 weeks after watering was reduced to once per week. This is supported by the results presented in Figure 6.2 that show a decrease at that time in root dry weight, with a continued increase in shoot dry weight. The proportion of biomass allocated to roots then tends to decrease again, at least until the fourth harvest. This decline in the proportion of resources allocated to roots was particularly marked for strawberry clover. The proportion of resources allocated to reproductive dry weight is much smaller than that allocated to roots and shoots for all populations throughout the experiment.

(a)



(b)

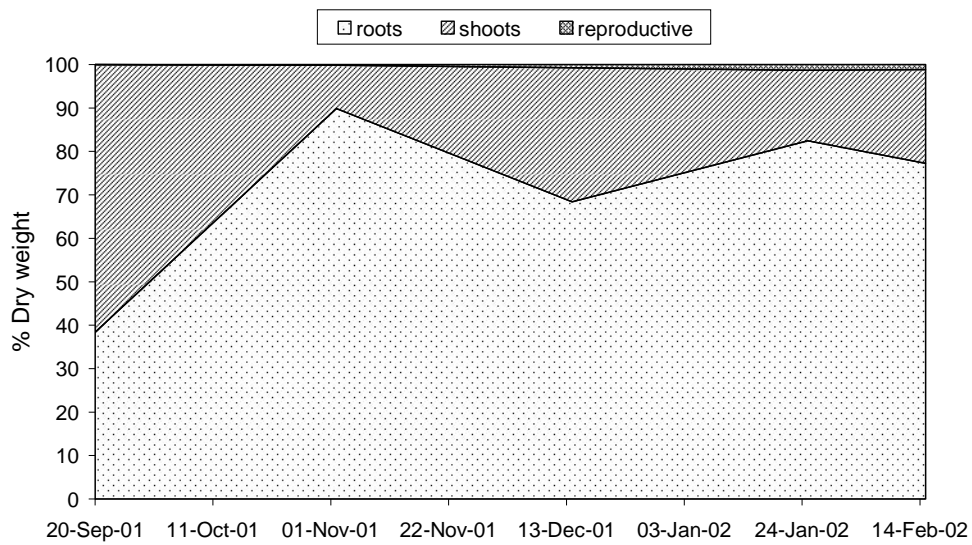
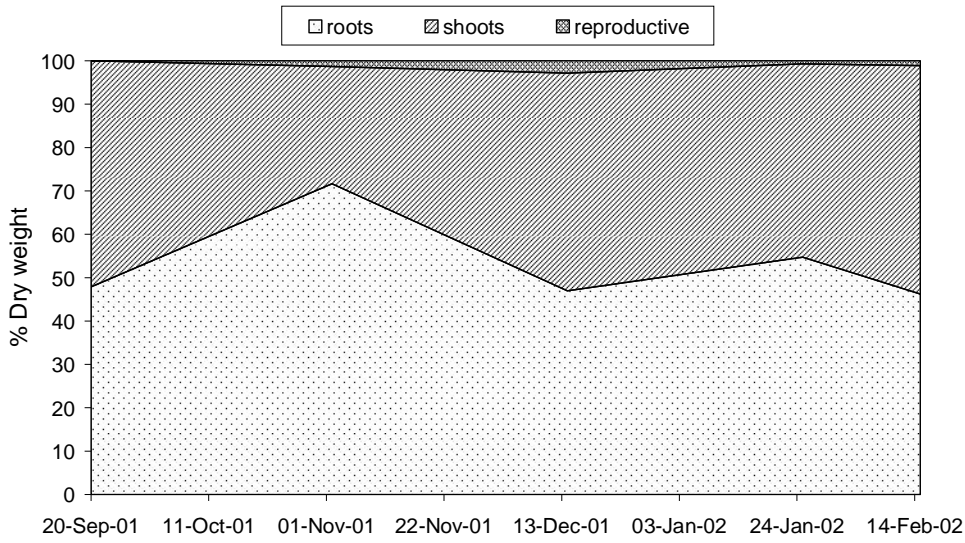


Figure 6.3. Proportions of root, shoot and reproductive (flowers and seeds) dry weight over the duration of the experiment for (a) *Trifolium alpestre*, and (b) lucerne. Watering was reduced to once per week from 1<sup>st</sup> December.

(a)



(b)

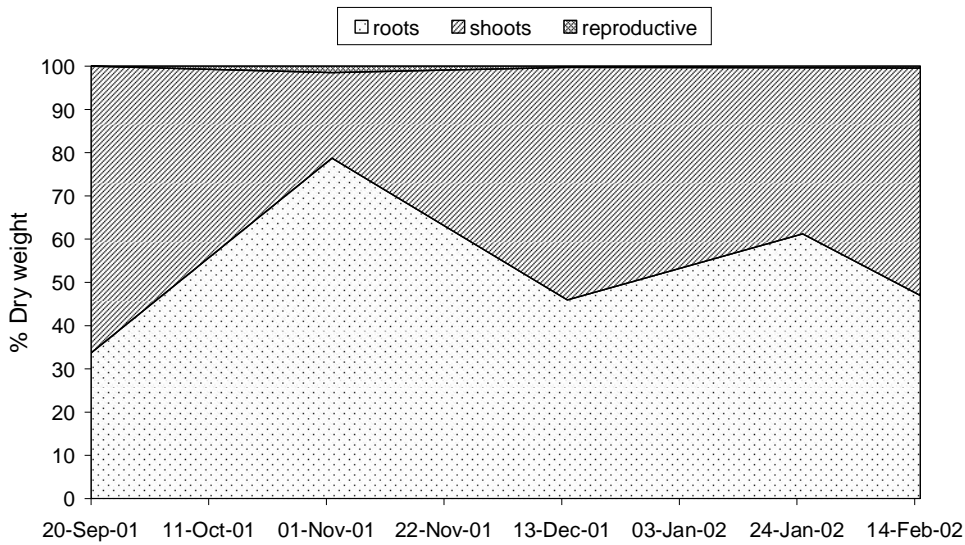


Figure 6.4. Proportions of root, shoot and reproductive (flowers and seeds) dry weight over the duration of the experiment for (a) *Trifolium uniflorum* from Turkey, and (b) *Trifolium uniflorum* from Greece. Watering was reduced to once per week from 1<sup>st</sup> December.

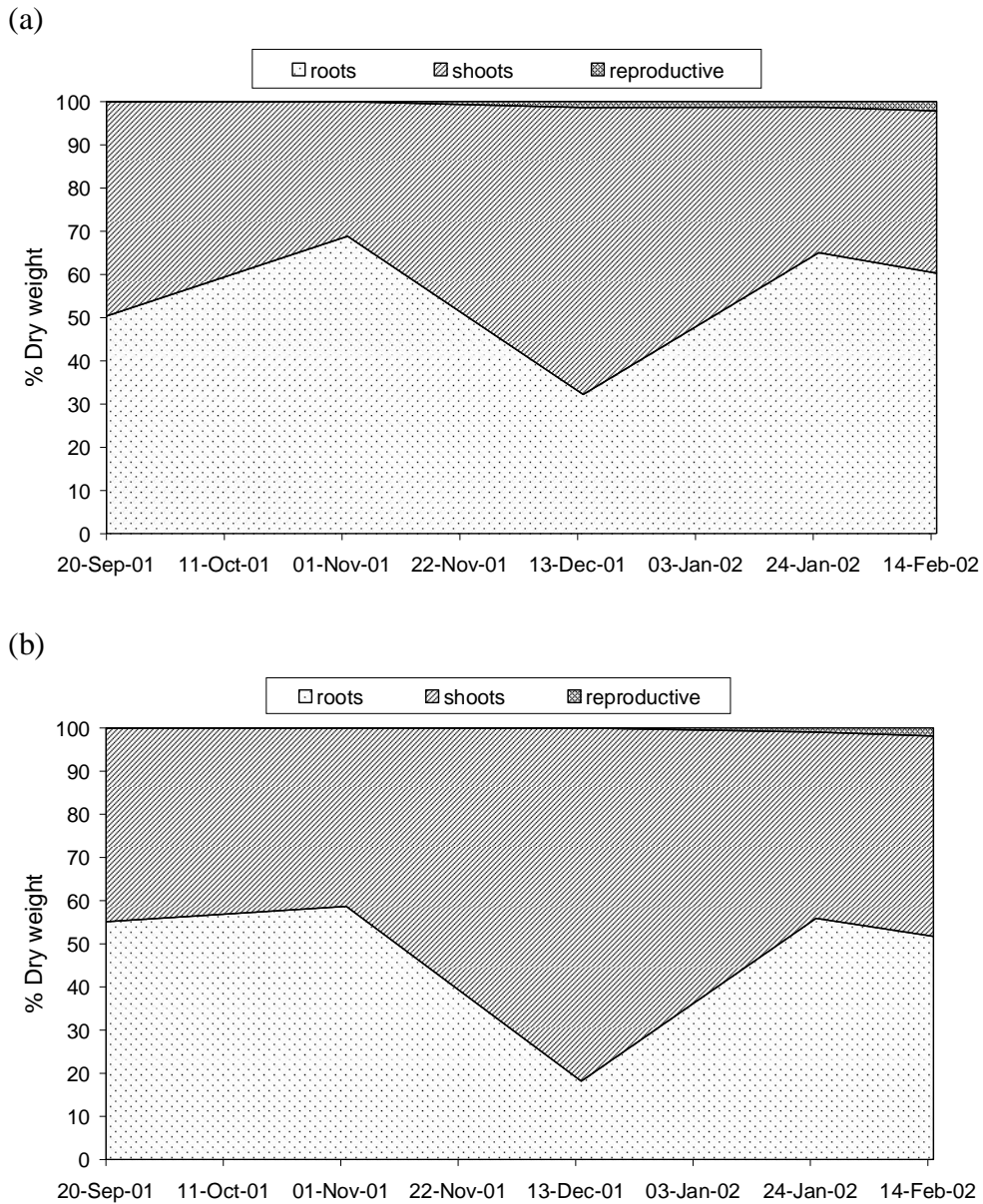


Figure 6.5. Proportions of root, shoot and reproductive (flowers and seeds) dry weight over the duration of the experiment for (a) strawberry clover from Badgingarra, and (b) strawberry clover from Spain. Watering was reduced to once per week from 1<sup>st</sup> December.

Lucerne roots reached the bottom of the planting tubes (100 cm) by the third harvest (18 weeks) and by the fourth harvest (24 weeks) the roots of both populations of strawberry clover had also reached 100 cm (Figure 6.5). Roots of lucerne penetrated down the soil at an average rate of 0.79 cm/day until reaching the bottom of the pots, while the strawberry clover roots penetrated at an average rate of 0.59 cm/day. None of the other populations had reached the bottom of the tubes by the end of the experimental period. *T. alpestre* had the slowest root growth and roots penetrated at an average rate of 0.40

cm/day. Greek *T. uniflorum* roots penetrated at an average rate of 0.43 cm/day and Turkish *T. uniflorum* at 0.44 cm/day.

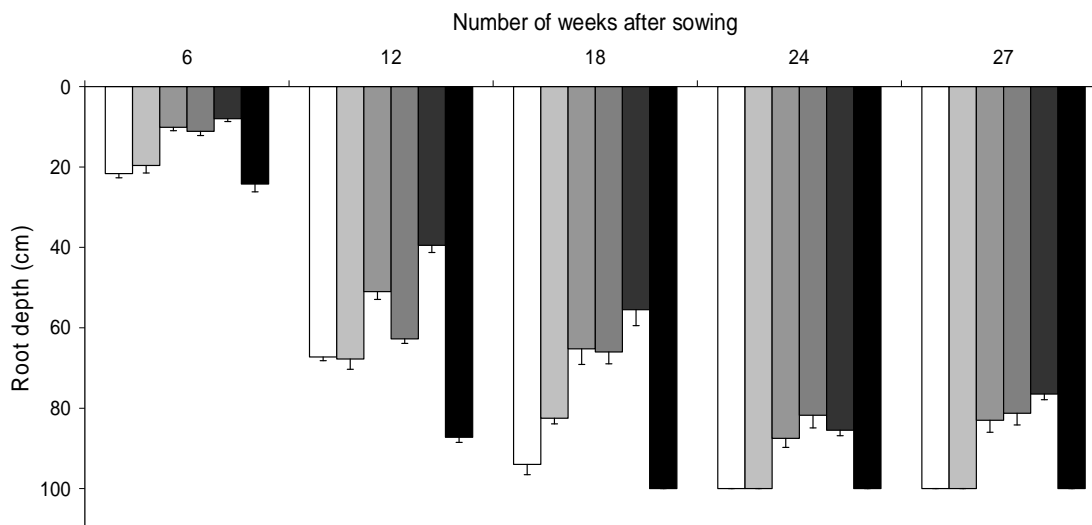


Figure 6.6. Depth of longest roots down the planting tubes at each harvest for strawberry clover from Badgingarra (□) and Spain (■), *T. uniflorum* from Turkey (■) and Greece (■), *T. alpestre* from Switzerland (■) and lucerne (■). Note that the bottom of the tubes was at 100 cm. Watering was reduced to once per week from 16 weeks after sowing.

Leaf area tended to plateau for both *T. uniflorum* populations after harvest 2 and for *T. alpestre* and Badgingarra strawberry clover after harvest 3. Lucerne leaf area increased steadily throughout the experiment, while leaf area for Spain strawberry clover leveled out after harvest 2 and then began to increase again by harvest 4 (Figure 6.7). From the first harvest onwards, lucerne and Badgingarra strawberry clover had significantly higher leaf areas than the other populations. There was a large difference in leaf area between the two strawberry clover populations, but not shoot dry weight, because most of the shoot dry weight for the Spanish strawberry clover was due to thicker and larger stems rather than bigger or more leaves as was the case for the Badgingarra strawberry clover. There was more variation in leaf area for lucerne and Badgingarra strawberry clover than for *T. alpestre*, *T. uniflorum* and the Spanish strawberry clover (data not shown).



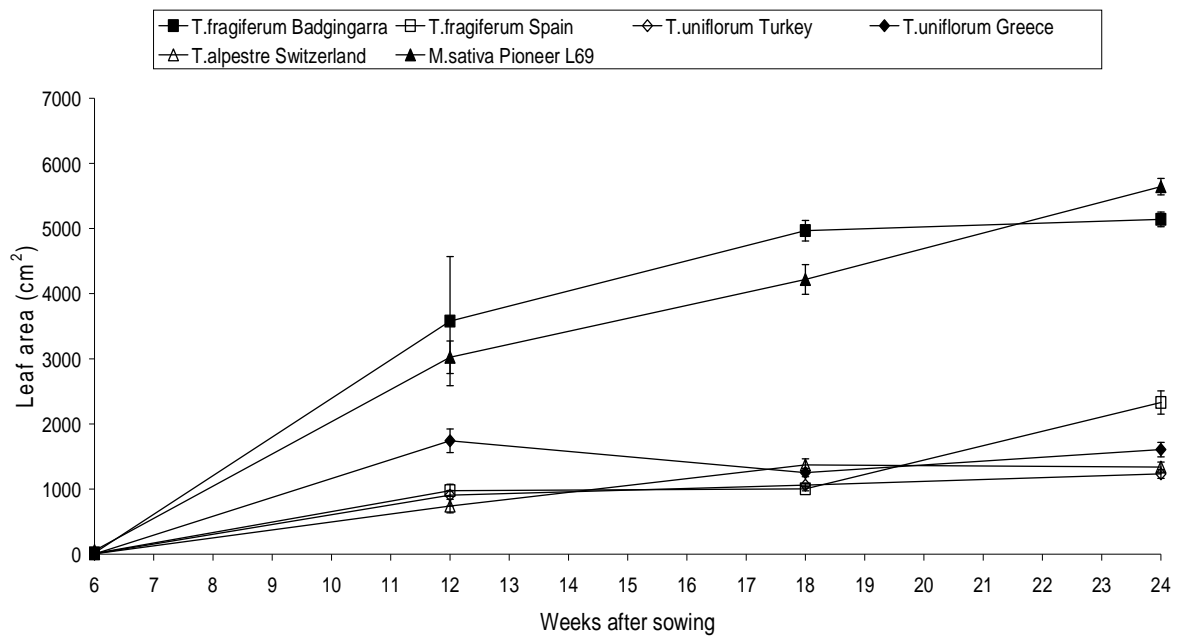


Figure 6.7. Leaf area at each harvest for *T. alpestre* from Switzerland, *T. uniflorum* from Turkey and Greece, strawberry clover from Spain and Badgingarra, and lucerne cv Pioneer L69. Watering was reduced to once per week from 16 weeks after sowing.

## 6.5 Discussion

Resource allocation patterns and root depth in the perennial *Trifolium* were not particularly similar to that of lucerne. There was also no evidence that specific resource allocation patterns in perennial *Trifolium* can be associated with specific habitats of origin, instead the results show that resource allocation patterns are species determined. The two species that were represented by two populations from different environments (strawberry clover and *T. uniflorum*) showed almost identical resource allocation patterns within the species. A similar conclusion was reached about the reproductive strategies of 11 species of annual *Trifolium* by Norman *et al.* (1998) who found that populations of a species collected from different locations had the same reproductive strategies.

The strawberry clover population from a Spanish grassland with a low annual rainfall of 350 mm and the population from an intensively managed pasture at West Badgingarra in Western Australia with a high annual rainfall of 650 mm showed little difference in the proportion of resources allocated to roots, shoot and reproductive structures (Figure 6.4). Their final root-shoot ratios and reproductive allocation (flowers and seeds)/vegetative allocation (roots and shoots) (RA/VA) ratios were not significantly

different from each other at 1.11 and 0.024, respectively, for the Spanish population and 1.61 and 0.018, respectively, for the Badgingarra population. Similar results were found for the pair of populations of *T. uniflorum*. The population from Greece originated in an alpine forest environment at an altitude of 1800 m above sea level, while the population from Turkey was located on a coastal hillside at an altitude of 50 m above sea level. Despite originating from such contrasting habitats, both populations exhibited similar resource allocation patterns, with their root-shoot and RA/VA ratios not different from each other ( $P < 0.05$ ) (Table 6.2). Also, the population of *Trifolium alpestre* from the Swiss Alps did not show resource allocation patterns particularly similar to the alpine population of *T. uniflorum* but, for the final harvest ratio results at least, instead showed patterns very similar to those of lucerne.

The RA/VA ratios for all populations were not different from each other and were also very small (Table 6.2), highlighting that reproduction via flowers is not a priority for the survival of any of these perennial *Trifolium* species. Turkington and Cavers (1978) drew a similar conclusion from a study that investigated the RA and VA of four different legumes – *Medicago lupulina* L., an annual, and three perennials; lucerne, *Trifolium pratense* L. (red clover) and white clover. *M. lupulina* had a RA/VA ratio of 0.445 after 18 weeks while the lucerne was only 0.004. While the other two perennials had higher RA/VA ratios than lucerne, neither was as high as the annual, even when the white clover RA included stolon weight. A study of strains of wild rice, *Oryza perennis* Moench., also supports these results with perennial types having very low proportions of seed weight to total dry weight while annual strains had up to 50% of their total dry weight apportioned to seed weight (Sano and Morishima 1982). These results highlight the role of seeds in annual plants to enable populations to survive summer droughts (non-growing season) and other environmental constraints as a bank of dormant seeds. This is obviously not so important in perennial species where the individual plants themselves live from year to year.

After the first harvest, lucerne had a greater proportion of resources allocated to root biomass throughout the duration of the experiment, ranging from a high of 90% at the second harvest to a low of 68% at the third harvest, and finishing with 77% at the final harvest. These results are higher than for any of the other populations, with the exception of *T. alpestre* at the fourth and fifth harvests where around 76% of resources were allocated to root biomass. As well as having a high proportion of root biomass,

lucerne also had a faster root depth penetration rate over the duration of the experiment than any other population and along with both populations of strawberry clover, the longest roots (Figure 6.6). Similar results were obtained by Bell (2005) where lucerne cv. Sceptre had a higher root weight ratio as plant size increased, and had faster root depth penetration, than either *Dorycnium hirsutum* L. or *D. rectum* L. Bell (2005) concluded that lucerne was more suited to the optimal growing conditions provided in the glasshouse, and that the slower growing root system of *D. rectum* could explain its poor seedling survival in the field during summer drought conditions.

It has been suggested that higher root density at depth and deeper penetrating roots are characteristics likely to improve the water use capability and survival of plants in drought conditions (Asch *et al.* 2005; Erice *et al.* 2006; Ho *et al.* 2004; Ho *et al.* 2005). As summer drought is an integral part of southern Australian climatic conditions, these are characteristics that would then be important for the survival of perennial legumes in southern Australian farming systems. Lucerne has already proven it has the capability to survive in these farming systems, albeit not particularly well in acidic soils and in waterlogged and saline conditions (Humphries and Auricht 2001), and with similar root growth rates it would be easy to conclude that perhaps strawberry clover would have a similar adaptability. However, at harvest 3 (18 weeks) all populations showed a decrease in the proportion of resources allocated to root biomass (Figures 6.3, 6.4, 6.5) and indeed showed a decrease in total root biomass itself (Figure 6.2), with both populations of strawberry clover showing the greatest decreases. Prior to harvest 3 the watering regime was changed to once a week in conjunction with the onset of higher temperatures in the glasshouse to more closely mimic field conditions at the commencement of summer. These results suggest that the strawberry clover populations may have less ability to deal with a sudden reduction of soil moisture, which could leave them vulnerable to summer droughts in southern Australia.

An increase in root biomass in response to drought is itself not likely to aid in survival of a plant over a drought period. In a study of the perennial forage grass *Trichloris crinita* P., Greco and Cavagnaro (2002) found that root-shoot ratio and dry matter partitioning to different organs was not affected by the water stress treatment they applied and so increased root dry matter would not be considered an adaptation for survival in drought conditions. Similar results have been obtained for rice (*Oryza sativa* L.) (Asch *et al.* 2005). However Asch *et al.* (2005) also measured root depth and found

that while no additional biomass was partitioned to the roots, roots of upland rice grew deeper under drought stress. Price *et al.* (2002) also found that rice plants, when subjected to an early water deficit, had increased root length, but they also showed major increases in root mass (total and at depth) and root-shoot ratio when compared to plants subjected to a “normal” water regime, in contrast to other studies. Improved performance of wheat wild relatives in dry conditions was also associated with an increased partitioning of root mass to deeper profiles and an increased ability to extract water from these depths, rather than a larger overall investment in root dry weight (Reynolds *et al.* 2007). Ho *et al.* (2005) and Yu *et al.* (2007) also conclude that rooting depth of the roots having the highest length-density and allocation of root biomass to these would be more vital than maximum rooting depth for maintaining water uptake under dry soil conditions.

Thus, while a greater root depth would most assuredly be an adaptation to survival in drought conditions, increased allocation to root biomass is unlikely to be as important – it is more important to have more roots at a greater depth, than to have more roots overall. Interestingly, when Denton *et al.* (2006) compared 2 Australian native perennial legumes, *Kennedia prorepens* and *Lotus australis*, to lucerne cv. Hunterfield in a glasshouse trial, the natives had a more even distribution of roots to one metre depth and hence a greater proportion of root biomass at depth. Indeed, lucerne had nearly 60% of roots in the top 5 cm, compared with ~ 20% for *L. australis* and ~ 5% for *K. prorepens*. Drought tolerance in southern Australian conditions has not previously been selected for in many lucerne breeding programs (Humphries and Auricht 2001) and it is possible that these natives exhibit a resource allocation pattern more suited to drought prone conditions. Further research is required to confirm this hypothesis.

Thus for the perennial legumes included in this study, assuming both root depth and root biomass at depth are important factors for adaptation to southern Australian farming systems (summer drought), then lucerne and strawberry clover are among the better adapted species. Unfortunately measurements of where the greatest root length density occurred for each of these were not collected in the current study. This would be useful work to further our understanding of the drought tolerance of perennial legumes and to determine which species are most suitable for further development.

While the reduction in watering frequency after week 15 did not cause an immediate decrease in shoot biomass for any of the populations, it did appear to reduce the shoot biomass of the Greek *T. uniflorum* after the 3<sup>rd</sup> harvest (week 18). Leaf area of all populations except the Greek *T. uniflorum* and Spanish strawberry clover also remained unaffected by the reduced watering frequency. However, root dry weights of all populations were reduced at this time. It seems likely that there was a loss of root dry weight and (in some populations) of shoot dry weight in response to the sudden onset of drier and warmer conditions, before the plants adapted and recovered for subsequent harvests. In a study on the comparative effects of repeated cycles of water stress on strawberry clover, Johnson and Raguse (1985) found that while leaf weight was reduced when plants were water stressed, there was no effect on stolon weight. This appears consistent with the results for Badgingarra strawberry clover where an increasing shoot dry weight after harvest 3 did not correspond to an increase in leaf area.

The root dry weight of the strawberry clover populations appeared more reduced by the reduction in available water after watering frequency was reduced to once per week than shoot dry weight. This may reflect their drought tolerance being poorer than that of lucerne. While *T. alpestre* ended the experiment with similar root-shoot ratios to lucerne, it is unlikely to be of use in the southern Australian farming systems, due to its poor productivity and slower and shallower root growth. It is interesting to note that although the proportion of resources allocated to root biomass was greatly reduced for all populations at harvest 3, no population showed a decrease in root depth over the duration of the experiment. This may indicate that it was the smaller, lateral branches of roots closer to the soil surface that were lost and not the deeper ones. Indeed, previous work has shown droughted rice plants and common bean (*Phaseolus vulgaris* L.) to have a higher percentage of root dry matter in the deeper layers than plants not in drought conditions (Asch *et al.* 2005; Ho *et al.* 2005).

The exception to the general conclusion that resource allocation is species determined and not as a result of adaptation to habitat of origin is leaf area (as opposed to shoot dry weight). The Badgingarra strawberry clover leaf area remained unaffected in response to the reduced watering regime while the leaf area of the Spanish strawberry clover, while not reduced, was certainly stalled. This may show an adaptive advantage of the Badgingarra population in comparison to the Spanish population. The Badgingarra population also maintained a leaf area up to three times greater throughout the duration

of the experiment even though the Spanish population had a slightly higher shoot biomass. Presumably the Spanish population of strawberry clover had thicker or heavier stems, contributing to the higher shoot biomass compared to the population from Badgingarra. That the Badgingarra population has a higher leaf area and less stem than the Spanish population is not surprising as it originated with a genotype selected for grazing purposes where leaf would be more important than stem, whereas the Spanish population was an unselected “wild” population. The Spanish population was also collected from a low rainfall site and it is possible that lower leaf area may be an adaptation to low rainfall/drought conditions to avoid excessive loss of water through transpiration of leaves over a greater surface area. The higher rate of shoot growth over the summer period of the Spanish population compared to the Badgingarra strawberry clover also suggests that the Spanish population has greater adaptation to low rainfall/drought conditions. It has been found that populations of St Johns Wort from lower latitudes (warmer and drier sites) exhibited a lower leaf area than those from higher latitudes (Maron *et al.* 2007) suggesting that lower leaf area may in fact be an adaptation to the warmer and drier conditions found in the south of the northern hemisphere.

The very high temperatures recorded in the glasshouse for the duration of the experiment were generally higher than those that would be recorded in the field. This, coupled with the more regular watering regime than would be experienced under natural rainfed conditions, may have affected resource allocation patterns in the species. It is likely that growth of leaf and stem would have been enhanced under these conditions, and it is also likely that allocation of resources to roots may have been reduced. It is likely to be true that plants that are watered regularly from the top have reduced need to expend energy by putting resources into deep root systems, as water and nutrients are more easily accessed from the surface. While the conditions in the glasshouse may not truly reflect field conditions, it is unlikely that the broad patterns of resource allocation investigated in this study would greatly differ when measured in the field.

In conclusion, this chapter shows that the resource allocation patterns in three species of perennial *Trifolium* generally do not vary in response to the environment in which they evolved but are species defined. All species had significantly different shoot and root biomasses and root/shoot ratios, however individual populations of the same species did not vary greatly from each other. An exception to this was leaf area, where the two

strawberry clover populations had very different leaf areas consistent with differences in their habitats of origin. The populations of *Trifolium* could not match the growth and resource allocation pattern of lucerne with most having slower and/or shallower root growth and less allocation of resources to roots. Apart from the populations of strawberry clover, they also could not match the shoot growth of lucerne. They are thus unlikely to be as widely adapted to southern Australian farming systems. However, there is certainly the scope for these species to fit into niche habitats within the farming systems and thereby aid in the inclusion of perennial species into these areas. Indeed, strawberry clover has been identified as a top priority for investigation and targeted seed multiplication and characterisation for legumes for discharge areas (Hughes *et al.* 2008). It has proven successful on a site with occasional waterlogging and mild summer salinity in Western Australia, producing amongst the highest biomass in the second year after sowing and having the highest legume plant frequency in year 3 (Nichols *et al.* 2008). With its tolerance to waterlogging and mild salinity (Townsend 1985; Chapter 5) strawberry clover has the capability to improve the productivity and sustainability of pastures in areas affected by waterlogging and salinity. The deep and extensive root system of strawberry clover may also aid in short-term drought tolerance over the dry summers in southern Australia.

# CHAPTER 7

## General Discussion

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### **7.1 Introduction**

Strawberry clover (*Trifolium fragiferum* L.) is a perennial legume previously described as tolerant of waterlogging (Hoveland and Mikkelsen 1967) and, anecdotally, mild salinity (Rumbaugh *et al* 1993); attributes that make it a possible useful plant for application in mildly saline discharge areas affected by waterlogging and/or inundation. Few resources have been allocated to investigation of the agronomy and breeding of strawberry clover in Australia to date and little is known of the variability that may be exploited by breeding programs to improve the productivity and usefulness of strawberry clover for agriculture in southern Australia.

Genetic variation in plants allows populations to adapt to better fit the environment in which they are growing. Genetic variation in pasture plants is not only important as a source of breeding material but also to ensure the survival of a pasture in a variable environment. Farming systems in southern Australia experience a high degree of climatic variability, especially those with a Mediterranean climate with hot, dry summers.

This study showed high levels of genetic variation within and between populations of strawberry clover naturalised in Western Australia, and also in wild populations collected in the Mediterranean Basin (Chapters 3-6). This variation could be exploited by breeding programs to develop new cultivars of strawberry clover better suited to growth in Australian conditions than those currently available. In this chapter, the results of the 4 experimental chapters in this thesis are considered together. Points of interest are discussed in relation to the variability that exists in strawberry clover, the origin of this variability, and its applicability to the improvement of the adaptation of strawberry clover to farming systems on mildly saline discharge areas in Western Australia, as well as the broader role of strawberry clover in farming systems in Western Australia.



## **7.2 Strawberry clover germplasm is very variable**

Chapter 3 showed that strawberry clover pastures have adapted to widely varying environments in Western Australia and while each of the populations in the study was originally sown as the cultivar Palestine, they all now differ from each other quite markedly. A similar response in the outcrossing perennial legume white clover (*Trifolium repens* L.) was reported from New Zealand by Caradus *et al.* (1990) who found white clover populations from wet and dry hill country were morphologically very different. Unfortunately, it was not stated whether these populations all originated from the same cultivar. A high level of variation would be expected in a population of strawberry clover due to its outcrossing nature. It is likely that other outcrossing pasture legumes would behave in a similar manner when introduced to the varying environments inherent in southern Australian farming systems, although no studies have as yet examined this issue.

In the study of strawberry clover populations from Western Australia (Chapter 3), certain traits appear to have been selected for in each of the environments that the populations had been growing in and those plant types had, presumably, come to dominate at that location. For example, at the two sites where grazing pressure was greatest (Badgingarra and Brookton), plants were typically smaller and more prostrate with smaller leaves and shorter stems than those from the other locations. Intensive grazing has been associated with a more prostrate growth habit in grasses in Europe (Mysterud and Mysterud 2000) and in capeweed (*Arctotheca calendula* L.) in southwestern Australia (Dunbabin 2001). In the current study, the sites which had experienced the heaviest grazing pressure were also the drier sites, making the impacts of grazing pressure and water availability impossible to separate. More prostrate and smaller leafed plant types were also observed in strawberry clover populations collected from drier sites in the Mediterranean Basin (Chapter 4).

Variation within and between populations was also found to be high for populations of strawberry clover collected from the Mediterranean basin (Chapter 4). The proportion of variation attributed to within population for various plant traits was almost double that in the Western Australian populations (Chapter 3). This is not surprising given that the populations from Western Australia all originated from the one cultivar whereas those collected in the Mediterranean came from widely varying locations, had not been

actively domesticated and were generally found growing wild and not placed under the uniform selection pressure that a systematic grazing regime as part of defined agricultural system would impose.

Strawberry clover populations also exhibited variation in their growth in waterlogged and saline conditions (Chapter 5). Three of the four populations studied showed adaptation to growth in waterlogged conditions, while growth in saline and waterlogged + saline conditions, whilst generally reduced, did vary markedly between populations. However, there was little variation in the allocation of resources between two populations of strawberry clover (Chapter 6), except in the case of leaf area, where one population had a much higher leaf area over the duration of the experiment than the other, despite having very similar total shoot (stem plus leaf) dry weights. When considered together, however, the response to salinity and waterlogging (Chapter 5) and the allocation of resource studies (Chapter 6) do further highlight the high level of variation present within and between strawberry clover populations.

This high level of variation found within and between populations of strawberry clover in both naturalised Western Australian pastures and in the Mediterranean Basin provides ample opportunity for the breeding of improved cultivars for use in southern Australia. By targeting and selecting desirable characteristics such as vigorous seedling growth, high biomass production and good salinity and waterlogging tolerance, new cultivars of strawberry clover will be able to provide farmers with a tool to increase production on mildly saline and waterlogged soils; the potential area of best adaptation of strawberry clover.

For instance, it may be possible to breed strawberry clover cultivars better adapted to use in mildly saline discharge sites in Western Australia using material selected only from targeted naturalised populations of strawberry clover from Western Australia, in view of the level of variation within these populations. Strawberry clover, like lucerne (*Medicago sativa* L.), is predominantly self-incompatible (Morley 1963) and this is presumably a mechanism to prevent inbreeding. Even so, many outcrossing species including lucerne can often suffer inbreeding depression when the genetic base of a population is small (e.g. Rumbaugh *et al.* 1988) so it is important not to limit the genetic base from which new cultivars are developed. Therefore it may be wise not to limit strawberry clover genetic material in breeding programs even though ample variation

appears to exist within the local populations. It is likely that naturalised populations from elsewhere in Australia may exhibit traits useful to be included in new cultivars, and Mediterranean populations (Chapter 4) could also provide useful traits, especially if collection of new populations is targeted to areas with similar characteristics to those for which better adapted cultivars of strawberry clover are targeted. Indeed, the use of ecogeographic data to enable climate matching between sources of plant diversity and target environments in Australian farming systems has been suggested as an important method for targeted collection of new germplasm (Bennett 1999; Ehrman and Cocks 1990; Ghamkhar *et al.* 2007).

The high levels of within and between population variation described in the naturalised Western Australian populations of strawberry clover also raises the issue of cultivar integrity. While it was known that each of the five populations were all originally sown as cultivar Palestine, it is not known how different the seed sources for each of these was in the beginning. While the cultivar sown had the same name it may not have been originally identical because of the outcrossing nature of strawberry clover. The seed source could already have begun to adapt to the site at which seed production occurred. Thus seed in different years from the same paddock would be expected to differ and the longer the time between seed collection the larger the differences likely to be encountered. This raises a question about defining the genetic status of a ‘cultivar’. From a practical perspective there would be advantages in seeking seed of a ‘cultivar’ from a source as similar in characteristics as the target area to be sown, in a similar manner as to that described above in searching out new sources of germplasm from ecogeographical data systems. It also indicates that in future studies of strawberry clover the origin of any cultivars used should be described with as much detail as possible.

### ***7.3 Adaptation of strawberry clover to waterlogged and mildly saline conditions***

Strawberry clover populations from Western Australia and the Mediterranean showed evidence of adaptation to growth in waterlogged conditions (Chapter 5). Three of the four strawberry clover populations included in the study when grown under waterlogged conditions had either no reduction in shoot and root growth or increased shoot and root growth. It was also found that some populations of strawberry clover showed more

tolerance to salinity than others, i.e. the presence of salinity in the absence of waterlogging did not decrease their growth to the same extent as for more susceptible populations.

Many other *Trifolium* species from the section Vesicaria are also tolerant of waterlogging, including *Trifolium tomentosum* L., a widely found naturalised annual cover in Western Australia (Gibberd and Cocks 1997). Variation in salinity tolerance has also been reported for a number of *Lotus* and *Trifolium* species (Schachtman and Kelman 1991) and for strawberry clover (Rumbaugh *et al.* 1993). While Schachtman and Kelman (1991) conducted their study in soil, as was the experiment reported on in Chapter 5, the experiment carried out by Rumbaugh *et al.* (1993) was in a substrate-free nutrient solution. The effects of waterlogging on plant growth can vary considerably in different soils and can be confounded by interaction with nutrient availability. For example, the ranking of six varieties of wheat for waterlogging tolerance changed under different soils (Khabaz-Saberi *et al.* 2006). In acid waterlogged soils, shoot concentrations of iron, manganese and aluminium were increased two – 10-fold, and in some cases were at toxic concentrations compared to plants grown in drained conditions. Concentrations of these elements were decreased or unchanged in shoots of plants grown in waterlogged neutral soils (Khabaz-Saberi *et al.* 2006). The authors concluded that their results supported the importance of screening for waterlogging tolerance in soils from the environment into which new varieties would be grown, for accurate germplasm characterisation to occur. While demonstration of adaptation to waterlogging and salinity stress in the target environment is important, these stresses tend to be spatially and temporally highly variable in the field. Use of soil to screen for variability within a population is often confounded by this variability so a controlled environment such as a nutrient solution is preferable. Once differences have been established, these then need to be confirmed in field soils in the target environment.

One of the few studies to examine the effect of both salinity and waterlogging on the growth of species of *Trifolium* reported that the species showed different responses in root porosities for salinity and waterlogging combined compared to waterlogging or salinity alone (Rogers and West 1993). Root porosity of strawberry and white clover was highest with salinity and waterlogging combined. Balansa clover (*Trifolium michelianum* L.) had its highest root porosity when subjected to waterlogging alone while subterranean clover (*T. subterraneum* L.) recorded its highest porosity when

subjected to salinity alone. In this experiment 60 mM NaCl was used for all salinity treatments, and the waterlogging treatment was induced by flooding pots for 15 days. Variation in root porosity in response to salinity and waterlogging treatments was also found between the different populations of strawberry clover included in Chapter 5, highlighting the variation between the populations. There was also a lack of correlation between high porosity and good waterlogging tolerance in Chapter 5. Root porosity response was so variable over all populations and treatments that there was no clear pattern to be discerned. This result conflicts with studies between species where increases in root porosity were highly correlated with waterlogging tolerance (Armstrong *et al.* 1991; Barrett-Lennard 2003; Gibberd *et al.* 1999; Gibberd *et al.* 2001). It may be that for strawberry clover other mechanisms of waterlogging tolerance are more important in maintaining plant growth in waterlogged and flooded conditions and further investigation into what these may be is warranted. Root porosity therefore does not appear to be suitable as a trait for use in screening strawberry clover populations for genetic variability in waterlogging tolerance.

The two populations of strawberry clover from Western Australia investigated for salinity and waterlogging tolerance in Chapter 5 had similar levels of variation to the other two Western Australian populations investigated in Chapter 3. However, both populations used in Chapter 5, while exposed to some waterlogging in the pastures from which they were collected, would not have been exposed to salinity and thus may not have possessed the highest variation available for salinity and waterlogging tolerance within Western Australian populations. In fact, as the Brookton population (not used in Chapter 5) was growing in a mildly saline, intermittently waterlogged site, it would have been interesting to have included this population in the salinity and waterlogging experiment described in Chapter 5. The two Mediterranean strawberry clover populations included in Chapter 5 were similarly variable to the remaining populations from Chapter 4. It is likely that other naturalised populations of strawberry clover in Australia could possess a greater variation for improved tolerance to waterlogging and salinity and it would be interesting to target those populations growing in periodically salty and/or waterlogged conditions for further investigation. This would then enable investigation into their inherent level of stress tolerance and the extent to which exposure to stress has increased the gene frequency of those genes conferring the tolerance. If the genes are present in all populations then the issue is to develop accurate and low cost screens to identify genotypes carrying those genes. *In situ*

adaptation, as discussed above, increases the frequency of expression of those genes and importantly increases the opportunity for combining genes where the tolerance is multigenic. These elite plants are the important starting point for further improvement in the hands of breeders.

Lucerne is also an outcrossing plant and consequently cultivars can often be quite variable. In Chapter 5 and 6, lucerne was represented with only one cultivar – Pioneer L69. Lucerne populations do show variation in salinity and waterlogging tolerance (Humphries and Auricht 2001). It is unlikely that the one population included in this thesis would have represented the full spectrum of the variation contained in the species for waterlogging and salinity tolerance.

The use of milder salinity levels in the salinity and salinity + waterlogging treatments than the 120 mM NaCl used in Chapter 5 may yield more interesting results on the variation contained in strawberry clover for tolerance to these treatments. Also, there has been little work on the variability in salinity and waterlogging over time in discharge zones in southern Australia. A greater knowledge of how these two factors vary in the field would allow design of more realistic salinity and waterlogging regimes for testing of populations under glasshouse conditions. The effect this might have on plant growth would also prove an interesting investigation.

#### ***7.4 Resource allocation patterns and adaptation to southern Australian farming systems***

There was little variation in resource allocation patterns within species (Chapter 6). Resource allocation patterns did not vary in response to the environment that populations originated from but were distinct for each plant species. Both populations of strawberry clover included in Chapter 6 had almost identical resource allocation patterns and plant growth characteristics, even though they came from widely varying environments (low-rainfall Spanish grassland and medium – high-rainfall managed pasture in Western Australia). Results from Chapters 3 and 4 showed morphological differences between the populations of strawberry clover so it is possible that if more populations of the one species had been included in the study reported in Chapter 6, differences within species may have occurred. However, as these morphological differences were only measured in leaf and stem characteristics it is not possible to

definitively conclude if this would have been the case. It is possible for the populations to be morphologically different to each other in their shoot characteristics and still exhibit the same resource allocation pattern. This was indeed the case for the two populations of strawberry clover included in Chapter 6, where the Spanish population consisted of overall smaller, more prostrate plants with thinner stems (Chapter 4) than the larger, less prostrate, Badgingarra strawberry clover (Chapter 3). The resource allocation patterns of the two strawberry clover populations were different to those shown for the other species included in the chapter.

Strawberry clover, *T. alpestre* and *T. uniflorum* did not have resource allocation patterns similar to lucerne and, in particular, had slower developing root systems and, in the case of *T. alpestre* and *T. uniflorum*, much shallower and less extensive root systems (Chapter 6). Overall, lucerne had a significantly higher root biomass and deeper penetrating roots than all other species studied. Strawberry clover roots did penetrate as deep as the lucerne roots by the end of the study, but were slower to get to their maximum depth. None of the other species reached the 1 m maximum depth during the time frame of the experiment. While the strawberry clover populations included in Chapter 6 had a root growth rate that was close to that of the lucerne cultivar they did exhibit a sudden decrease in root biomass and in the proportion of resources allocated to roots mid-way through the experiment. This effect was much less marked in lucerne. A reduction in watering occurred just prior to this and temperatures were increasing as spring was replaced by summer. This suggests that strawberry clover is not as drought tolerant as lucerne. Overall, the results from Chapter 6 indicate that it is unlikely that strawberry clover, *T. alpestre* or *T. uniflorum* will be as broadly persistent in southern Australian farming systems, with their hot dry summers, as lucerne.

While lucerne is the best current perennial legume option for inclusion in farming systems, it is far from ideal. Its lack of tolerance to acid soils and waterlogging are major impediments to its survival in the Western Australian wheatbelt, particularly in discharge zones (Humphries and Auricht 2001). Interestingly, while lucerne appears to be the most drought tolerant of the species investigated in Chapter 6, its resource allocation pattern is not necessarily similar to native plants that have evolved to survive conditions in southern Australia. In a study comparing lucerne to 2 native Australian herbaceous perennial legumes, Denton *et al.* (2006) found that the native legumes had a significant proportion of their root systems at depth, whereas lucerne had the majority of

roots concentrated in the surface soil. Deep penetrating roots and higher root density at depth are considered to improve the survival of plants in drought conditions (Asch *et al.* 2005; Erice *et al.* 2006; Ho *et al.* 2005). Summer drought is an integral part of the southern Australian climate, therefore these characteristics may enhance survival of a perennial pasture plant in the farming systems of this area.

A closer investigation of the root morphology of the species studied, by sectioning the tubes by depth, would have proved interesting. Native herbaceous perennial legumes originating in low rainfall environments, such as *Kennedia prorepens* F.Muell., have a greater proportion of their roots at depth, a characteristic that is likely genetically programmed to optimise water capture from depth (Denton *et al.* 2006). A more detailed study on the root morphology of each species would have allowed conclusions to be made on whether resource allocation to different sections or parts of the rooting system of the plants changed throughout establishment and when the watering regime was reduced. Very few studies exist on this topic. Increases in root growth at depth with no change in overall root biomass have been found in rice (Asch *et al.* 2005) and in wheat wild relatives (Reynolds *et al.* 2007) under moisture stress conditions. The ability to grow deeper roots and extract water from depth is a good adaptation for the survival of perennial plants over the period of summer drought often experienced in southern Australian farming systems.

Further investigation into defining what the optimal resource allocation pattern for a productive perennial legume in southern Australian farming systems should be a priority. There may also be more than one pattern or strategy for successful survival and production of perennial legumes. Rooting depth and proportion of roots concentrated at depth are likely to be important components (Denton *et al.* 2006) but this may not be the whole story. Woodier but less productive species may be better adapted to drought. *Dorycnium hirsutum* L. appears to have good drought tolerance. Its deep roots confer an ability to extract water from depth and thereby allow maintenance of green leaf during severe drought conditions; a useful trait in a dryland livestock production system (Bell *et al.* 2008). However, *D. hirsutum* is less productive than lucerne and has a poorer forage quality. Understanding of the optimal resource allocation pattern(s) for maximising survival and productivity, and of the trade-off that may exist between these two parameters, for perennial legumes in southern Australian



farming systems would aid in the targeting of suitable germplasm and improved selection decisions for breeding programs. Substantial further research is required.

### **7.5 What role for strawberry clover in farming systems in south-west Australia?**

Dryland salinity is an increasing threat to agricultural production in southern Australia. There are currently 5.7 million hectares of agricultural land in Australia estimated to be affected by dryland salinity or at risk from shallow watertables and this area is expected to increase to 17 million hectares by 2050 (Dolling *et al.* 2001). Also often associated with dryland salinity is an increased risk of waterlogging and inundation that result in additional stresses on plant growth (Barrett-Lennard *et al.* 1999). There is a need for future development of plants suited for growth in discharge areas prone to salinity and waterlogging not only to improve agricultural productivity but to aid in reducing the spread of dryland salinity and its impact on farm sustainability. Perennial plants have been suggested as an important tool to reach this goal due to their year round growth and deeper rooting habit enabling them to access more water than annual crops and pastures and so aid in reducing deep drainage (recharge) (Cocks 2001; Cocks 2003; Dear *et al.* 2003). It is important to develop perennial pastures adapted to growth in all landscapes and farming systems in southern Australia, but, no one plant will contain all the necessary traits. A diversity of perennial pasture options will ensure productivity over a wide range of environments and will reduce the risk of disease or insects wiping out farming systems relying on a single perennial pasture.

In a review of work conducted by researchers from the Co-operative Research Centre for Plant-based Management of Dryland Salinity (CRC Salinity), Dear *et al.* (2008) concluded that strawberry clover only appeared to have a role in high rainfall southern latitudes where summers are less harsh. They also concluded that strawberry clover cv. Palestine performed poorly in most locations despite its reputation as being adapted to saline, waterlogged conditions, in an evaluation at 5 discharge sites across southern Australia over 3 years. Three of these sites were classified as highly saline in summer, the other 2 being moderately saline, while in winter one site was classified as moderately saline, 2 had low salinity and 2 were non-saline (Nichols *et al.* 2008). Perennial legumes that were identified with potential for discharge sites through these evaluations included *Melilotus siculus* (Turra) Vitman ex B.D. Jacks., *M. sulcatus* Desf.,

and *Lotus tenuis*; all species yet to be subjected to any significant breeding or selection in Australia.

However, this conclusion does not take into consideration the high variation inherent in strawberry clover due to its outcrossing nature. This thesis has shown significant levels of variation within and between populations of strawberry clover, including for waterlogging and salinity tolerance, which could be exploited in breeding programs to improve its performance. Dear *et al.* (2008) also stated that productivity of strawberry clover was considered low. At the Badgingarra, Western Australia, collection site productivity of the pasture was observed to be very high (McDonald, unpublished data) and this pasture was predominantly strawberry clover with kikuyu (*Pennisetum clandestinum* Hochst. ex Chiov.) and annual ryegrass (*Lolium rigidum* Gaud.). The site is located in a medium rainfall zone but had access to fresh water at depth for much of the year. In a 3 year study including 24 perennial legumes from 20 species, strawberry clover was also found to be one of the better performing perennial legumes for persistence and biomass production at Cranbrook, Western Australia, a site with occasional waterlogging and a summer salinity estimated at a moderate 6.9 dS/m in the surface 10 cm of soil (Nichols *et al.* 2008). These instances highlight the potential usefulness of strawberry clover in suitable situations, and the possibility that with improved cultivars this potential may be further exploited. Variation in productivity and waterlogging and salinity tolerance are worth further investigation as there are so few other available options that do not require more extensive breeding (Cocks 2001; Dear *et al.* 2003). Dear *et al.* (2008) did include strawberry clover in a list of plant species with cultivars suitable for immediate use and for further investigation in both recharge sites (heavy clays and poorly drained soils lower in the landscape) and discharge sites (areas with high levels of soil moisture over summer and mild salinity). This thesis supports these recommendations.

Strawberry clover has previously been under-resourced for breeding and development activities. With its tolerance to waterlogging and mildly saline soils, improved cultivars of strawberry clover could prove to be a very productive and useful niche plant on discharge sites. This thesis has shown there is ample genetic variation in strawberry clover to warrant further investigation into improving agronomic characteristics such as seedling vigour and biomass production as well as waterlogging and salinity tolerance and adaptation to the environments in the southern Australian wheatbelt.

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