Evaluation of *Dorycnium* spp. as Alternative Forage Plants.

by

Simon Roger Davies

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University of Tasmania
Hobart

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Declaration

I declare that this thesis contains no material which has been accepted for the reward of any other degree or diploma in any other tertiary institution and, to the best of my knowledge and belief, contains no copy or paraphrase previously published or written by any other person except where due reference in made in the text of the thesis.

Simon R. Davies

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Publications From this Project

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Abstract

The genus *Dorycnium* L. consists of a number of species of perennial leguminous shrubs known to be relatively drought tolerant. Low rainfall areas (i.e <600 mm annually) of Australia under agricultural use are subject to periods of feed shortage during summer and autumn, and hence animal production is limited at this time. *Dorycnium* spp. have been identified as having the potential to be integrated into Australian grazing systems as a source of forage when little or no other feed is available. This project was established to further investigate a number of key issues related to the agronomic and forage characteristics of this potentially important genus. Research was undertaken into *Dorycnium* spp. to examine three important factors associated with the evaluation of a legume, seed germination characteristics, the nutritional value of the forage, and rhizobial associations.

Dorycnium hirsutum Ser. accessions TAS1002 and TAS2001 were subjected to a range of germination experiments examining the level of pod maturity, harvest season, and the effect of pre-germination treatments. The seed coat of *D. hirsutum* was found to influence germination behaviour, with the use of pre-germination scarification treatments improving germination behaviour by increasing the percentage germination (PG) and lowering the mean time to complete germination (MTG) and percentage hard seed. Mechanical scarification of TAS2001 for 20 seconds was found to increase ($P<0.05$) PG from 86 to 96 %, lower the MTG from 6.0 to 2.7 days, and reduce the percentage hard from 13.6 to 1.9 % in relation to untreated seed. Mechanical and chemical scarification techniques were found to be the most effective in promoting rapid and uniform germination, were simple to apply and were repeatable. In general, inherent differences in seed lot germination characteristics were believed to be associated with the influence of environmental factors and the natural characteristics of selected accessions with indeterminate flowering.

Established plots of *D. rectum* Ser., *D. hirsutum* and *D. pentaphyllum* Scop. were sampled along with an area of lucerne (*Medicago sativa* L.) on a regular basis throughout the spring/summer period of 2001/2002. Samples were analysed using

near infrared reflectance spectroscopy (NIRS) and wet chemistry for crude protein (CP), neutral detergent fibre (NDF) and dry matter digestibility (DMD) and metabolisable energy (ME). Over the course of the sampling period forage of *Dorycnium* spp. generally displayed decreases in CP, ME, DMD and increases in NDF. Typical CP values ranged from 4 – 18 % of dry matter (DM), NDF 21 – 72 % of DM, DMD 32 – 75 %, and ME 4.1 – 11.0 MJ/Kg/DM. The nutritive value of *Dorycnium* spp. forage appeared to be influenced by environmental and developmental characteristics, with the growth stage identified as a useful tool for predicting forage quality. Although *Dorycnium* plants were of lower forage value than lucerne, their forage can provide livestock with an important source of nutrition in areas of low rainfall and during periods where there are feed gaps.

Experimental plots of *Dorycnium* spp. at three Tasmanian sites were sampled every six weeks throughout the spring/summer period of 2002/2003 and analysed using a modified butanol-HCl method for condensed tannins (CT). The CT content of *D. hirsutum* was found to fluctuate from 3.2 to 16.6 % of the DM. *Dorycnium rectum* and *D. pentaphyllum* were found to contain CT levels of at least 7.7 and 6.8 % of DM respectively during the sampling period. The CT levels observed were considered to be high in general, with only *D. hirsutum* containing levels that may be considered to be low and possibly beneficial at certain stages of development. Increases in CT levels were associated with the initiation of flowering, and interactions between the environment and species were observed, although no common factor was identified as influencing CT levels.

A glasshouse experiment was undertaken to assess the nitrogen fixing ability of the commercial *Lotus corniculatus* L. inoculant SU343 with *Dorycnium* spp. against a range of alternative inoculants. The host/rhizobia interactions of *Dorycnium* spp. along with six important pasture legumes and a range of inoculants was assessed. Strains WSM1284, WSM2323 and WSM2338, along with SU343 were found to be suitable inoculants for *Dorycnium* spp. examined. However, negative interactions between these inoculants and important pasture legumes were identified. Inoculant strains, WSM1284, WSM2323, WSM2338 and SU343 were selected to undergo evaluation under Tasmanian field conditions with *D. hirsutum* and *D. rectum*. In the field all strains were found to fix adequate amounts of atmospheric nitrogen.

Inoculant SU343 was confirmed to be a suitable inoculant for *D. rectum* in terms of performance and commercial viability, however, WSM2338 and WSM1284 were found to be equally suitable. The inoculation of *Dorycnium hirsutum* with the Tasmanian isolate WSM2323, was found to be a significantly ($P<0.05$) superior strain to SU343 in terms of nitrogen fixation. The inoculation of *D. hirsutum* did not affect ($P>0.05$) plant DM production in the field. The ability of the inoculants to compete with a background population of root nodule bacteria was found to be of concern, and may have serious implications for the long-term performance from a single inoculation event. It was proposed that a combination of rhizobial strains may be more effective as a commercial inoculant rather than relying on the single *L. corniculatus* inoculant SU343.

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Chapter 1. Introduction

The maintenance of pasture systems and subsequent animal production throughout the year presents grazing industries with a challenge in drought prone and semi-arid areas of Australia. The ability to sustain animal production during summer and early autumn is very difficult due to poor persistence and production of pasture species when soil moisture and rain are limited. The leguminous genus *Dorycnium* has been identified as possessing traits which enable forage production under such conditions. Therefore, this project was established, and funded by Rural Industries Research and Development Corporation (RIRDC) to investigate the potential of *Dorycnium* spp. as a source of forage under low rainfall conditions.

Australia's temperate and Mediterranean climatic regions present a wide variety of conditions, such as, low winter temperatures, high summer temperatures and variable rainfall. Feed shortages or feed gaps are common during summer and early autumn and have led to research into a range of alternative plants for inclusion in grazing systems. Alternative forage species have been sought from around the globe that display tolerance of Australia's harsh climatic conditions. *Dorycnium* is one of many genera identified from the Mediterranean region and currently being investigated throughout Southern Australia for use in low rainfall (i.e <600 mm annually) regions. *Dorycnium* spp. also known as canary clovers are perennial leguminous shrubs which are believed to be tolerant of drought conditions. *Dorycnium* spp. has been identified as having the potential to be integrated into Australian agricultural systems as a source of nutritional forage for utilisation when little or no other feed is available.

The evaluation of a new pasture legume presents a range of issues relating to the agronomic, physical, chemical and biochemical properties of the plant. The potential for research is extensive and continuous. Evaluation is concerned with identifying traits which make a species or cultivar useful, or increasing the knowledge of its characteristics (Laidlaw and Reed, 1993). The evaluation of a forage plant should take into account the following traits, ease and rate of establishment, nutrition levels and presence of toxins, ease of harvest and effect on the environment (Laidlaw and

Reed, 1993). In addition to this, Howieson (1995) stated that the examination of new pasture legumes requires substantial research into identifying well adapted root nodule bacteria. Legumes provide high quality forage as well as a source of nitrogen (Raven *et al.,* 1992; Howieson, 1995).

The survival of a species is reliant on the process of seed germination (Campbell, 1993; Bell, 1999). The time taken for seeds to emerge is critical in relation to crop establishment and may determine the eventual yield (Parera and Cantliffe, 1992). When investigating the germination behaviour of seeds about which little is known, a number of crucial questions need to be considered, such as, are there any specific treatments required for germination to occur (Baskin and Baskin, 1998). Hard seed coats can be a major limiting factor in the establishment of some forage legumes (Hick *et al.,* 1989).

The evaluation of forage quality is of great importance in determining the nutritional value it presents for grazing animals (Schneider and Platt, 1975). Chemical analysis is a means by which the potential for the delivery of a particular nutrient in a food can be determined, however, the actual amount obtained by an animal may be affected by losses through the digestive system i.e faeces (Schneider and Platt, 1975; Minson, 1990). Consideration must also be given to anti-nutritional factors such as condensed tannins. Condensed tannins can affect forage quality and cause toxicity (Kumar and Singh, 1984) with changes in digestive processes (Barry and McNabb, 1999).

The lack of well adapted root nodule bacteria is a hindrance to the commercialisation of new pasture legumes (Howieson, 1995). Brockwell *et al.*, (1995) stated that the symbiotic function of legumes is often overlooked by agronomists. The selection and evaluation process for new strains involves, strain isolation, assessment of nitrogen fixing ability, field evaluation and rhizobial survival (Howieson *et al.* 2000a).

The scope of this project was very broad, as extensive research into the *Dorycnium* genus had not been undertaken. The goal of this project was to evaluate three *Dorycnium* spp., *D. rectum*, *D. hirsutum* and *D. pentaphyllum*, which displayed suitable physiological and agronomic traits as alternative forage plants. The

undertaking of this project and extended industry work are aimed at forming the basis for determining the suitability of *Dorycnium* spp. for inclusion into Australian grazing systems, and identifying future research into this genus.

Overview of Thesis Structure

This PhD project aimed to identify a number of key issues related to the agronomic and forage characteristics of *Dorycnium* spp. Four main sections of work form the thesis;

1. A literature review of *Dorycnium* spp.
2. Examination of the germination characteristics of *Dorycnium* spp. seed.
3. Evaluation of *Dorycnium* spp. forage quality and condensed tannin content.
4. Examination of *Dorycnium* spp./rhizobia associations.

The three experimental sections also contain background literature reviews.

Chapter 2. Literature Review – The Genus *Dorycnium*

2.1 Introduction

The undertaking of this evaluation project into *Dorycnium* spp. has initiated research into a relatively unknown genus. The ability to draw on all previous research and literature pertaining to this subject is critical in forming the basis for conducting future research. This will further our understanding of the plants characteristics and potential. Therefore an extensive literature search was initiated as part of this project to meet these objectives. The following is a literature review of the major publications pertaining to *Dorycnium* spp. and legumes in general.

2.2 Legumes

Fabaceae (formerly Leguminosae) is considered to be the second largest family of flowering plants based on the number of species it contains (Allen and Allen, 1981). The Fabaceae family comprises three subfamilies, Caesalpinioideae, Mimosoideae and Papilionoideae (Dénarié *et al.,* 1992). Brockwell *et al.* (1995) stated that the family of legumes is as diverse as it is large. Within the family Fabaceae there are believed to be approximately 750 genera and between 15,000 and 20,000 species (Allen and Allen, 1981; Pepper and Upchurch, 1991; Salisbury and Ross, 1992; Dénarié *et al.,* 1996). The distribution of this family is relatively wide with legumes found from equatorial to arctic regions (Brockwell *et al.,* 1995). The Fabaceae family contains many plant forms including, woody vines, shrubs, trees, annual or perennial herbs (Allen and Allen, 1981).

2.2.1 Legumes in Agriculture

The use of legumes in agriculture has been undertaken for many centuries (Pepper, 1991). Brockwell *et al.* (1995) stated that in agriculture approximately 100 or less legumes were of importance. The use of legumes in modern agriculture involves the rotation of leguminous crops with non-leguminous crops where the leguminous crops not only increase the nitrogen content in the soil by fixation and decomposition of plant material, but can be harvested for feed (Raven *et al.,* 1992). Legumes have many uses in agriculture and can provide many products including, forage, green

manure (Pepper, 1991), firewood, pharmaceuticals, honey, shelter belts and reclamation of degraded land (Brockwell *et al.,* 1995).

2.2.2 Nitrogen Cycling

The mineralisation of soil organic matter and plant residues is the means by which most plants obtain the nitrogen which they require. Legumes have the ability to thrive in areas where there is a minimal supply of nitrogen from the soil by forming symbiotic relationships with rhizobia, which biologically fix nitrogen on the roots of the legume (Allen and Allen, 1981). Legumes may be used to serve a number of purposes including, reduce the use of fertilisers, improve ground cover, and reduce residual soil nitrates, which may contaminate ground water (Zachariassen and Power, 1991).

2.3 *Dorycnium* Species.

Dorycnium is a genus of plants consisting of approximately 12 species (Allen and Allen, 1981; Slavik, 1995), which are broadly classified as herbaceous perennials and deciduous sub-shrubs (Allen and Allen, 1981; Wills and Douglas, 1984). From research conducted in New Zealand, *Dorycnium* spp. have been described as low growing perennial legumes that are frost and drought tolerant (Wills, 1983; Rys *et al.,* 1988; Wills, 1994), with severe frosting experienced during winter having little or no effect (Wills *et al.,* 1989a). *Dorycnium* spp. are generally found on soils that are of low fertility, light and sandy in texture (Allen and Allen, 1981). It is also reported that *Dorycnium* spp. have been found to be suited to the following climatic conditions: high winds, low winter temperatures and low rainfall (Wills and Douglas, 1984). In addition to this, *Dorycnium* has been found to have good fire resistance (Wills and Douglas, 1984).

2.3.1 Origin of *Dorycnium* spp.

Dorycnium spp. originated from the Mediterranean region (Rys *et al.,* 1988; Wills *et al.,* 1989a; Wills, 1994; Slavik, 1995) and are found from the Canary Islands to the Caspian Sea and from Central Europe to Northern Africa (Slavik, 1995). *D. pentaphyllum* and *D. hirsutum* are native to the Mediterranean basin (Wills *et al.,*

1989a; Alegre *et al.,* 1998), whereas, *D. rectum* originated from the Canary Islands (Douglas *et al.,* 1996a).

2.3.2 Germination and Emergence

Germination tests performed *in vitro* revealed that both *D. hirsutum* and *D. pentaphyllum* had greater than 80% germination after 28 days (Wills *et al.,* 1989b) with scarification found to improve the percentage (Wills *et al.,* 1989a+b). The scarification and pelleting of seed prior to planting has been found to enhance seedling establishment (Wills, 1983; Wills and Douglas, 1984; Sheppard and Douglas, 1986).

Germination trials undertaken by Douglas and Foote (1994) where average soil temperatures ranged from 8 to 24 ^{o}C, found that the number of days for the emergence of *D. hirsutum, D. pentaphyllum* and *D. rectum* were 13.7, 11.3 and 11.3 respectively. The percentage emergence for *D. hirsutum, D. pentaphyllum* and *D. rectum* were 10.9, 31.3 and 36.2 respectively.

2.3.3 Field Establishment

Dorycnium spp. seed should be planted in the field in autumn or early spring when the soil is likely to be moist or frost may not be a problem (Wills and Douglas, 1984). Douglas *et al.* (1996b) found that the spring sowing of *D. hirsutum* and *D. pentaphyllum* seeds resulted in 2-5 times greater emergence than sowings in autumn in the field, with this being attributed to warmer soil temperatures. The average annual rainfall for the experimental site was 850mm (Douglas *et al.,* 1996b), however, temperatures were not specified. Roder (1992) examined the establishment of the temperate legume species *D. hirsutum* in Bhutan and found that germination or establishment failure led to the species not being successful.

In the field *Dorycnium* can be established from either seed or seedlings grown in containers (Wills and Douglas, 1984; Sheppard and Douglas, 1986). Careful management of *Dorycnium* is required to ensure good establishment and survival (Wills, 1983).

2.3.4 Fertiliser Response

Low soil fertility is tolerated by most legumes, however, the addition of fertilisers can be beneficial (Wills, 1986), with the use of fertilisers such as superphosphate helping plants attain their maximum vigour (Sheppard and Douglas, 1986). Wills *et al*. (1999) found that *D. hirsutum* seedlings had taller vegetative growth and greater branching when treated with 20-10-0-13 fertiliser, in comparison with superphosphate (0-7-0-28) and no fertiliser treatments.

2.3.5 Vegetative Growth and Dry Matter Production

Wills *et al.* (1989b) found that *Dorycnium* spp. exhibited excellent long-term performance with comprehensive vegetative ground cover year round. However, as a forage plant *Dorycnium* spp. will probably be most beneficial in autumn/spring. Rys *et al.* (1988) found that *D. pentaphyllum* and *D. hirsutum* displayed different persistence at various New Zealand trial sites. The main vegetative growth phase for *D. hirsutum* and *D. pentaphyllum* occurs in spring, with a secondary phase often occurring in autumn/early winter and autumn respectively (Wills *et al.,* 1989a). Wills and Douglas (1984) and Wills *et al.* (1989b) found that at all of their New Zealand trial sites *D. hirsutum,* and to a lesser extent *D. pentaphyllum,* had spread some distance from the parent plants, with nodulation occurring effectively. Rys *et al.* (1988) found that the first year yields of *D. pentaphyllum* was approximately half that of *D. hirsutum*, however, after three years the yields of the two were approximately the same.

2.3.6 Flowering

Dorycnium spp. begin their growth early in spring with flowering occurring early in summer. Large numbers of seedlings have been found to establish around parent plants due to the prolific flowering of *Dorycnium* spp., even though seed set is considered to be low in the field (Wills, 1983; Wills and Douglas, 1984). Field trials in New Zealand have shown that where grazing of *Dorycnium* spp. has not been managed plants may continue to mature and die off following flowering (Wills, 1983).

2.3.7 Grazing and Management

Wills (1983) found that once established *Dorycnium* accessions can withstand hard grazing, however, the palatability of feed is highly variable. Wills (1983) and Wills and Douglas (1984) found that establishment of *Dorycnium* from seed is slow and consequently two growing seasons are required prior to grazing (Wills, 1994), however, once well established, plants can withstand heavy grazing via livestock (Wills and Douglas, 1984; Sheppard and Douglas, 1986; Wills, 1994). The lower palatability of *D. hirsutum* may be an advantage during times of drought stress due to limited grazing, whereas, *D. pentaphyllum* may be less persistent under stress conditions due to greater palatability and lower competitive and seeding ability (Rys *et al.,* 1988). The failure to manage *D. pentaphyllum* and *D. hirsutum* correctly may lead to the continued maturation of the plant and death may occur (Wills and Douglas, 1984; Wills, 1994). The agronomic characteristics of *D. hirsutum* and *D. pentaphyllum* are still relatively unknown and therefore the appropriate establishment, climatic and edaphic conditions need to be characterised (Wills *et al.,* 1989b). Douglas and Foote (1994) stated that the domestication of *Dorycnium* spp in New Zealand was in the early stages and improvements could be made with appropriate breeding strategies.

2.3.8 Soil Requirements

Trials in New Zealand's South Island have suggested that *Dorycnium* spp. are best suited to light, Brown Grey Earth soil types. Trials on New Zealand's South Island have also demonstrated that *Dorycnium* spp. are able to tolerate high soluble salt concentrations and soil pH's of 5.4 to 8.6 (Wills and Douglas, 1984; Wills *et al.,* 1989b; Wills, 1994). Free draining weakly acid to alkaline soil types are most suitable for *D. hirsutum* and *D. pentaphyllum,* with heavy wet soils not tolerated by these species (Wills *et al.,* 1989a). *Dorycnium rectum* is able to survive on damp soils (Douglas *et al.,* 1996a). Generally, soils high in clay content and that are wet result in poor growth of *Dorycnium* spp. (Wills and Douglas, 1984).

2.3.9 Aluminium Toxicity

The hill country of New Zealand is characterised by poor pasture persistence and production and this is believed to be associated with low soil fertility and subsoil acidity. As well as soil acidity, toxic levels of aluminium are believed to be

responsible for this (Wheeler and Dodd, 1995). Deficiency or toxicity of particular nutrients associated with soil acidity limits the growth of plants in many parts of the world. Investigations into the effect of soil acidity on the bacterium *Bradyrhizobium japonicum* found that Al toxicity is the most severe stress inhibiting growth in addition to soil acidity (Cline and Kaul, 1988). Cline and Kaul (1988) found that below pH 4.6 Al toxicity and acidity limited the growth of *B. japonicum*, however, acidity was the main limiting factor at pH's above 4.6.

Aluminium toxicity is a factor known to be limiting growth of plants in soils with a pH of less than 5.5 (Edmeades *et al.,* 1991). Edmeades *et al*. (1991) concluded that temperate grasses and legumes are sensitive to micro molar concentrations of aluminium. Wheeler and Dodd (1995) found that a range of temperate legume species grew well in the absence of aluminium, with no obvious nutritional disorders. Wheeler and Dodd (1995) measured the activity of aluminium (μM) at which root yield was reduced by 50%, and found that for *D. pentaphyllum* and *D.hirsutum* the aluminium activity (μM) was 1.5 - 2.0 and 1.4 - 2.4 respectively. Breeding of aluminium tolerant legumes needs to be done in order to improve the generally low tolerance of aluminium in the field.

2.4 *Dorycnium hirsutum*

2.4.1 Description and Characteristics

Dorycnium hirsutum or hairy canary clover, occurs naturally in the Mediterranean region and Southern Portugal (Allen and Allen, 1981; Sheppard and Douglas, 1986; Wills, 1986; Woodman *et al.,* 1992). This species is a perennial leguminous plant which belongs to the tribe Loteae and is closely related to the genus *Lotus* L. (Brockwell and Neal-Smith, 1966; Sheppard and Douglas, 1986; Wills *et al.,* 1989a).

Dorycnium hirsutum exhibits a wide range of phenotypic characteristics (Wills, 1983; Wills and Douglas, 1984). These plants produce erect stems covered in silvery hairy leaves (Wills and Douglas, 1984) and can grow up to about 0.5m in height and 1.0m in diameter (Sheppard and Douglas, 1986; Wills *et al.,* 1989a) (see Figure 2.1).

Dorycnium hirsutum characteristics include, lax branches, penta-foliate leaves, with greyish-green oblong to ovate densely hairy leaflets (Sheppard and Douglas, 1986). This plant produces large numbers of flowers (Alegre *et al.,* 1998), with flowering occurring in late spring/early summer (Sheppard and Douglas, 1986). White or pink flowers are produced (Wills and Douglas, 1984), grouped together in globular clusters (Sheppard and Douglas, 1986; Wills *et al.,* 1989a). *Dorycnium hirsutum* produces pods which are 6-12mm in length and contain about four seeds (Sheppard and Douglas, 1986), which when ripe are spread up to 1-2m from the parent plant (Wills *et al.,* 1989a). This species flowers prolifically allowing for adequate natural regeneration material (Rys *et al.,* 1988; Wills, 1994).

Dorycnium hirsutum has a deep taproot (Sheppard and Douglas, 1986) and is most suited to drought prone areas (Wills *et al.,* 1989a). The characteristics of this legume suggest the potential for use on hill faces where erosion has occurred (Sheppard and Douglas, 1986). Woodman *et al.* (1992) found that *D. hirsutum* was extremely drought tolerant and survived such conditions in the field. The results from their trials confirm that *D. hirsutum* is a legume suitable for use on drought prone lower slopes with sunny aspects in New Zealand.

Figure 2.1 *Dorycnium hirsutum* plant.

2.4.2 Forage Production

Dorycnium hirsutum showed early promise as a forage species and as a plant for controlling soil erosion in California (Brockwell and Neal-Smith, 1966). *Dorycnium hirsutum* has displayed the potential for being used as a 'fodder bank' species due to its drought tolerance (Chapman *et al.,* 1989). Brockwell and Neal-Smith (1966) found that *D. hirsutum* when examined in Canberra was found to be persistent and productive. *Dorycnium hirsutum* was found to be resistant to pests and was palatable to grazing stock (Woodman *et al.,* 1992), and once established could withstand heavy grazing (Wills and Douglas, 1984). Further research into *D. hirsutum* is required (Wills, 1983) with this plant displaying a great deal of promise due to its drought and frost tolerance (Sheppard and Douglas, 1986). Chapman *et al.* (1989) concluded that *D. hirsutum* is one of a number of legumes that may become a part of sustainable farm systems.

2.4.3 Soil Requirements

The growth of *D. hirsutum* on heavy clayey soils or on soils with high levels of soluble salts may limit plant growth by problems such as root rot during winter. The establishment of *D. hirsutum* may be done on free draining drought prone slightly acid to alkaline soils (Sheppard and Douglas, 1986). In the Mediterranean region *D. hirsutum* is frequently found in areas of higher elevation with calcareous soils with low soil fertility or close to the sea on light sandy soils (Brockwell and Neal-Smith, 1966).

2.4.4 Growth and Development

Douglas and Foote (1994) stated that the relative low emergence of *D. hirsutum* when compared with species such as *Medicago sativa* may be due to the species being prone to weed infestations or competition by other species. Vegetative growth begins early in the spring from the base of the plant, slowing during flowering, and resuming after seed ripening in autumn (Sheppard and Douglas, 1986). Wanjiku *et al.* (1997) found that the non-woody component of *D. hirsutum* decreased during summer, and that the high relative productivity was due to the production of woody material. Grasses and herbs may be established within the canopy of *D. hirsutum* due to its open growth habit (Wills, 1994).

2.4.5 Nitrogen Fixation

Wanjiku *et al.* (1997) found that the seasonal uptake of nitrogen from the atmosphere by *D. hirsutum* did not vary significantly, however, nitrogen fixation by *D. hirsutum* was greatest during winter/spring (July to November) and summer/winter (February to July) periods. The above ground amount of nitrogen fixed was 71 kg/ha/year, and the mean annual percentage of nitrogen uptake from the atmosphere was 98 for *D. hirsutum.*

2.5 *Dorycnium pentaphyllum*

2.5.1 Description and Characteristics

The common name of *D. pentaphyllum* is prostrate canary clover (Wills and Douglas, 1984; Keoghan, 1985; Wills, 1986; Woodman *et al.,* 1992; Wills, 1994). *Dorycnium pentaphyllum* is native to central and southern Europe (Sheppard and Douglas, 1986).

Dorycnium pentaphyllum is described as a leguminous sub-shrub (Wills *et al.,* 1989a) with a prostrate habit (Wills, 1983; Wills and Douglas, 1984; Rys *et al.,* 1988; Wills, 1994). This plant grows up to about 0.3 m in height (Wills and Douglas, 1984) and 1.0 m in diameter (Wills *et al.,* 1989a) (see Figure 2.2). The leaves of these plants are glabrous (Wills *et al.,* 1989a) and are palatable (Wills, 1983; Wills and Douglas, 1984; Wills, 1994). Ground cover provided by *D. pentaphyllum* is excellent due to its spreading growth habit (Wills, 1994), however, its open and prostrate growth habit allows for the growth of other herbs and grasses within the canopy (Wills and Douglas, 1984).

Dorycnium pentaphyllum flowers in late spring/early summer producing white flowers grouped in globular clusters (Wills and Douglas, 1984; Wills *et al.,* 1989a). *Dorycnium pentaphyllum* has small 2-4 mm pods that contain one seed which is propelled a short distance from the parent plant following ripening (Wills *et al.,* 1989). *Dorycnium pentaphyllum* reseeds poorly (Rys *et al.,* 1988; Wills, 1994), however, Herranz *et al.* (1998) found that *D. pentaphyllum* had the ability to regenerate via vegetative regrowth allows for less dependence on reproduction by

seeds. *Dorycnium pentaphyllum* can be propagated vegetatively very easily (Alegre *et al.,* 1998).

Dorycnium pentaphyllum is a facultative resprouter following fire, which suggests the plant is able to resprout from below the ground via vegetative buds. *Dorycnium pentaphyllum* seed treated at 120^{o}C for ten minutes, was found to have significantly higher germination over control seeds (Herranz *et al.,* 1998).

Dorycnium pentaphyllum has a deep taproot and is best adapted to drought prone areas (Wills *et al.,* 1989a). Alegre *et al.* (1998) found that *D. pentaphyllum* stem cuttings were able to form roots under cold (2 ^{o}C) and dark glasshouse conditions, however, rooting ability was improved when low night temperatures were avoided. The roots of *D. pentaphyllum* are substantially stimulated by treatment with auxin with the length of the rooting zone being affected (Alegre *et al.* 1998).

The flowers and fruits of *D. pentaphyllum* are almost indistinguishable from *Anthyllis fulgurans* Porta. (Lassen, 1979), with leaf morphology very similar to some species of *Lotus* (Wills and Douglas, 1984; Wills *et al.,* 1989a).

Figure 2.2 *Dorycnium pentaphyllum* plants

2.5.2 *Dorycnium pentaphyllum* ssp. herbacium and germanicum

The Czechoslovak flora refers only to *D. pentaphyllum* with references to the sub-species *germanicum* Rouy. and *herbaceum* Vill. These sub-species are often confused and referred to under the same name of *D. pentaphyllum* (Slavik, 1995). The two sub-species *germanicum* and *herbaceum* are found in two clearly different climatic localities with *herbaceum* being a far more ecologically tolerant species. *Germanicum* is found in dryer, warmer regions than *herbaceum.* The two *D. pentaphyllum* sub-species *germanicum* and *herbaceum* may be considered to be two *Dorycnium* species in their own right (Slavik, 1995).

Dorycnium pentaphyllum ssp. *germanicum* may be found in a range of habitats from, forests and woodlands, river canyons, rocky, dry, and grassy hill slopes (Slavik, 1995). *Dorycnium pentaphyllum* ssp. *germanicum* produces a deep root system that is able to draw on water deep within the soil profile. Warm, slightly shaded areas are recommended for growth, with most of the plants foliage being maintained during winter, and leaf replacement occurring during the following spring (Slavik, 1995).

Dorycnium pentaphyllum ssp. *herbaceum* may be found in a wide variety of habitats including pine and oak forests, dry grassy slopes and pastures. *Dorycnium pentaphyllum* ssp. *herbaceum* has been found on podsol soils, but is mainly found on brown earth soils in central Europe. The deep penetrating root system of *D. pentaphyllum* ssp. *herbaceum* allows the plant to tolerate dry skeletal soils, with slightly shaded warm areas preferred for growth. When compared to *D. pentaphyllum* ssp. *germanicum*, *D. pentaphyllum* ssp. *herbaceum* is more suited to higher rainfall and lower temperatures (Slavik, 1995). Slavik (1995) stated that previous research by V. Vacek found that *D. pentaphyllum* ssp. *germanicum* and *D. pentaphyllum* ssp. *herbaceum* were considered to be poor in terms of fodder due to a number of factors, including, lignification and hairy leaves. The description of the two sub-species by Slavik (1995) conflicts with that of Wills *et al.* (1989a) who described *D. pentaphyllum* as glabrous. This suggests that the two sub species *D. pentaphyllum* ssp. *germanicum* and *D. pentaphyllum* ssp. *herbaceum* may in fact be separate species in their own right.

2.6 *Dorycnium rectum*

2.6.1 Description and Characteristics

Dorycnium rectum, commonly known as erect canary clover is described as a perennial sub-shrub 0.1 m in diameter and 0.5 m tall (Chapman *et al.,* 1989) (see Figure 2.3), however it has been reported to grow over 2 m in height (Waghorn *et al.* 1998; Dear *et al.,* 2003). *Dorycnium rectum* has a well developed tap-root system, and is most likely tolerant of low fertility sites (Waghorn *et al.,* 1998).

Dorycnium rectum is native to northern Africa, temperate Asia and Southern Europe. In these locations *D. rectum* is usually found in damp areas (Dear *et al.,* 2003). Waghorn and Molan (2001) states that *D. rectum* originated from moist areas within the Mediterranean, Portugal and Spain.

Figure 2.3 *Dorycnium rectum* plants

2.6.2 Forage Production

Douglas *et al.* (1996a) found that *D. rectum* performed poorly at a dry site in New Zealand, with the plants being chlorotic despite having healthy nodules on the roots. It was concluded from this trial that *D. rectum* would be suitable for providing forage at seasonally dry sites.

Dorycnium rectum is able to regrow after intense defoliation (Douglas *et al.*1996a). Waghorn *et al.* (1998) stated that 3-4 cuts were possible per year for *D. rectum*, with yields of 20 t DM/ha possible (Douglas and Foote, 1994). Waghorn *et al.* (1998) described high levels of crude protein and condensed tannins in the leaves (1 8% and 19 % respectively), with lower levels of crude protein in the stems (5 %).

Despite containing high levels of condensed tannins, *D. rectum* displayed moderate palatability in sheep feeding trials conducted by. The use of this species is also of interest, as the condensed tannin content of the plant is believed to reduce the effects of gastrointestinal parasitism in sheep (Waghorn and Molan, 2001).

2.7 Other *Dorycnium* spp.

Several other species of *Dorycnium* are reported in the literature. The main species are *D. suffruticosum* which originates from the west Mediterranean, *D. jordanii* and *D. anatolicum* from Asia Minor and *D. haussknechtii* from the Armenian Highlands (Slavik, 1995).

Dorycnium axilliflorum found in Anatolia has almost sessile inflorescences and is closely related to *D. pentaphyllum* in terms of inflorescence and habit (Lassen, 1979).

2.8 Potential Uses of Alternative Legumes

Areas that limit plant establishment and growth due to factors such as temperature or soil moisture may be inhabited by a number of legumes for soil conservation/revegetation purposes (Douglas and Foote, 1994) and are a focus of research into alternative legumes (Douglas, 1993). The reduced ability to establish

plants for late autumn, winter and early spring grazing has led to the evaluation of a number of alternative legume and grass species (Keoghan, 1985). Also, the decline in the use of expensive fertilisers and an increase in soil erosion has led to a greater interest into a range of alternative legumes (Douglas, 1993).

Woodman *et al.* (1992) stated that in the South Island hill and high country of New Zealand the production of insufficient quality feed during winter and early spring is a major constraint in terms of meeting animal feed requirements. The lower sunny faces in the high country of New Zealand are areas where conventional species and cultivars are difficult to establish due to a number of factors, including, poor survival, persistence and spread. The establishment of persistent legumes on these slopes has the potential to provide cost effective feed during times of shortage (Chapman *et al.,* 1989).

2.9 Legumes in Arid Regions

A number of legumes are suited to growth in arid and semiarid areas, with legumes found in most deserts (Pepper and Upchurch, 1991). Legumes have a high adaptation to arid and semi arid environments, ability to fix nitrogen, grow on poor soils and are a source of forage (Ibañez, and Passera, 1997). An important part of sustainable agricultural systems in arid and semiarid areas is the use of multipurpose trees and shrubs that provide an important source of forage for stock and wildlife (González-Andrés and Ortiz, 1996) and nitrogen to the agricultural system. Legumes adapted to such environments are of interest to many Australian scientists due to the ability to rehabilitate soils and provide a source of forage in the low rainfall regions of Australia where grazing occurs.

A common approach to the problem of stock feed shortages is to reduce stock numbers prior to periods of low rainfall. Alternatively forage species that are productive under stress conditions may be established (Rys *et al.,* 1988). The ability of some species to inhabit adverse areas is due to the ability of these plants to tolerate drought conditions and cycle nutrients in poor soils and to stabilise hill slopes (Lambert *et al.,* 1989). In arid and semi-arid areas throughout the world the use of

multi-purpose trees and shrubs act as a source of forage during times of feed shortage or are collected and stored for use in times of feed shortfalls (Lambert *et al.,* 1989).

The revegetation of semi-arid land may be done with a number of leguminous species (Wills, 1986), with the use of shrubs tolerant of drought conditions invariably found in association with dry land grasses (Brosnan, 1987). The establishment of leguminous species has a number of advantages, such as, the ability to fix nitrogen via symbiotic relationships with rhizobium, provide fodder for bees, and provide ground cover (Wills, 1986).

2.10 Revegetation and Soil Conservation

Dorycnium spp. are potentially suitable for the revegetation of sites where there is low soil moisture and fertility (Douglas and Foote, 1994). *Dorycnium hirsutum* has shown considerable promise in California and Australia for the control of soil erosion and as a source of forage (Brockwell and Neal-Smith, 1966). The main advantage associated with the growth of *Dorycnium* is the ability to establish plants under such conditions and maintain green plant material during autumn and winter. Hand seeding of *Dorycnium* on 45^{o} + slopes with shallow soils have formed good ground cover in this harsh environment of the North Island of New Zealand (Wills *et al.,* 1989a).

The biological control of soil erosion involves the use of live vegetation via the establishment of plants on problem sites, which is an economical method of land rehabilitation and is ideal where natural revegetation does not occur or is very slow (van Kraayenoord, 1986). The establishment of plants in areas where erosion is a problem can be beneficial in a number of ways, such as, soil stabilisation via plant roots, increasing soil fertility via nitrogen fixation and by providing a physical barrier to the soil (van Kraayenoord, 1986). Brosnan (1987) found that in the Kurow area of New Zealand during a drought period led to the overgrazing of pastures, with clovers and weeds dying, which resulted in erosion problems occurring.

The establishment of shrubs in drought prone areas can serve a number of purposes when grown in association with grasses. Shrubs serve to transfer nutrients from deep

in the soil profile to the topsoil and provide canopy shelter from wind (Brosnan, 1987). Also the dense ground cover via the establishment of grasses and legumes absorbs the impact of raindrops and hence reduces the effects of runoff (van Kraayenoord, 1986). The improvement in soil fertility via nitrogen fixation allows for favourable conditions for soil micro-organisms and hence the whole soil ecosystem is improved which allows for continued maintenance of plant ground cover (van Kraayenoord, 1986).

The ability to survive and spread that has been displayed by *Dorycnium* suggests there is potential to be used for soil conservation (Wills and Douglas, 1984; Wills *et al.,* 1989a). Wills and Douglas (1984) and Douglas (1993) stated that the evaluation of *Dorycnium* spp. has shown that they have the potential for revegetation and soil conservation purposes, where *Dorycnium* spp. have been under examination in New Zealand for soil conservation purposes since the 1970's (Wills *et al.,* 1989b). Terrill *et al.* (1992) stated that the persistence of *Dorycnium* spp. may lead to them being useful for nitrogen fixation and soil conservation purposes.

D. hirsutum and *D. pentaphyllum* have displayed a great deal of promise for the revegetation of drought prone semi-arid regions (Wills *et al.,* 1989b) due to the plants tolerance of drought conditions, wind and frost (Wills and Douglas, 1984). These species have been identified as having the potential for the conservation, re-vegetation and rejuvenation of soil in dryland pasture, semi-arid and drought prone areas of New Zealand (Wills *et al.,* 1989a).

2.11 Forage, Hay and Other Uses

Wills (1983) concluded that *Dorycnium* spp. not only helps to provide protection against soil erosion, but also have the potential to provide forage in drought prone areas. The establishment of a combination of grass and shrub cover has a number of benefits, as it not only provides feed and shelter at the same time, but dramatically increases the potential for the provision of animal feed in the form of browse fodder (Brosnan, 1987). Throughout any year, a number of feed gaps can occur at particular times, such as, the end of summer, during winter and at the end of spring due to growth restrictions in relation to climate (Rios *et al.,* 1989). The use of *Dorycnium*

spp. has been primarily for revegetation purposes, however, there is now interest in using these plants as a source of forage (Wills 1983) during these periods of shortages.

Wills (1983) concluded that in addition to the use of *Dorycnium* spp. in drought prone areas, these plants may also be useful in higher rainfall areas for their multiple forage uses. The establishment of grasses and other appropriate shrubs not only can provide feed in the form of 'hay' but can serve a number of other purposes, which include, shelter during lambing, soil conservation, fodder for bees and a habitat for other wildlife (Brosnan, 1987). The flowers of *Dorycnium* spp. are very attractive to bees and consequently they are worked heavily (Wills, 1994). *Dorycnium* spp. may also be used as plants for specialist bee crops in times when feed is traditionally in short supply (Wills, 1983). *Dorycnium* spp. may also be used as ornamentals (Wills and Douglas, 1984) and have been introduced to many ornamental gardens in Buenos Aires (Allen and Allen, 1981).

Chapter 3. Germination Literature Review

3.1 Introduction

The survival of most species is dependent on the process of seed germination (Bell, 1999). Germination is a critical stage in the life of a seed as it is exposed to a number of external factors that may be detrimental to the health of the seed (Campbell, 1993). Conservation and dissemination are the biological purpose of seed, and consequently conditions need to be suitable for germination to occur in order to achieve this (Quinlivan, 1971). The germination responses of flora within Australia to ensure survival are varied due to the range of environmental conditions experienced (Bell, 1999). The time taken for seeds to emerge is critical in relation to crop establishment and may determine the eventual yield (Parera and Cantliffe, 1992).

3.1.1 Requirements for Germination

A number of internal and external factors are important for the germination of seeds. The external factors include environmental triggers, such as oxygen, water, temperature, and in some cases light (Raven *et al.,* 1992). Seeds must imbibe water before they can germinate (Baskin and Baskin, 1998). Generally, mature seeds contain approximately 5 to 20 % moisture, and therefore the process of germination is not possible unless the seed undergoes the process referred to as imbibition, the uptake of water by the seed, which is driven by the low water potential within the seed (Campbell, 1993). Imbibition provides the seed with the water required for metabolic activities. Water activates enzymes already present within the seed, which are used for the production of new enzymes, and the utilisation of food reserves stored within cotyledons of the legume embryo (Raven *et al.,* 1992; Campbell, 1993).

3.1.2 Seed Enlargement and Germination

The process of cell enlargement and division is initiated in the embryo, with each species having its own characteristic pattern (Raven *et al.,* 1992). Following the initial enlargement and division, any further growth requires a continuous supply of

water and nutrients (Raven *et al.,* 1992), with the nutrients provided directed towards the growing regions of the embryo (Campbell, 1993).

The radicle generally is the first organ to emerge from the germinating seed (Campbell, 1993) and is considered by many as the event that defines germination (Khan, 1980; Hilhorst and Toorop, 1997; Baskin and Baskin, 1998). In some cases the shoot appears first, or the shoot and root simultaneously (Baskin and Baskin, 1998).

3.2 Seed Production

Background knowledge pertaining to the germination characteristics of production crops is very important, and therefore many studies aim to improve the quality of seed (Hilhorst and Toorop, 1997). The production of seed is suggested to be done in low fertility soils to avoid the excessive production of vegetative growth and subsequent reduction in seed yield, however careful attention must be paid to the provision of certain essential nutrients. The provision of sufficient bees for insect pollinated species is also a crucial issue for the greater uniformity of seed ripening (Desai *et al.,* 1997).

Seed produced from forage legumes adapted to dry conditions are characteristically difficult to harvest due to seed shedding, which occurs very easily after ripening (Desai *et al.,* 1997). In order to harvest the maximum number of mature seeds the colouration of the pods needs to be monitored. For example, to avoid the spontaneous loss of large quantities of *Lotus corniculatus* seed by dehiscence the pods should be harvested when 20-30% have turned a dark violet or black colour (McDonald and Copeland, 1997).

The harvest of seed generally occurs by wind-rowing of green plant material and allowing the pods to change colour and seeds harden (Desai *et al.,* 1997; McDonald and Copeland, 1997). Once the windrowed crop has been allowed to dry in the field threshing occurs and minimal quantities of seed are lost via dehiscence (McDonald and Copeland, 1997). The use of defoliants, such as, diquat may be useful as this reduces the amount of green material harvested (Desai *et al.,* 1997).

The storage of seed between harvest and germination tests should be done in a well-ventilated cool room or refrigerator. However, some seeds do not store well and therefore should be tested as soon as possible (Machanicek, 1991). The storage of seeds at low temperatures allows for the slowing of the rate of physiological changes. Dry storage at low temperatures can result in differences in the germination responses of some seeds (Baskin and Baskin, 1998).

3.3 Seed Maturity

Baskin and Baskin (1998) stated that fully ripened seeds should only be collected, as immature seeds generally will not germinate. In most cases seeds will change in colour from green when they are mature. Seed should be collected when natural dispersal occurs (Baskin and Baskin, 1998). Baskin and Baskin (1998) stated that the germination requirements and percentages of immature and mature seeds of the same species may be different due to factors such as dormancy. The level of hardseededness and subsequent rate of softening is influenced by the environment in which the seed ripens (Quinlivan and Millington, 1962). Generally, physical dormancy (hardseededness) is due to the seed being physiologically immature which results in the inability of water and oxygen to penetrate the seed (Raven *et al.,* 1992).

3.4 Physical Dormancy

Dormancy is a common reason that some seeds fail to germinate even though conditions may be favourable (Raven *et al.,* 1992). Various dormancy mechanisms prevent the germination of seeds until the timing and environmental conditions are suitable (Quinlivan, 1971). Dormant seeds may need a period of after ripening which may be triggered by a number of factors including low temperatures, removal of seed coat inhibitors, mechanical cracking or extreme heat (Raven *et al.,* 1992).

Seeds from species found in environments with unpredictable climate conditions have the ability to remain dormant for long periods (Teketay, 1996). The seeds of plants found in dry regions are generally characterised by a high level of seed dormancy which results in germination occurring over a long period of time or not at all. The prevailing dry conditions and unreliability of weather in many parts of

Australia means that some form of seed dormancy is essential for seeds to survive (Quinlivan, 1971).

3.4.1 Hard Seed Coats

Where the testa of the seed prevents the uptake of water, the seed is said to be 'hard' (Ellis *et al.,* 1985a) and 'impermeable' (Baskin and Baskin, 1998). Imbibition of the seed is dependent on the permeability of the seed to water (Bansal *et al.,* 1980). The thick testa of certain hard seeds may prevent germination due to the restriction of water and oxygen uptake (Bell, 1999), and act as a barrier to radicle emergence (Ellis *et al.,* 1985a). Seeds that are surrounded by an impermeable seed coat will not be able to allow a seed to imbibe no matter what the conditions (Bansal *et al*., 1980).

The imposition of seed coat dormancy is a means by which germination is delayed under certain conditions that may not be suitable for germination and increases the chances of the seed germinating and completing its life cycle. The seed coat dormancy needs to be broken in order for rapid and uniform germination to occur (Teketay, 1996).

According to Quinlivan (1971) the rate and degree to which seed coat impermeability occurs is dependent on atmospheric moisture. Fairbrother and Pederson (1993) suggested that rainfall during seed production can influence hardseededness, where moisture stress is believed to cause plants to 'dry out.' Factors such as, stage of seed development and genetics can also play a role in determining seed coat impermeability (Baskin and Baskin, 1998).

3.4.2 Permeability of Seed

The palisade layer of cells is the region of the cell identified as causing the impermeability of the seed to water, which is mainly due to the suberin and cutin components (Quinlivan, 1971). Martin *et. al* (1975) stated that the waxy or pectic materials associated with legume seeds were found to exclude water. The impermeability of the seed coat increases as the seed dries where the moisture content can drop to 2-21 % (Baskin and Baskin, 1998). In some species of legumes, when the moisture content of the seed is reduced below 15 % the seed becomes impermeable to water. The further loss of moisture may occur via the hilum,

however, moisture is prevented from being taken up via this part of the seed (Ellis *et al.,* 1985a).

Hardseededness may appear in two forms, reversible and irreversible. Reversible hard seeds can soften under conditions of high humidity where the moisture content of the seed is >10 %. When the moisture of a hardseeded seed is reduced to approximately 5-7 % the seed is said to be irreversibly hard. The problem of irreversible hardseededness is most likely going to be a problem in seeds from wild accessions. Hardseededness exhibited by seeds in dry regions means that seeds can dry to a low level and maintain a low moisture content (Ellis *et al.,* 1985a). Seeds with an impermeable seed coat buried in the soil may germinate over a number of years (Baskin and Baskin, 1998).

3.4.3 Legumes and Hardseededness

Members of Fabaceae differ in terms of the testa structure, which may render the seed impermeable or hard (Ellis *et al.,* 1985a+b). Bansal *et al*. (1980) found that most leguminous seeds examined were impermeable to water, which was due to a hard seed coat. When dealing with forage legume seed, the proportion of hard seed is an important consideration (Zimmermann *et al.*, 1998), as hardseededness can be a major limiting factor in the establishment of some forage legumes, such as, alfalfa (*Medicago sativa*) and sweet clover (*Melilotus alba* Desr.) (Hick *et al*., 1989).

3.5 Seed Pre-treatments

Hardseededness in legumes has been reduced via various means of scarification, such as, mechanical and chemical, and the use of hot water treatments (Hick *et al.*, 1989). The use of scarification methods is known to consistently achieve rapid, uniform and high germination (Cavanagh, 1987). Teketay (1996) concluded, with 20 leguminous species collected from Ethiopia and examined for germination characteristics, dormancy imposed by the seed coat prevented the uptake of water, and therefore mechanisms were required to overcome this barrier.

The success of the seed treatment is dependent on the species and treatment method (Baskin and Baskin, 1998), and duration (Hicks *et al.,* 1989; Teketay, 1996). Teketay

(1996) stated that differences in species requirements for seed pre-treatments is probably associated with differences in seed coat thicknesses. Excessive treatment duration may result in damage to the seed and hence reduce the potential for germination and seedling vigour. Teketay (1996) concluded that it is not possible to apply a single pre-treatment that is effective for all species.

3.5.1 Mechanical Scarification

'Notes' made in the The Journal of the American Society of Agronomy (1947) stated that mechanical scarification of seeds is a means of increasing germination. Mechanical scarification involves the movement of seeds over an abrasive surface (Singer and Pitman, 1988), which damages the testa, and can be done using a range of methods (Ellis *et al.,* 1985a; Hick *et al.,* 1989). Water can enter a seed following the scarification of the impermeable seed coat with instruments, such as, sand paper, files, razor blades etc. (Baskin and Baskin, 1998). Mechanical scarification should be done on the seed coat sufficiently to break the 'dormancy' condition without damaging the embryo (ISTA, 1999). González-melero *et al.* (1997) and Hick *et al.* (1989) found that the germination of *Coronilla* spp. L. and alfalfa respectively were improved by mechanical scarification.

3.5.2 Acid Scarification

The use of acid is often done for the scarification of seeds in experiments, however, it is difficult to apply to large quantities of seed (Singer and Pitman, 1988). The soaking of seeds in concentrated sulphuric acid and subsequent washes, is the means by which acid scarification is undertaken (Baskin and Baskin, 1998), with the length of soaking dependent on the species (Quinlivan, 1971; Ellis *et al.,* 1985a; ISTA, 1999). The use of acid on seeds is a severe treatment and consequently if the concentration, or soaking time is too great, the seeds can easily be damaged (Ellis *et al.,* 1985a). Hick *et al.* (1989) demonstrated that the chemical scarification of crown vetch (*Coronilla varia* L.) has been successful in the improvement of germination characteristics and stand establishment.

3.5.3 Organic Solvents and Scarification

Organic solvents have been used as scarification treatments as the solvent dissolves part of the waxy cuticle (Ellis *et al.,* 1985a). For example, the impermeability of seed coats has been found to be reduced using alcohol in some legumes (Quinlivan, 1971).

3.5.4 Water Treatments

Many seeds with hard seed coats will germinate more readily after being soaked in water for a period of time (ISTA, 1999). The exposure of seeds to extremes of temperatures in water is another means of overcoming the problem of hardseededness (Ellis *et al.,* 1985a). Boiling water is a simple and safe means of scarifying seeds, however, it is not suitable for some sensitive seeds, and boiling for too long can be lethal (Teketay, 1996).

3.5.5 Imbibition Injury

In addition to the problem of hardseededness faced by some legumes, imbibition injury may occur by the rapid movement of water into the seed (Ellis *et al.,* 1985b). The rapid uptake of water by dried seeds may lead to the leaking of soluble compounds such as sugars, where membranes have not fully recovered (Anonymous, 2003). Therefore, some legume seeds may benefit from humidification prior to germination (Ellis *et al.,* 1985b).

3.6 Germination Trial Methodology

When investigating the germination behaviour of seeds about which little is known, a number of crucial questions need to be considered; what stage is the seed in its lifecycle, what conditions are the seeds exposed to in their habitat, what are the temperature conditions between maturation and germination, and finally, are there any specific treatments required for germination to occur (Baskin and Baskin, 1998). When undertaking a germination test, a sufficiently large sample needs to be taken in order for seed lots to be uniformly represented (Machanicek, 1991).

3.6.1 Germination Testing in the Field

Field tests examining germination are difficult to repeat with any reliability, and therefore laboratory testing is done where some, or all environmental factors can be

controlled (Gordon and Edwards, 1991). Germination trials must be replicated, preferably with a number of small replications for each treatment. Historically it has been suggested that three replications of 50 seeds be used (Baskin and Baskin, 1998), however, it is suggested by ISTA (1999) that well spaced seed lots of 100 seeds per replicate are placed on an appropriate medium in order to minimize any effect on nearby seeds.

3.6.2 Germination Conditions

Petri dishes are suitable for use in germination studies for a number of reasons, such as, reduction of water loss, and to allow penetration of light (Baskin and Baskin, 1998). Seeds may be placed in or on any number of mediums including, paper, sand or soil (ISTA, 1999), all of which have advantages and disadvantages (Baskin and Baskin, 1998). For small seeds the use of filter paper is particularly useful as seeds can easily be found and checked. The filter paper should be saturated, with excess water removed as germination may be inhibited by too little, or too much water. The amount of water can interact with other factors affecting germination, including light and temperature (Baskin and Baskin, 1998). A major problem associated with the use of paper as a substratum is that of mould (Gordon and Edwards, 1991), where the warm, moist conditions seeds are exposed to during germination tests are more favourable for the growth and spread of fungal pathogens. Such problems with mould are not normally experienced in the field (Singer and Pitman, 1988).

Sufficient aeration and moisture must be provided within the substrate in order to allow germination to occur. Most seeds will germinate in darkness or in light, however, if light is required it is suggested that indirect sunlight or artificial light is used, as better seedlings are produced (ISTA, 1999). The use of light when germinating seeds is recommended, however, in many cases there is little or no evidence that light is required (Gordon and Edwards, 1991). In some cases the germination trial may need to be undertaken where the seeds are treated in order to promote germination i.e where physiological dormancy or inhibitory substances are an issue (ISTA, 1999).

3.6.3 Trial Duration

The duration of a germination test needs to be long enough to allow germination to occur, but not so long that hot or cold stratification occurs relative to a seeds specific requirements for germination. Germination tests may be extended to periods of three or four weeks if germination percentages are increasing at the end of two weeks (Baskin and Baskin, 1998).

3.6.4 Seeds Not Germinated

Seeds that have not germinated at the conclusion of the trial should be classified as either, hard, fresh, dead or into other appropriate categories (Gordon and Edwards, 1991; ISTA, 1999), and should be checked for viability (Baskin and Baskin, 1998). According to Gordon and Edwards (1991) hard, fresh and dead seeds should be defined as follows; hard seeds are those that have not taken up water, usually restricted to the family Fabaceae, fresh are those that are not hard or germinated. For hard or fresh seeds, the conditions presented during the germination test are considered to have been suboptimal. Dead seeds are those that are not hard or fresh and are often soft, discoloured and mouldy.

3.6.5 Analysis of Germination Data

Baskin and Baskin (1998) stated that an understanding of seed germination ecology requires knowledge of physiological and morphological characteristics of seeds and the changes that occur with these characteristics during germination, and the environmental conditions required for these changes to occur. In order to obtain these data, observations need to be made on seeds in relation to seed maturation, germination, plant life cycle, and environmental influences.

González-melero *et al.* (1997) stated that variation in germination response between samples of the same species is frequently seen in wild plants and therefore extrapolation of data is limited. The results of germination research often cannot be extrapolated to the field, and therefore should be done with caution (Baskin and Baskin, 1998).

Data from germination studies in many cases are arcsin transformed, with any significant differences analysed using analysis of variance (ANOVA). The rate of

germination provides valuable information on how favourable the germination conditions were and the degree of dormancy (Baskin and Baskin, 1998).

Chapter 4. Germination Characteristics of *Dorycnium* spp.

4.1 Introduction

Seed germination is a critical stage in the establishment of a crop (Campbell, 1993). The time taken for planted seeds to germinate and seedlings to emerge influences the stand density, plant uniformity and the ensuing crop yield and quality (Parera and Cantliffe, 1992). A number of physiological and biochemical factors are important for the germination of seeds. External to the seed a number of stimuli or environmental factors are required for germination, such as, oxygen, water, temperature, and in some cases light (Raven *et al.,* 1992).

Viable seeds which fail to germinate, despite environmental conditions being favourable are said to be dormant. Dormancy in seeds is generally in the form of physiological immaturity or seed coat impermeability to water and oxygen (Raven *et al.,* 1992).

Seed coat imposed dormancy in legumes has been overcome by scarification, involving mechanical and chemical treatments (Hick *et al.*, 1989), which 'scratch' or reduce the thickness of the seed coat testa (Ellis *et al.,* 1985a; Hick *et al.,* 1989). Water uptake is possible following the scarification of the impermeable seed coat (Baskin and Baskin, 1998). Once the barrier to water imbibition is overcome seed lot germination is generally rapid, uniform and high (Cavanagh, 1987).

Previous experience with *Dorycnium* spp. in Tasmania (Hall pers. comm., 2001) revealed that seed lot germination was extremely variable and that the rate and level of germination were a limiting factor to establishing plants in the field. Additional evidence highlighted a large variance in seed morphology and colour within seed lots. The observed evidence provided prior to the commencement of this experimental work suggetsed that seed maturity and hard seed coat imposed dormancy were likely causes of poor seed lot quality.

A series of experiments were conducted to investigate factors likely to be influencing seed quality and germination behaviour of *Dorycnium* spp. This work aimed to investigate two primary questions:

- The effect of pod maturity and seasonal variation on germination characteristics.
- Effectiveness of pre-germination treatments on germination characteristics.

The overall aim was to improve seed lot germination performance by considering seed maturity and the use of pre-germination treatments.

4.2 Materials and Methods:

A preliminary experiment was conducted to establish the germination characteristics of three species of *Dorycnium* and to select a single species for more detailed investigation. Seed lots from two accessions of each of the three species of *Dorycnium* under investigation were obtained from the Tasmanian Institute of Agricultural Research (TIAR) pasture and forage germplasm collection. The seed lots were from *D. rectum* TAS1274 and TAS2206, *D. hirsutum* TAS1002 and TAS2001, and *D. pentaphyllum* TAS2207 and TAS1179. Sub samples of seeds from each of the accessions were germinated in accordance with ISTA guidelines for *Lotus corniculatus* (20 ^{o}C on top of filter paper) (ISTA, 1999). *Lotus corniculatus* was selected due to botanical similarities with *Dorycnium* spp.

By the end of 12 days the level of germination for the six seed lots ranged from 28 % (*D. pentaphyllum* TAS1179) to 8 3% (*D. rectum* TAS1274). *Dorycnium hirsutum* TAS1002 and TAS2001 germination percentage after 12 days was 33 and 49 % respectively.

Based on the low level of germination recorded and their superior agronomic traits, the two *D. hirsutum* accessions, TAS1002 and TAS2001, were selected for further experiments. A number of experiments were undertaken to examine factors influencing seed lot quality using the chosen accessions. The factors examined included; harvesting according to pod maturity, seasonal variation in germination characteristics, and the extent of seed coat imposed dormancy and methods of overcoming it.

4.2.1 Collection of Seeds

Unless stated otherwise, seeds were collected from mature plants grown at Mt Pleasant, Launceston (E511500, N5409000). Seed was harvested over a period of approximately three weeks by hand, with only pods considered ripe being removed each time. The pods tended to change colour just prior to dehiscence of the seed, and hence were considered ripe. Pods were placed in a paper bag and stored at 40 ^{o}C for three months prior to cleaning. Following this drying period all of the pods had shattered and the seed was cleaned using sieving and compressed air to remove debris. Seeds were then stored at 4 ^{o}C prior to laboratory germination experiments.

4.2.2 Standard Germination Test Procedures

Germination tests were conducted using petri dishes fitted with two pieces of filter paper (Double Rings™, 7 cm, 102 ash less). Distilled water was added to the filter paper until it was saturated and free water appeared on the surface. Fifty seeds were added to each petri dish and spread evenly over the surface of the filter paper. This was replicated three times for each treatment. Petri dishes were randomly placed in a large plastic container that was sealed to prevent evaporation, which inturn was placed in a germination cabinet maintained at 20 ^{o}C without light. *Dorycnium hirsutum* seeds were found to germinate in the absence of light.

Seeds were considered to have completed germination when the radicle reached a length of 1mm. The number of germinated seeds was recorded daily for 28 consecutive days from the start of imbibition. When required, additional water was added to the filter paper to maintain it at saturation.

Results of germination experiments after 28 days were calculated on the basis of percentage germination (PG), mean time to complete germination (MTG), coefficient of uniformity of germination (CUG), and classification of non-germinants. Percentage germination was expressed as a percentage of the seeds germinated relative to the total number of seeds included in each replicate. The percentage hard seed i.e no imbibition and a complete seed, was determined from the remaining non-germinants and expressed as a percentage of the total numbers of seeds included in each replicate. MTG was calculated using Equation 4.1:

Equation 4.1: MTG (days) = $\sum (t_x.n_x)/ \sum n$

Where, n_x is the number of seeds that germinated on day t_x. The MTG value gives an average time taken for each seed to germinate.

CUG was calculated using Equation 4.2:

Equation 4.2: CUG = $\sum n/ \sum[(MTG-t_x)^2 n_x]$

Where, n is the number of seeds that germinated. MTG is defined as the mean time to complete germination, n_x is the number of seeds that germinated on day t_x. CUG measures the spread of germination.

4.2.3 Pod Maturity and Seasonal Variation

The aim of this experiment was to investigate the effect of pod maturity and the season of harvest on germination characteristics.

Six seed lots of *D. hirsutum* TAS1002 and TAS2001 were collected at various stages of maturity in 2002 and 2003. Table 4.1 below describes the accession, pod maturity and the means by which the seeds were harvested. The average pod maturity describes the percentage of mature pods at the time of harvest.

Table 4.1 Description of seed lots examined in germination assessment experiments.

Accession	Average Pod Maturity	Harvest Method and Timing
TAS1002	0 %	*All flowering stems were harvested, where 0 % of the pods were mature*
	10 %	*All flowering stems were harvested, where 10 % of pods were mature*
	50 %	*All flowering stems were harvested, where 50 % of pods were mature*
	100 %	*Seed pods were picked by hand, where 100 % of pods were mature*
TAS2001	100 %	*Seed pods were picked by hand, where 100 % of pods were mature*
	100 % (dehiscent)	*Dehisced seed collected from weed matting around the plants*

The plants from which seed was obtained could not be harvested as a replicated trial and therefore the treatments could not be replicated in this trial or related studies (see

also 4.2.6). Consequently seed lot/pod maturity was used as a blocking factor, and significant block effects taken as an indication of seed lot/pod maturity effect (Gates, 1991).

4.2.4 Breaking Dormancy

Dehisced TAS1002 seed collected in March 2002, an accession which had displayed a higher incidence of dormant seeds in previous experiments, was selected to examine the efficacy of five separate treatments commonly employed to break seed coat imposed (physical) dormancy. This seed lot was selected as it was naturally dehisced and seen as being physiologically and biochemically mature. The treatments were applied to seeds immediately prior to the commencement of the experiment and are described below:

Acid Scarification: Seeds were soaked in 98 % H_2SO_4 for 5 minutes, followed by thorough rinsing with water.
Boiling Water: Seeds were soaked in boiling water for 1 minute.
Mechanical Scarification: Seeds were mechanically scarified for 20 seconds in a small drum 'custom made' scarifier. The inside of the drum was lined with 60-grit sand paper.
Nicking: A small section of seed coat was removed exposing the embryo using a dissecting scalpel.
Stratification: Seeds were stored at 2 ^{o}C on saturated filter paper for five days prior to the commencement of the experiment.

Visual assessments of seed coat damage were made of all seed lots pre-treated with scarification techniques. For each particular treatment the level of damage was determined.

The results were analysed with ANOVA, with pre-germination treatments as the treatment effect in a completely random design.

4.2.5 Refining Scarification Treatments

Acid and mechanical scarification pre-germination treatments stimulated the greatest increase in seed lot performance in the previous trial. This experiment was

undertaken to optimise the duration of exposure of seeds to the scarification treatments for enhanced seed germination capacity, rate and uniformity.

Seed collected from accession TAS2001 (harvested off the plant where 100 % of pods were mature, see Table 4.1), which had previously displayed a higher level of hardseededness was selected and was subdivided to produce 30 sub seed lots each containing 50 seeds. Each sub seed lot was subjected to either 1, 5 or 10 minutes of acid scarification or 10, 15, 20, 25, 30, 45 or 60 seconds of mechanical scarification (techniques as described in 'Breaking Dormancy'). Each treatment was replicated three times. The application of the scarification treatments was consistent with the previous descriptions. The germination characteristics of the treated seeds were compared with an untreated control seed lot.

The results were analysed with ANOVA, with mechanical and chemical scarification as the treatment effect in a completely random design.

4.2.6 Application of Pre-germination Scarification Treatments

Soaking of seeds in 98 % H_2SO_4 for 5 minutes and mechanical scarification for 20 seconds were identified as being the optimum methods for improving the germination characteristics of a single seed lot. The efficacy of these two scarification methods was compared with an untreated control on the six seed lots used in the 'Pod Maturity and Seasonal Variation' germination experiments.

The results were analysed with ANOVA, with seed lots/pod maturity as blocks and scarification techniques analysed as treatment effects.

4.2.7 Statistical analysis

Experimental results were analysed using the analysis of variance (ANOVA) procedure in SPSS (SPSS Inc. 1989-2000). Fishers least significant difference (LSD) was calculated to compare treatment means when a significant difference at the 95 % confidence limit was recorded. Percentage values were angularly transformed using the arcsin transformation when the values ranged from 0-20 and 80-100 (Steel and Torrie, 1980) prior to the ANOVA test. Standard deviations were calculated for

percentage germination, mean time to complete germination, coefficient of uniformity of germination and percentage hard seed data.

4.3 Results

4.3.1 Pod Maturity and Seasonal Variation

There were no significant (P>0.05) block or treatment effects.

Results were highly variable. The PG ranged from 82 to 99 % in 2002, whereas, in 2003 the range was from 63 to 96% (see Table 4.2). The relatively low PG in 100 % mature TAS2001 seed was due to a high incidence of mould infestation (data not shown). The MTG ranged from 6.2 to 10.9 days for the 2002 seed lots, and in the 2003 seed lots ranged from 6.7 to 11.0 days. CUG values ranged from 0.04 to 0.10 for seed lots harvested in 2002, and values ranged from 0.03 to 0.12 in 2003.

Table 4.2 Percentage germination (PG), mean time to complete germination (MTG), coefficient of uniformity of germination (CUG) of *D. hirsutum* TAS1002 and TAS2001 seeds harvested at different levels of maturity in 2002 and 2003. Values are the mean ± SD, where n=3.

Accession	Pod Maturity	PG (%)				MTG (Days)				CUG			
		2002		2003		2002		2003		2002		2003	
TAS1002	0 %	82	±4.1	95	±1.1	10.9	±0.8	6.7	±0.2	0.04	±0.01	0.12	±0.02
	10 %	83	±4.7	96	±1.9	9.0	±0.8	7.2	±0.6	0.05	±0.02	0.06	±0.01
	50 %	88	±7.4	95	±1.1	9.2	±0.5	10.0	±0.3	0.06	±0.01	0.10	±0.02
	100 %	98	±0.0	93	±3.4	7.1	±0.6	7.3	±0.2	0.09	±0.03	0.11	±0.04
TAS2001	100 %	89	±5.0	63	±7.5	6.2	±0.1	11.0	±1.2	0.10	±0.04	0.03	±0.0
	100 % (deh.)	99	±1.2	88	±4.8	8.4	±0.5	10.5	±0.6	0.05	±0.02	0.05	±0.01

4.3.2 Breaking Dormancy

The PG after 28 days for *D. hirsutum* TAS1002 seed ranged from 34 to 100 % for all pre-germination treatments (see Table 4.3). The highest (P<0.05) PG values were obtained with mechanically scarified and nicked seeds. The lowest (P<0.05) PG of 34 % was obtained with seeds boiled for one minute. There was no significant difference (P>0.05) in the PG values for the control, acid scarified, mechanically scarified and stratified seeds.

The MTG values varied greatly with a range from 3.6 to 8.0 days. The lowest (P<0.05) MTG value was recorded where the seed coat was nicked with a scalpel,

whereas the control seed had the highest (P<0.05) MTG value. There was no significant difference (P>0.05) in the MTG values for the boiled, mechanically scarified, and stratified seeds. There was no significant difference (P<0.05) in the MTG for the acid scarified, mechanically scarified and boiled seeds.

There were no significant differences (P>0.05) between the CUG values for any of the pre-germination treatments of *D. hirsutum* TAS1002.

There were no hard seeds present after 28 days for any of the pre-germination treatments. The untreated control seeds were found to have a low percentage of hard seed (2.7 %) at the end of the experiment.

Table 4.3 The effect of pre-germination treatment on percentage germination (PG), mean time to complete germination (MTG), coefficient of uniformity of germination (CUG), and percentage hard seeds of *D. hirsutum* TAS1002 dehiscent seed collected in March 2002. Values are the mean of three replicates. Standard deviations are shown where data was not statistically analysed or there was no significant difference (P>0.05). LSD and T groupings are displayed where there were significant differences (P<0.05).

Pre-Germination Treatment	PG (%)		MTG (Days)		CUG		Hard (%)	
Control	97	B	8.0	A	0.05	±0.03	2.7	±1.2
Acid Scarification 5 Minutes	95	B	5.4	C	0.06	±0.03	0	
Boiling Water 1 Minute	34	C	6.3	BC	0.10	±0.05	0	
Mechanical Scarification 20 secs	98	AB	6.1	BC	0.06	±0.01	0	
Seed Coat Nicking	100	A	3.6	D	0.15	±0.17	0	
Stratification	96	B	6.5	B	0.06	±0.04	0	

A visual assessment of those seeds with physiological pre-treatments revealed varying degrees of seed coat scarification. The soaking of seeds in acid for five minutes appeared to soften the seed coat. The seed coat was softened dramatically with seed swelling occurring following the boiling treatment. Mechanically scarified seeds displayed scratching of the seed coat with fragments of seed coat removed. Nicked seeds had a complete section of the seed coat removed with the embryo partially exposed.

4.3.3 Refining Scarification Treatments

Significant differences ($P<0.05$) were detected in the PG of *D. hirsutum* TAS2001 subjected to a range of pre-germination treatments, with values ranging from 80 to 99 % (see Table 4.4). Seed untreated and mechanically scarified for 10 seconds had the lowest ($P<0.05$) PG, while the highest ($P<0.05$) PG was the seed acid scarified for 1 and 5 minutes, or those mechanically scarified for greater than 20 seconds.

There were significant differences ($P<0.05$) in MTG values, with a range from 2.4 to 7.1 days. The mechanically scarified seeds for 10 seconds had the highest ($P<0.05$) MTG, whereas, the lowest ($P<0.05$) MTG was observed with seeds mechanically scarified for 25 and 45 seconds.

There were no significant differences ($P>0.05$) in CUG values for *D. hirsutum* TAS1002 with pre-germination treatments.

The percentage hard seed ranged from 0 to 15.7 %. No hard seeds were recorded for the acid scarified seed lots or those seeds mechanically scarified for 25 seconds or longer. Seed scarified for 10 seconds had the highest percentage of hard seed, with the control seeds similarly high. There was a great deal of variation in the percentage hard seed between each replicate for the seed lot/treatment combinations with remaining hard seed.

Table 4.4 The effect of pre-germination treatments on percentage germination (PG), mean time to complete germination (MTG), coefficient of uniformity of germination (CUG), and percentage of hard seed of *D. hirsutum* TAS2001. Each value was the mean of three replicates with standard deviations where data was not statistically analysed or there was no significant difference (P>0.05). LSD and T groupings are displayed where there were significant differences (P<0.05).

Pre-Germination Treatment	PG (%)		MTG (Days)		CUG		Hard (%)	
Control	86	DE	6.0	B	0.10	±0.05	13.6	±7.2
Acid 1 Minute	98	ABC	2.7	E	1.36	±0.70	0	
Acid 5 Minutes	97	ABC	3.0	D	0.38	±0.41	0	
Acid 10 Minutes	95	BC	2.9	D	0.50	±0.12	0	
Scarified 10 Seconds	80	E	7.1	A	0.04	±0.01	15.7	±7.2
Scarified 15 Seconds	94	CD	3.5	C	0.10	±0.11	3.9	±2.0
Scarified 20 Seconds	96	ABC	2.7	E	0.46	±0.57	1.9	±2.0
Scarified 25 Seconds	98	ABC	2.4	G	1.43	±3.0	0	
Scarified 30 Seconds	98	ABC	2.6	EF	0.40	±0.33	0	
Scarified 45 Seconds	99	A	2.5	FG	0.95	±1.3	0	
Scarified 60 Seconds	99	AB	2.6	EF	1.02	±0.96	0	

A visual assessment of the acid scarified seeds revealed that the seed coats were softened by the treatment. Seeds soaked for 10 minutes appeared to have their seed coat largely removed. Seeds mechanically scarified for 10 to 20 seconds displayed seed coat scratching with minor fragments of the seed coat removed. Scarification for 25 to 60 seconds resulted in larger seed coat fragments being removed, through to almost complete removal of the coat for the longest treatment of 60 seconds.

4.3.4 Application of Pre-germination Scarification Treatments

The mechanically scarified seed had the highest PG (P<0.05) (see Table 4.5). There was no significant (P>0.05) difference in PG between the control and acid scarified seed. The PG for control and pre-treated *D. hirsutum* TAS1002 and TAS2001 seeds after 28 days ranged from 80 to 100 % for the six seed lots.

There were significant (P<0.05) differences in PG in relation to pod maturity. TAS1002 seed harvested where 0 and 10 % of pods were mature had the lowest PG (P<0.05). TAS1002 seed harvested where 100 % of pods were mature and dehiscent TAS2001 seed had the highest PG (P<0.05). There was no significant difference (P>0.05) in PG between TAS1002 50 % pod maturity and TAS2001 100 % pod maturity.

Table 4.5 Percentage germination (PG) after 28 days of *D. hirsutum* TAS1002 and TAS2001 seeds harvested at different levels of pod maturity. Seeds were treated with acid scarification for five minutes and mechanical scarification for 20 seconds. Seeds were germinated for 28 days at 20 °C. Each value was a mean of three replicates, with the standard deviation calculated. LSD and T groupings are displayed where there were significant differences (P<0.05).

Accession	Pod Maturity	Control		Acid 5 Minutes		Scarified 20 Seconds		Mean	T
TAS1002	0%	82	±4.1	85	±6.7	84	±3.0	84	**C**
	10%	83	±4.7	80	±6.7	89	±5.6	84	**C**
	50%	88	±7.4	89	±2.4	93	±3.2	90	**B**
	100%	98	±0.0	100	±0.0	100	±0.0	99	**A**
TAS2001	100%	89	±5.0	93	±5.1	97	±1.1	93	**B**
	100% (dehisced)	99	±1.2	99	±1.2	100	±0.0	99	**A**
	Mean	90		91		94			
	T	**B**		**B**		**A**			

The percentage hard seed for TAS1002 and TAS2001 seed after 28 days ranged from 0 to 10.8 % (see Table 4.6). A number of seed lot and pre-germination treatment combinations recorded no hard seed at the conclusion of the trial. TAS2001 seed harvested where 100% of pods were mature had the highest percentage hard seed with 10.8 %. There was a reduction in percentage hard seed with the application of scarification techniques, with no hard seed present in the seed lots mechanically scarified.

Table 4.6 Percentage hard seed of *D. hirsutum* TAS1002 and TAS2001 seeds harvested at different levels of maturity. Seeds were treated with acid scarification for five minutes and mechanical scarification for 20 seconds. The percentage hard seed was determined from the non-germinants. The percentage hard seed was classified as 0 %, where at least two replicates contained no hard seed. Seeds were germinated for 28 days at 20 °C. Each value was a mean of three replicates, with standard deviations shown where at least two replicates contained hard seed.

Accession	Pod Maturity	Control		Acid 5 Minutes		Scarified 20 Seconds
TAS1002	0%	6.8	±1.0	3.4	±2.5	0
	10%	10.2	±1.8	0		0
	50%	10.7	±6.3	2.7	±2.9	0
	100%	0		0		0
TAS2001	100%	10.8	±5.0	4.0	±2.0	0
	100% (dehisced)	0		0		0

The untreated control seed lots from *D. hirsutum* TAS1002 and TAS2001 had the highest MTG values (P<0.05), whereas, the mechanically scarified seed had the lowest (see Table 4.7). The MTG ranged from 2.4 to 10.9 days for TAS1002 and

TAS2001 after 28 days. The lowest mean MTG value was observed with TAS1002 seed harvested when 100% of pods were mature and the seed was pre-treated with mechanical scarification. The control seed for TAS1002 where 0 % of pods were mature had the highest mean MTG with 10.9 days.

TAS1002 seeds where 0 and 10 % of pods were mature had the highest MTG values ($P<0.05$). TAS1002 and TAS2001 seed harvested where 100 % of pods were mature or had dehisced had the lowest MTG values ($P<0.05$).

Table 4.7 Mean time to complete germination (MTG) of *D. hirsutum* TAS1002 and TAS2001 seeds harvested at different levels of maturity. Seeds were treated with acid scarification for five minutes and mechanical scarification for 20 seconds. Seeds were germinated for 28 days at 20 ^{o}C. Each value was a mean of three replicates, with the standard deviation calculated. LSD and T groupings are displayed where there were significant differences ($P<0.05$).

Accession	Pod Maturity	Control		Acid 5 Minutes		Scarified 20 Seconds		Mean	T
TAS1002	0 %	10.9	±0.8	6.4	±0.8	5.5	±0.5	7.6	**A**
	10 %	9.0	±0.8	6.5	±1.6	4.8	±0.7	6.8	**AB**
	50 %	9.2	±0.5	5.9	±0.3	4.1	±0.2	6.4	**B**
	100 %	7.1	±0.6	2.9	±0.3	2.4	±0.2	4.1	**C**
TAS2001	100 %	6.2	±0.1	3.8	±0.4	2.7	±0.1	4.2	**C**
	100 % (dehisced)	8.4	±0.5	4.4	±0.9	2.5	±0.3	5.1	**C**
	Mean	8.7		5.0		3.7			
	T	**A**		**B**		**C**			

There was no significant difference ($P>0.05$) in block or treatment effects for CUG.

The CUG values ranged from 0.04 to 0.52 for TAS1002 and TAS2001 seed after 28 days with mechanical and chemical scarification (see Table 4.8). The lowest mean CUG values were observed with TAS1002 where 0 and 10 % of pods were mature for the control and acid scarified seeds respectively. Mechanically scarified TAS1002 seed harvested where 100 % of pods were mature had the highest mean CUG. There were large standard deviations in the CUG values within seed lot/treatment combinations.

Table 4.8 Coefficient of uniformity of germination (CUG) of *D. hirsutum* TAS1002 and TAS2001 seeds harvested at different levels of maturity. Seeds were treated with acid scarification for five minutes and mechanical scarification for 20 seconds. Seeds were germinated for 28 days at 20 ^{o}C. Each value was a mean of three replicates, with the standard deviations calculated.

Accession	Pod Maturity	Control		Acid 5 Minutes		Scarified 20 Seconds	
TAS1002	0 %	0.04	±0.01	0.05	±0.05	0.14	±0.21
	10 %	0.05	±0.02	0.04	±0.05	0.08	±0.01
	50 %	0.06	±0.01	0.06	±0.04	0.13	±0.07
	100 %	0.09	±0.03	0.45	±0.26	0.52	±0.11
TAS2001	100 %	0.10	±0.04	0.18	±0.09	0.32	±0.14
	100 % (dehisced)	0.05	±0.02	0.07	±0.05	0.39	±0.82

4.4 Discussion

4.4.1 Pod Maturity and Seasonal Variation

The statistical analysis revealed that there were no significant (P>0.05) block or treatment effects. The absence of significant (P>0.05) block effect suggests that pod maturity had no effect on germination characteristics. According to Baskin and Baskin (1998), only fully ripened seed should be collected, and this is shown by the dehiscence of seed by the plant. The natural dehiscence of seed is an indication that the seed is physiologically and biochemically mature and ready to germinate.

This experiment also examined the effect of season on germination characteristics, where 'identical' seed lots were harvested in 2002 and 2003. There was no significant difference (P>0.05) between the years in which the seed was harvested in relation to germination characteristics. The data presented in Table 4.2 revealed large variations between replicates highlighted by the standard deviations. The 'wild' nature of this population of plants where no extensive selection or breeding has occurred may have contributed to variation in germination behaviour. This experiment also highlighted the difficulty associated with employing certain techniques to produce consistent seed lots from year to year when the inherent environmental factors can fluctuate greatly e.g temperature, rainfall, humidity…

4.4.2 Breaking Dormancy

The results of this experiment suggested that hardseededness may not have been as great an issue as first perceived. There were significant differences (P<0.05) in the PG and MTG for the pre-germination treatments applied to the *D. hirsutum* seed over

the 28 day experimental period. There appeared to be little difference between treatments apart from those seeds that were boiled. The data suggested that when provided with a long enough germination period, the majority of seeds will germinate, providing the health of the seed has not been compromised. The control seeds were the only treatment to have hard seed in the non-germinant fraction. Theoretically, if the experimental period was only 14 days the hard seed fraction may be higher within treatments and there may be greater differences in PG and MTG as was the case in the preliminary work described in the Materials and Methods. The results demonstrated that the application of pre-germination treatments can improve germination characteristics, however, given time, most of the seeds will eventually germinate. The application of scarification techniques does not necessarily improve the overall germination, but the rate at which germination occurs.

The inability, or reduced ability of seeds to imbibe and germinate due to seed maturity may also affect the rate and uniformity of germination, leading to longer and more irregular establishment periods. When dealing with forage legume seed, the proportion of hard seed is an important consideration (Zimmermann *et al.*, 1998). The presence of a hard seed fraction translates to a reduced germination potential and uniformity of germination. Physical dormancy imposed by a hard seed coat is due to the seed being physiologically immature which results in the inability of water and oxygen to penetrate the seed (Raven *et al.,* 1992). Hick *et al*. (1989) stated that hard seed coats can be a major limiting factor in the establishment of some forage legumes. This is very important in terms of the health of the seeds, crop establishment and production potential.

The scarification of the seed coat, which included the nicked seeds, displayed excellent germination characteristics, whether it was the PG or MTG. The nicked seeds had the highest ($P<0.05$) and lowest PG and MTG respectively of all the treatments. The improved MTG values of scarified seed over the control seeds suggest that compromising of seed coat integrity promotes water uptake, and hence imbibition and germination occurs more rapidly. These techniques applied to the surface of the seeds appeared to be highly effective in reducing the thickness of the seed coat, or removing a section of it completely. However, the health and integrity of the embryo must be considered to promote germination and successful seedling

establishment when applying seed coat treatments. Scarification should be sufficient to break the 'dormancy' condition without damaging the embryo (ISTA, 1999).

The PG of the stratified seeds was not significantly ($P>0.05$) different to the control, mechanically or chemically scarified seed. Stratification of the seed was designed to examine the possibility that the seeds may require a physiological stimulus for germination to occur. Stratification of seed improved germination characteristics relative to the control seeds. This is believed to be due to the five days of stratification under moist conditions prior to the commencement of the experiment allowing for imbibition to occur to some extent.

The boiling of *D. hirsutum* was highly successful in softening the seed coat, but produced the lowest ($P<0.05$) PG and appeared to be detrimental to seed health. The majority of the boiled seeds were 'cooked,' with limited germination occurring. The majority of seeds imbibed, but failed to germinate. Boiling inferred a negative response in terms of seed health and germination. Ellis *et al.* (1985a) stated that soaking seeds in boiling water is a means of overcoming hardseededness, however, Teketay (1996) suggested that boiling is not suitable for some seeds and may be lethal.

4.4.3 Refining Scarification Treatments

The 'Breaking Dormancy' experiment identified the use of acid and mechanical scarification as suitable techniques for promoting germination, and that seed coat imposed dormancy may be a controlling factor in the germination of *D. hirsutum* seed. Teketay (1996) stated that seed coat dormancy needs to be broken in order for rapid and uniform germination to occur. The nicking of the seed coat was a highly effective means of breaking the seed coat barrier and highlighted its role in relation to the uptake of water. The commercial viability of the scarification techniques was an important consideration, and techniques such as nicking the seed coat are not viable options. Therefore it was decided to focus on refining the mechanical and chemical scarification of the seed lots.

There was no significant difference ($P>0.05$) in the PG between the acid scarification treatments, but, the seeds soaked in acid for one minute had a significantly lower

MTG value than the five or ten minute treatments. All of the acid treatments removed the presence of the hard seed fraction. The scarification of seeds by soaking in acid for five minutes was selected for further application for a number of reasons. This treatment consistently achieved excellent germination characteristics (PG and MTG) whilst maintaining seed coat integrity and there was no apparent advantage in longer scarification. Seeds soaked in acid for longer durations displayed signs of seed damage from over exposure to the concentrated acid and removal of the seed coat. A five minute treatment time provided a reduction in the seed coat thickness without directly damaging the embryo, whilst having the highest ($P<0.05$) PG, and second lowest MTG for the acid treatments.

There was no significant difference ($P>0.05$) in PG between mechanically scarified seed for >20 seconds, whereas, the 25 and 45 second treatments had the significantly lowest ($P<0.05$) MTG values. Despite the higher ($P<0.05$) MTG than other mechanical scarification timings and the presence of a hard seed fraction, the mechanical scarification for 20 seconds treatment was selected to undergo further application. The degree to which the seed coat was scarified and subsequent germination characteristics identified this treatment as promoting germination whilst limiting the extent to which seed health was compromised.

Chemical and mechanical scarification are used with legumes to reduce hardseededness (Hick *et al.,* 1989). The 'Refining Scarification Treatments' experiment examined in greater detail the use of chemical and mechanical scarification and demonstrated the importance of treatment timing. The extent to which the seed coat is damaged will determine the subsequent rate or ability to take up water. Mechanical and chemical scarification methods were highly successful due to their ability to be controlled relatively accurately and be repeated.

4.4.4 Application of Pre-germination Scarification Treatments

The higher ($P<0.05$) PG, lower MTG and elimination of hard seed demonstrated that the mechanical scarification technique was effective in improving germination characteristics. The results of these experiments provides support for the findings of Wills *et al.* (1989a+b), who stated scarification improved the germination percentage of *Dorycnium* spp. This was also supported in part by the results of the acid

scarification treatment, which reduced the hard seed fraction and MTG, however, it was not as effective across the range of seed lots as the mechanical scarification treatment. The differences in germination characteristics between control seeds and those scarified, suggested that a hard seed coat is a controlling factor, and improvement in germination characteristics can be achieved by compromising the seed coat integrity. The physical 'damage' to the seed coat allows for the more rapid and even imbibition and subsequent germination. In the field this translates to a more uniform and rapidly established crop, and reduces the potential for compromising seed health and vigour.

Mechanical scarification of the seed coat improved ($P<0.05$) germination characteristics over the acid scarified seed. However, acid scarification did improve some germination characteristics over the untreated seeds. Acid scarifies the whole seed surface, whereas mechanical scarification is more random and potentially severe on the seed coat. This suggests that the seed coat acts as a regulator of water uptake and the rate of water uptake is proportional to seed coat thickness. Visual assessments of mechanically scarified seed revealed that the seed coat was completely removed in parts, and hence there was no restriction to the uptake of water. Despite the removal of complete sections of the seed coat in some cases, their appeared to be no damage to the embryo.

Despite the improvement in germination behaviour across all of the seed lots with the application of scarification techniques, there was room for improvement with some of the seed lots. Teketay (1996) concluded that it is not possible to apply a single pre-treatment that is effective for all species. This suggests that further investigation into seed pre-treatment may be required for some seed lots. The application of simple cost effective techniques such as mechanical scarification can have a dramatic impact on the survival and establishment of seeds, which translates to the overall success of the crop. However, this may not necessarily translate to a positive relationship in terms of seedling health and survival, and therefore consideration must be given to ensuring seed maturity and the health of the embryo where severe scarification techniques have been used.

There was a significant ($P<0.05$) block effect suggesting that pod maturity influenced germination characteristics. The results suggest that the more mature seed lots for both TAS1002 and TAS2001 had superior germination characteristics than less mature seed. The TAS1002 seed where 100 % of pods were mature and the dehiscent TAS2001 had the highest PG, lowest MTG and eliminated hard seed in the untreated and scarified seed. This suggests that pod maturity may be an important factor in achieving desirable germination characteristics, such as, reducing hard seed and MTG, and increasing the PG, before considering the subsequent application of pre-germination treatments.

4.5 Conclusions

Detailed examination of *D. hirsutum* TAS1002 and TAS2001 seed concluded that a hard seed coat may not be as significant issue with germination characteristics as first thought. The seed coat of *D. hirsutum* was found to influence germination behaviour, with the use of pre-germination scarification treatments improving germination behaviour by increasing the PG and lowering the MTG and percentage hard seed. Mechanical and chemical scarification techniques were found to be the most effective in promoting rapid and uniform germination. In particular the mechanical scarification of seed for 20 seconds was found to optimise germination characteristics on a range of seed lots without physically damaging the embryo. These scarification techniques were found to be simple and repeatable.

Inherent differences in seed lot germination characteristics between seasons and pod maturity were believed to be associated with the influence of environmental factors and the inherent characteristics of a ‘wild’ population of plants producing the seed. However, the harvesting of seed using pod maturity as an indicator may be a useful tool in optimising germination characteristics, with the most mature seed highly desirable.

4.6 Acknowledgements

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Chapter 5. Forage Quality Literature Review

5.1 Animal Production

Domestic ruminants contribute approximately 70 % of total animal protein and 10 % of natural fibre used by humans (Minson, 1990). Ruminants are herbivorous animals that mainly rely on plants as a source of nutrition (Maynard and Loosli, 1979). Muscle and fibre growth, fat deposition or milk production by grazing animals are all affected by the quality and balance of nutrients in the forage consumed (Poppi *et al.*, 1997).

5.2 Feed Production

Browse shrubs and trees have the potential to provide grazing stock with quality forage, however, the management of these plants can be difficult (Oppong *et al.*, 1996). Forage as a source of feed is considered to be highly variable in terms of nutrient composition due to the effects of soil fertility, stage of growth, management practices and weather. The cultivar and date of maturity affects the nutritive value of legumes (Kellaway *et al.*, 1993). Minson *et al.* (1960) stated that the type of herbage and stage of development are the two main factors determining the extent of digestion of material by ruminants. Understanding herbage and its stage of development may also lead to better sward management, with the efficient use of forage only achieved when these factors are understood (Minson, 1990). Grazing animals display selective preference for young plant material over mature leaf and stem (Minson, 1990). Maturing material leads to a decline in nutritive value, which is likely to be due to changes in the stem dry matter digestibility rather than leaf, as well as an increase in the stem:leaf ratio (Kellaway *et al.*, 1993).

5.3 Nutrient Requirements

The building and renewal of body components must be done with the supply of nutrients via food (Maynard and Loosli, 1979). The concentration of nutrients in forage can be defined as the nutritive value (Kellaway *et al.*, 1993). The production of animal products, such as, eggs, and meat require certain compounds to be in the diet, and if they are not in sufficient quantities production may be limited (Schneider and Platt, 1975).

5.4 Protein

Proteins are complex high molecular weight compounds consisting of amino acids in various proportions. The provision of amino acids in the diet will affect nearly all nitrogen based compounds in the animal body (Chesworth, 1992). The majority of nitrogen required by an animal is for the synthesis of animal proteins, with most of the intake of nitrogen in the form of proteins (Minson, 1990).

5.4.1 Protein Requirements

Protein is required for ruminant maintenance and reproduction and is a major component of their products. Protein is the main limiting nutrient of young ruminants relying on lactation (Minson, 1990). Black *et al.* (1982) stated that the nitrogen requirement of animal tissues is dependent on a number of factors including, body weight, energy intake, genotype and physiological state. Protein deficiencies in sheep may be identified by a number of physiological and behavioural symptoms, such as, poor growth and development and reduced feed intake (Anonymous, 1975).

The active tissues of the plant, primarily consist of protein, and are therefore found in higher proportions in the leaves than in the stem. As the plant matures protein is moved from the vegetative parts to the seeds (Maynard and Loosli, 1979).

5.4.2 Protein Determination

The determination of the protein content of plant material is done by measuring the nitrogen content using the Kjeldahl technique (Minson, 1990; Shenk and Westerhaus, 1994). This method assumes that all of the nitrogen contained in the food is in the form of protein (Minson, 1990), and hence this is referred to as the crude protein (CP). The determination of the crude protein value from the nitrogen content is calculated using the formula as described by Minson (1990):

$$CP\ (g/kg) = gN/kg \times 6.25$$

5.5 Carbohydrates

The principal component of the plant as a whole is carbohydrate (Maynard and Loosli, 1979; Moore and Hatfield, 1994). The form of the carbohydrate in the plant

can vary greatly depending on its function, whether it be structural or for reserve. Starch is generally the reserve carbohydrate found primarily in seeds, whilst in the stems, and to a lesser extent the leaves, cellulose is the primary structural carbohydrate (Maynard and Loosli, 1979). Carbohydrates are the principal source of energy in the ruminants diet (Moore and Hatfield, 1994), and are found in the animal body principally in the form of glucose and glycogen and are found mainly in the liver, muscles and blood (Maynard and Loosli, 1979).

5.5.1 Energy

The dry matter intake and composition of the feed determine the metabolisable energy (ME) available to the animal (Black *et al*., 1982). The energy requirements of ruminants depend on a number of factors, including, size, age, reproductive stage, growth and the provision of other nutrients such as protein (Anonymous, 1975). Energy in the animals diet tends to be the regulator of production (Anonymous, 1975), with a continuous response to change often observed to the amount supplied (Minson, 1990). Sheep suffering from energy deficiencies may display a number of physiological symptoms, which may be further complicated by deficiencies in protein, minerals and/or vitamins (Anonymous, 1975). High concentrations of metabolisable energy can be found in forages grazed in the leafy vegetative state (Barry and McNabb, 1999).

5.5.2 Energy Determination

Carbohydrates, fats and proteins are sources of energy in feeds (Schneider and Platt, 1975). The metabolisable energy of a feed can be determined by chemical analysis, and based on the composition, with equations used to calculate the energy value (Minson, 1990). Energy for ruminants is primarily expressed as megajoules (MJ) of metabolisable energy per kilogram of dry matter (Anonymous, 1990). The simplest means of determining the digestible energy (DE) of a feed is to account for the energy lost through faeces (F), which is subtracted from the gross energy (GE) i.e DE = GE – F. Metabolisable energy may also be predicted by the assessment of digestibility by fermentation *in vitro* of the organic matter in the food (Minson, 1990).

5.6 Energy/Protein

Miller (1982) stated that the physiological state of the animal determines the energy and protein value required from feed, where protein and energy are interrelated and it is difficult to separate the two in terms of animal response (Poppi *et al.,* 1997). The supply of energy to the animal is known to influence the catabolism and synthesis of protein in the tissues, and the efficiency by which absorbed amino acids are utilized (Miller, 1982). Miller (1982) stated that high levels of production, require both high levels of protein and energy, whereas, moderate levels of production can be achieved by either high protein/low energy or low protein/high energy.

5.7 Digestibility

Poppi *et al.* (1997) stated that the feeding value of a particular feed is determined by intake, digestibility and the efficiency of use of nutrients absorbed. The chemical composition of a food greatly affects the digestibility (Minson, 1990). Given two feeds with the same chemical composition the feed with the greater digestibility is of higher nutritive value assuming that they require the same amount of energy for digestion and metabolism (Schneider and Platt, 1975). According to Kitessa *et al.* (1999) the energy content of animal feeds is closely related to the digestibility of the nutrient components, with the digestibility of a feed an extremely important parameter in terms of the nutritive value. The digestibility of a particular food can be defined as the amount that is not lost through faeces, and hence, is assumed to be absorbed by the animal. The digestible fraction of a feed is the only part that can provide energy and allow for the production of animal products (Minson, 1990).

5.7.1 Digestibility and Plant Maturity

The maturity of forage, both grasses and legumes, is the major factor affecting the quality of forage (Nelson and Moser, 1994). The fall in dry matter digestibility (DMD) is associated with the decrease in the proportion of leaf sheath to stem (Nelson and Moser, 1994) and flowering head. Digestibility begins to plateau when flowering heads emerge (Minson, 1990). The reduction in crude protein also contributes to a reduction DMD, and with a number of forages, maturity leads to increases in lignin and hemicellulose, primarily in the stems (Nelson and Moser, 1994). Minson (1990) stated that the DMD of mature forage is dependent on the

proportion of low digestible stem. Field studies of lucerne and grasses that were water stressed, had a similar or higher DMD than well watered plants. This was attributed to the reduction in stem material and the higher digestibility of leaf material (Minson, 1990).

Cellulose and related compounds when found in high concentrations in plant material, reduce digestibility (Maynard and Loosli, 1979). As plant material matures lignin concentrations increase in the cell wall, where the ratio of cell wall to cell contents control the DMD of herbage samples (Smith *et al.,* 1998). The concentration of lignin in *Lotus uliginosus* Schkuhr. is negatively associated with DMD (Miller and Ehlke, 1994).

5.8 Fibre

Fibre exerts the greatest influence over the digestibility of a food, with both the amount and chemical composition important (Minson, 1990). Modern feed analysis techniques allows for the fractionation of the plant cell. Forages heated with neutral detergent solution fractionate into cell contents and a residue known as the neutral detergent fibre (NDF) which is cell walls. The cells walls can then be separated into hemicellulose, and cellulose and lignin (known as acid detergent fibre or ADF) by extraction in the presence of acid detergent solution (Minson, 1990; Flinn, 1991).

5.9 Forage Characteristics of *Dorycnium* spp.

The feed value of *Dorycnium* spp. is relatively unknown, however, the ability of these plants to be grazed under adverse conditions is encouraging in terms of using these plants as a source of forage (Wills and Douglas, 1984). The inclusion of browse plants, such as *D. rectum* in pastures, can supplement forage during early autumn and summer (Oppong *et al.,* 2001).

5.9.1 Palatability

Under grazing, *Dorycnium* accessions exhibit a wide range of palatability, the less hirsute types being most palatable (Wills, 1983). Rios *et al.* (1989) and Sheppard and Douglas (1986) described *D. pentaphyllum* as very palatable. Sheppard and Douglas (1986) have found that several accessions of *D. hirsutum* are palatable to sheep. The

less hirsute forms of *Dorycnium* are considered to be more palatable, however, during times of drought when feed is required less palatable accessions may be consumed (Wills, 1983; Wills and Douglas, 1984; Wills, 1994).

5.9.2 Forage Analysis

Terrill *et al.* (1992) found that the total N concentration in *D. hirsutum, D. pentaphyllum* and *D. rectum* was 2.60, 3.57 and 3.25 (% DM) respectively, and that the *in vitro* DMD for *D.hirsutum, D. pentaphyllum* and *D. rectum* was 73.0, 79.7 and 73.7 % respectively. Douglas *et al.* (1996a) similarly found that the average total N content of *D. rectum* was 25 g N/kg DM (2.5 %). Oppong *et al.* (2001) found that the concentration of nitrogen in *D. rectum* exceeded the recommended levels for a lactating ewe with a lamb.

5.10 *Dorycnium* spp. Forage Production

Douglas and Foote (1994) investigated the DM production of a range of perennial species including, lucerne, *D. hirsutum, D. pentaphyllum* and *D. rectum.* The plants were grown on a site considered to be subject to moisture stress on the North Island of New Zealand. The DM production for these four species was 1.02, 3.73, 0.31 and 3.83 t DM/ha respectively. Oppong *et al.* (1996) reported lower yields for *D. rectum* with 2.4 t DM/ha, of which 1.7 t DM/ha was able to be consumed.

5.11 Determination of Forage Quality

The evaluation of forage quality is of great importance for ensuring domestic animals diet meets their nutritional requirements (Schneider and Platt, 1975). The analysis of feed can have three stages; basic analysis of feeds examines the gross effects on animals through feeding experiments e.g live weight gains per unit forage consumed, quantifying individual components of the feed e.g percentage CP of DM, and finally the digestibility of each individual nutrient (Schneider and Platt, 1975). Chemical analysis is a means by which the potential for the delivery or a particular nutrient in a food can be determined, however, the actual amount obtained by an animal may be affected by losses through the system i.e faeces (Schneider and Platt, 1975; Minson, 1990).

5.12 Near Infra-red Reflectance Spectroscopy

Near infrared reflectance spectroscopy (NIRS) is a laboratory based means of estimating forage characteristics (Minson, 1990). The use of NIRS provides an instantaneous analysis of a wide range of chemical properties (Wrigley, 1999). Near infrared reflectance spectroscopy is one of the most widely used methods of food and beverage analysis (Wrigley, 1999), and is also used to measure a broad range of agricultural feed components (Smith *et al.,* 1998; Farrell, 1999; Kitessa *et al.,* 1999). The use of NIRS has a number of advantages including, rapidity (Barton, 1989; Smith *et al.,* 1998), repeatability (Kitessa *et al.,* 1999), chemicals are not required, and it is relatively inexpensive (Farrell, 1999). The equipment associated with NIRS is however, relatively expensive, but very versatile (Farrell, 1999).

NIRS was first used commercially in the 1960's for the analysis of moisture content, moving onto the rapid analysis of grain and oilseeds in 1968, which were found to exhibit specific NIR absorption bands. By 1976 NIRS could be used for the analysis of quality constituents in forage (Shenk and Westerhaus, 1994). Flinn (1991) stated that NIRS is one of the most significant advances in feed analysis in recent years.

5.12.1 NIRS Measurements

NIRS identifies the major components of feedstuffs by examining the near infra-red absorption characteristics (Flinn, 1991), with measurements reflecting the proportion of organic structures in the material examined (Minson, 1990). NIR instruments produce monochromatic light, that when passed through finely ground material results in absorption, reflectance, refraction and transmission (Shenk and Westerhaus, 1994). The near infra-red spectra is 700-2500 nm with absorptions primarily due to carbon (C), nitrogen (N) and oxygen (O) bonds with hydrogen (H) (Shenk and Westerhaus, 1994). The principle behind NIRS is the detection of the presence of spectral bands from overlapping overtones which correspond to various chemical bonds, such as, C-H, O-H etc. (Wrigley, 1999). NIR spectra of forage samples typically contain 7-10 peaks (Shenk and Westerhaus, 1994). The NIRS method of analysis is based on correlations between spectra and feed qualities (Wrigley, 1999). The moisture bands are by far the most prominent features of a forage spectrum (Shenk and Westerhaus, 1994).

The accurate calibration of equipment used for NIRS is essential (Farrell, 1999), and calibration must cover all sources of variation likely to be encountered during analysis (Kitessa *et al.,* 1999). Calibration is required against a set of standards that have been analysed by a reference method. Feed components analysed using NIRS that fall outside the calibration curves should not be analysed using this method, as the results may not be valid (Wrigley, 1999).

Smith *et al.* (1998) stated that equations associated with NIRS have been developed for the determination of DMD, NDF, lignin and CP. The measurement of protein using NIRS typically produces important peaks at 2060 nm and 2168-2180 nm, detergent fibres produces peaks at 2300, 2310 and 2340 nm due to cellulose, and peaks in the regions of 1600-1800 nm and 2200-2300 nm due to lignin (Shenk and Westerhaus, 1994).

5.12.2 Sampling for NIRS

The correct sub-sampling of the material in question is necessary to obtain chemical analysis that is representative. Samples should be dried at 65 ^{o}C to minimise any chemical changes, and then ground to <1 mm (Shenk and Westerhaus, 1994).

5.13 Condensed Tannins

Condensed tannins (CT) in plant material are polyphenols that vary in size and complexity (Haslam, 1981; Kumar and Singh, 1984), and are known to precipitate proteins in the diet (Min *et al.,* 1999). Tannins are water soluble that have a wide range of molecular weights being between 500 and 3000 (Kumar and Singh, 1984). Haslam (1981) stated that there are a number of theories for the occurrence of tannins in nature. The production of condensed tannins in plant material is believed to have evolved as a means of plant defence against bacterial or fungal attack (Barry, 1989).

Kumar and Singh (1984) stated that CT in forages can affect forage quality and cause toxicity. Condensed tannins in forage can affect the value of the diet via a number of factors, including, voluntary feed intake, digestive processes and the metabolism of

nutrients (Barry and McNabb, 1999). The presence of these compounds in plant material may inhibit some microbial processes in the rumen (Barry, 1989). Concentrations of CT considered to be high (>4 % of DM) in plant material are believed to reduce voluntary intake and the digestion of fibre. However, lower CT concentrations (20-40 g/kg DM) are believed to be beneficial by increasing the amount of protein available for digestion and absorption (Barry, 1989).

5.13.1 Beneficial Effects of Condensed Tannins

Kumar and Singh (1984) stated that CT may help in the prevention of bloat. The inclusion of species containing CT in some dairy pastures may prove to be beneficial, with grazed legumes containing CT reducing the occurrence of bloat in cattle and sheep (Douglas, 1993). Waghorn and Jones (1989) found that bloat in cattle was prevented when they were fed dock (*Rumex obtusifolius* L.), which contained CT. These findings suggest that low concentrations of condensed tannins in dock material (1.13 % of dock DM) altered ruminal conditions sufficiently to prevent bloat from occurring. The identification of the genes responsible for the production of CT in leaves is believed to hold the potential for insertion of these genes into other species in order to improve ruminant production (Barry, 1989).

The presence of CT in animal feed has been attributed to some improved performances in sheep (Douglas *et al.,* 1999). Min *et al.* (1999) found feeding *Lotus corniculatus* that contained CT increased reproduction efficiency and wool production in grazing ewes. Condensed tannins in ruminant feed are believed to reduce protein losses by forming complexes (Kumar and Singh, 1984), and improving the flow of protein to the small intestine (Douglas *et al.,* 1999). Barry and Manley (1984) found that a postruminal increase in protein absorption and decrease in DMD has been observed in fresh *L. uliginosus* forage containing CT. Barry and McNabb (1999) concluded that the efficiency of nitrogen digestion and productivity can be improved using temperate forages containing CT under certain conditions.

The food intake and live-weight gain of lambs may be depressed following subclinical Helminth infections (Poppi *et al.,* 1990). The inclusion of forage that contains CT in the diet of sheep has been shown to have significant effects on intestinal nematodes in some studies (Molan *et al.,* 2000). The effect of CT on the

parasites may either be associated with greater amounts of proteins reaching the small intestine and hence increasing resistance to the parasite, or by directly affecting the parasite (Molan *et al.,* 2000). Condensed tannins purified from *D. pentaphyllum* and *D. rectum* had an inhibitory activity against one month old *Trichostrongylus colubriformis* larvae of 63 % and 53 % respectively. The potential exists for the inclusion of these forages containing CT into ruminant diets in order to reduce the problem of intestinal parasites and reduce the need for antihelminth treatments (Molan *et al.,* 2000).

5.13.2 Detrimental Effects of Condensed Tannins

Waghorn and Jones (1989) found that the presence of CT in feed served to reduce the soluble protein in the rumen liquor, as the condensed tannins strongly bind to protein (Barry and McNabb, 1999) and cause precipitation of protein (Jones and Mangan, 1977). Miller and Ehlke (1994) found that condensed tannin concentrations in *L. corniculatus* in the range of 27 to 85 g catechin equivalent (CE)/kg of DM can reduce ruminal protein degradation, however, there appeared to be little or no corresponding reduction in DMD. Kumar and Singh (1984) found that tannins decreased the permeability of the gut wall, and hence, the uptake of nutrients is reduced.

Condensed tannins limit biodegradability and are limiting factors in many plant forages (Kumar and Singh, 1984), such as *L. uliginosus,* where there is a positive relationship between lignin and CT concentration (Barry and Manley, 1986). Kumar and Singh (1984) found that voluntary feed intake was reduced due to the unpalatability of astringent CT.

5.14 Sampling and Condensed Tannin Analysis

Terrill *et al.* (1994) described a field sampling method for CT, where forage was frozen immediately with dry ice, stored at –30 ^{o}C, freeze dried and then ground to <1 mm. Extractable CT in *Lespedeza cuneata* G.Don. were found to decrease when samples were oven dried rather than freeze dried (Terrill *et al.,* 1994).

The methods and results of CT analysis are varied due to the diverse nature of the chemical compound structures. Many of the methods used for tannin determination are based on the formation of coloured complexes, with other methods based on the formation of complexes with proteins where precipitation occurs (Wina *et al.,* 1999). Wina *et al.* (1999) concluded that there are a number of considerations that should be made when selecting a method for tannin analysis, including, simplicity, accuracy and cost.

5.15 *Dorycnium* spp. and Condensed Tannins

Terrill *et al.* (1992) concluded that total CT concentrations in *Dorycnium* spp. were in the range of 13-19 % DM, and considered not suitable for ruminant nutrition. Therefore *Dorycnium* spp. were more suitable for soil conservation purposes. The total CT for *D. hirsutum, D. pentaphyllum* and *D. rectum* have been found to be 18.7, 12.6 and 14.3 (% DM) respectively, and these relatively high CT concentrations may result in ruminant voluntary feed intake being restricted (Terrill *et al.,* 1992). Barry (1989) reported that *D. hirsutum* contained high concentrations of CT, however, *D. hirsutum* contained as little as 95 g/kg DM in leaf material.

Chapter 6. Forage Evaluation

6.1 Introduction

Dorycnium spp. are being evaluated in Tasmania as a potential source of forage in areas where average annual rainfall is <600 mm, and at times when there are feed gaps in the grazing system. The forage value of *Dorycnium* is relatively unknown, however, the ability of these plants to be grazed during adverse seasonal conditions is encouraging (Wills and Douglas, 1984).

Anecdotal evidence has shown that small plots of *Dorycnium* spp. in Tasmania are palatable to sheep, with stock readily grazing stands of the plants when little or no other forage is available. Preliminary work suggested that the forage quality of *Dorycnium* spp. was suitable for grazing livestock, however, there was no detailed information on the seasonal changes in quality of forage produced by these plants. The aim of this experiment was to monitor the changes in the forage quality of *Dorycnium* spp. throughout the growing season using lucerne as a reference plant.

6.2 Materials and Methods

6.2.1 Sampling Methods

The site used for this experiment was located at Swansea (E587000, N5333000, GDA 94) on the East Coast of Tasmania where small blocks of *D. rectum*, *D. hirsutum* and *D. pentaphyllum* had been established in 1995. The three species and accessions used for this experiment were *D. rectum* TAS1274, *D. hirsutum* TAS1002 and *D. pentaphyllum* TAS1273.

The plants had been grazed by sheep on an annual basis prior to the commencement of this experiment, but were not grazed during this experiment. The plants were mechanically cut to a height of approximately 10 cm above the soil surface using a 'brush cutter' in early Autumn 2001 to remove residual plant material from the previous seasons growth. Plants were then sampled at monthly intervals from September (2001) through to April (2002). This was designed to include the predominant growing season of these plants, and the summer/autumn period when they are likely to be grazed by stock.

Three plants of each species were randomly selected and the top 30 cm of vegetative growth was removed with secateurs. The erect growth habit, and relatively greater elongation of stems displayed by *D. rectum*, led to the samples being separated into <30 cm and >30 cm stem lengths once the stems were greater than 30 cm in length. Additional samples were taken at the September, December and March samplings of *D. hirsutum* and *D. rectum* (<30 and >30cm), which were separated into stem and leaf components to be analysed individually for nutritional attributes. The ratio of stem:leaf in terms of relative contribution to the DM yield was also determined. *D. pentaphyllum* was not subjected to this analysis due to the small size of the leaves and the difficulty in separating leaf and stem components.

At each sampling date the growth stage of the plants was described using a lucerne growth stage key (see Table 6.1 taken from Frame *et al.,* 1998), which was applied to *Dorycnium* spp.

Table 6.1 Summary of stages of development of lucerne plants (Frame *et al.,* 1998).

Stage 0:	***Early vegetative***: stem length up to 15 cm; obviously vegetative (no visible buds, flowers or seed pods); axillary buds not easily seen.
Stage 1:	***Mid vegetative:*** stem length 15-30 cm; obviously vegetative; axillary buds developing (with 1 or 2 leaves), especially at the mid stem.
Stage 2:	***Late vegetative***: stem longer than 30 cm but still vegetative; axillary buds beginning to elongate; inflorescence buds at apex enclosed by young leaves beginning to develop.
Stage 3:	***Early bud***: one or two nodes with developing buds near apex on main axis or on branches; no flowers or pods.
Stage 4:	***Late bud:*** three or more nodes with visible buds; no flowers or pods; clear separation of flower buds in raceme.
Stage 5:	***Early flower:*** one node with an open flower; no seed pods
Stage 6:	***Late flower:*** two or more nodes with open flowers; no seed pods; nodes with flowers spread around mid portion of stem.
Stage 7:	***Early seed pod:*** one to three nodes with green pods usually on inflorescences at lower nodes initially.
Stage 8:	***Late seed pod:*** four or more nodes with green seed pods; older stems highly branched; leaves falling off.
Stage 9:	***Ripe seed pod:*** most pods brown and mature; stem thick and fibrous; seed ready to harvest.

In addition to the sampling of *Dorycnium* spp., a paddock of irrigated lucerne was sampled at the University of Tasmania Farm on the corresponding spring/summer dates. The University Farm (E534500, N5260500) is located approximately 80km South of Swansea and provided a source of lucerne that could be easily accessed and

controlled. Three exclusion cages were erected to prevent the lucerne from being cut for hay or grazed, and allowed for the natural growth cycle to be followed. The lucerne was sampled and treated using the same procedures as for *Dorycnium* spp.

6.2.2 Preparation of Stem and Leaf Material

The harvested plant material was dried at 60 ^{o}C in a forced draught oven for approximately 48 hours until a constant weight was reached. The dried plant material was ground to <1 mm and sent to 'FEEDTEST' laboratories (Department of Primary Industries, Hamilton, Victoria) for analysis using NIRS (near infrared reflectance spectroscopy) and wet chemistry. Crude protein (nitrogen x 6.25), NDF and DMD were determined, and ME calculated (MJ/kgDM), from the DMD using Equation 6.1:

Equation 6.1 ME = {0.164 (DMD% + EE) – 1.61}

Where EE = Ether Extract (% of DM) but assumed to be 2% for all types of fodder (Anonymous, 2002).

6.2.3 NIRS Methodology

NIR spectra were collected on all samples using A Foss-NIRSystems 5000 scanning monochromator in conjunction with Infrasoft International (ISI) software. NIR calibrations for CP, NDF and estimated *in vivo* DMD had previously been derived on large sample populations using the procedures of Shenk and Westerhaus (1991) (Dalton pers. comm., 2005).

Reference methods used for NIR calibrations were as follows: CP using the Kjeldahl method, NDF by the method of van Soest and Wine (1967) but using ANKOM equipment and DMD using a pepsin-cellulase technique based on that of Clarke *et al*. (1982), with analytical values adjusted using a linear regression based on similar samples of known *in vivo* DMD. Any spectral outliers from the calibrations were analysed by the wet chemistry techniques as specified (Dalton pers. comm., 2005).

6.2.4 Wet Chemistry Methodology

Neutral Detergent Fibre (NDF) content was determined with samples weighed into filter bags and refluxed in a heated neutral environment for 1 hour. The samples

were then rinsed, dried and reweighed. The amount of sample remaining is the NDF and is expressed as a percentage dry matter (%DM) (Dalton pers. comm., 2005).

Nitrogen content in a feed sample is determined using a partially automated digestion/distillation process based on the Kjeldahl method of nitrogen determination. The amount of nitrogen determined has a mathematical relationship to crude protein and thus can be used to calculated crude protein (Dalton pers. comm., 2005).

Digestibility was determined using a two step method over four days that attempts to reproduce similar conditions to that of animal digestion. The sample is digested at 40^{o}C with acidified pepsin, heated to 80^{o}C and then digested at 40^{o}C with a buffered cellulase solution following pH adjustment to 4.6. Digestibility is measured as the disappearance of dry matter (or organic matter), adjusted using a linear regression based on similar samples of known *in vivo* digestibility (Dalton pers. comm., 2005).

The metabolisable energy (ME) of a feed is predicted from digestibility results using an equation such as that described by Equation 6.1 (Dalton pers. comm., 2005).

6.2.5 Analysis of Forage Data

The data could not be analysed statistically using ANOVA due to the design of the experiment, however, standard errors are presented for mean values. Figures for CP, NDF and ME include a reference data set indicating the requirements of a 60 kg ewe as she passes through the normal lambing cycle where lambing occurs early in spring, where a single lamb is being 'carried.' The values for CP, ME and NDF for this analysis were obtained from Anonymous (1975). The requirement levels displayed in the figures are correlated with the major stages of the reproductive and maintenance cycle of a ewe and the time of year. The determination of forage ME relative to the model requirements was determined from Anonymous (1990) where an average daily intake of feed was calculated based on the seasonal average DMD of the forage.

Regression analysis using SPSS (SPSS Inc. 1989-2000) was undertaken for the stage of growth and protein, and stage of growth and metabolisable energy data sets to

determine whether a linear relationship was present. Where a significant probability ($P<0.05$) of a linear relationship was found, the R^2 and equation of the line were determined.

6.3 Results

6.3.1 Climate Data

Rainfall and daily maximum temperatures were obtained from the Bureau of Meteorology and averaged for each month (see Figure 6.1).

The average monthly temperature gradually increased from 12.4 ^{o}C in September 2001 to 16.5 ^{o}C in February 2002. The monthly average temperatures were very similar to the long term average monthly temperatures throughout the sampling period. Average monthly temperatures for January, February and March 2002 were very similar, but by April 2002, the average temperature had decreased to 14.7 ^{o}C.

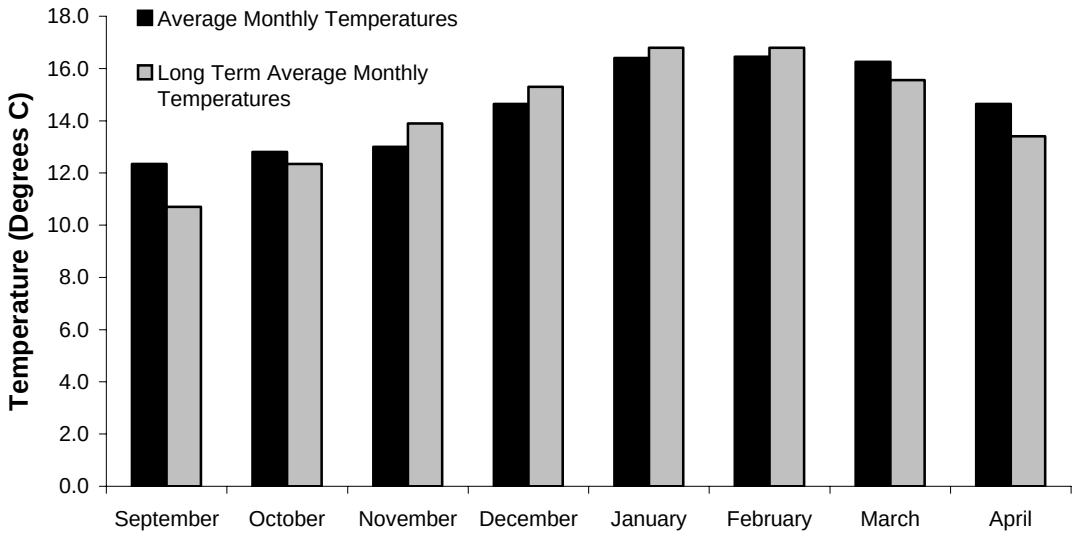

Figure 6.1 Average monthly temperature (2001/2002) and long term average monthly temperatures for Swansea.

The average monthly rainfall fluctuated greatly during the sampling period at the Swansea Trial site (see Figure 6.2). The average monthly rainfall was close to the long term average rainfall in the months of September, January, February and April. Rainfall for the months of October and November in 2001 far exceeded the longterm

monthly average, and for December 2001 and March 2002 was less than the long term monthly average.

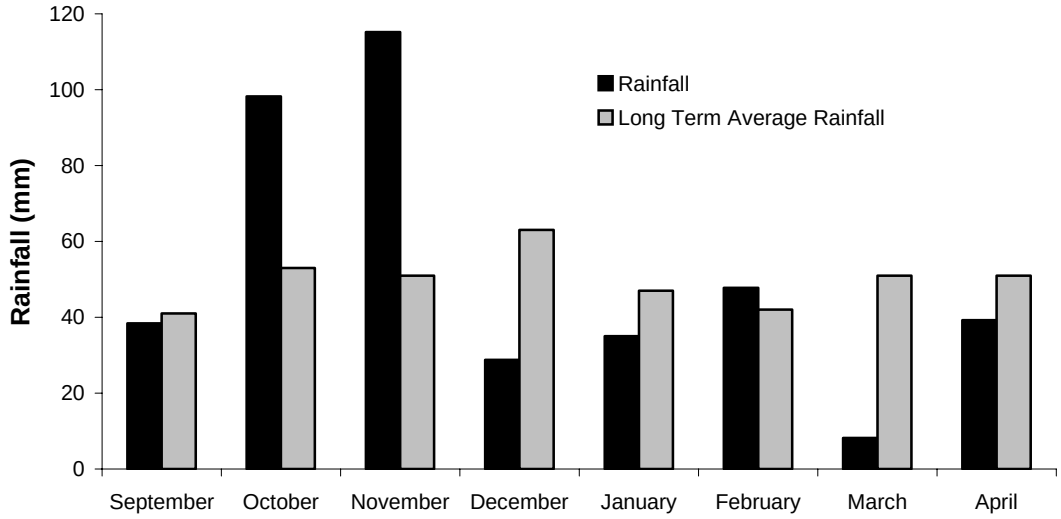

Figure 6.2 Monthly rainfall (2001/2002) and long term average rainfall for Swansea.

6.3.2 Growth Stages of *Dorycnium* spp.

At each harvest date the average stage of growth for each species was estimated using a lucerne stage of growth key taken from Frame *et al.* (1998) (see Figure 6.3). *Dorycnium rectum* when sampled in September was at the late vegetative stage and progressed through to the ripe pod stage at the end of the sampling period. Flowering in *D. rectum* occurred from December to January.

Dorycnium pentaphyllum displayed a very different pattern of development during the sampling period. *Dorycnium pentaphyllum* was in early flower when sampled in September and progressed to early seed pod stage in January, before initiating new vegetative growth in February, which continued to April. Flowering in *D. pentaphyllum* occurred from September through to January, with full flower (stage 6) in November and December.

Dorycnium hirsutum was in late bud/early flower stage in September and steadily progressed to ripe seed pod stage in April. Flowering occurred in October for *D. hirsutum*.

Lucerene displayed a similar pattern of development as *D. rectum* between September and January. Lucerne was in the late vegetative stage in September and progressed to late flower/early seed pod in April. Flower initiation occurred in December with plants in full flower (stage 6) in March.

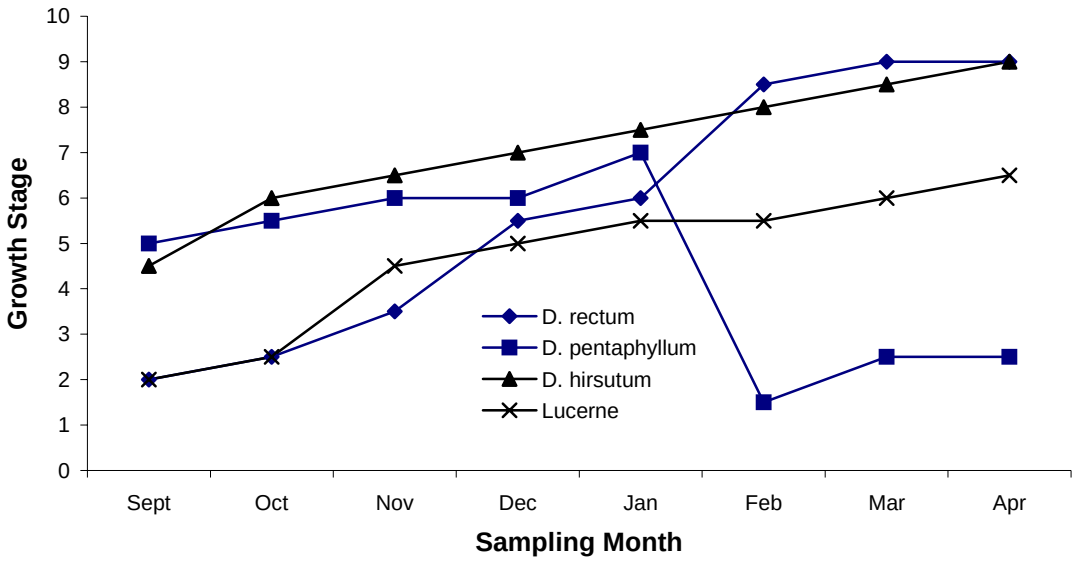

Figure 6.3 Average plant growth stages at each sampling date from September 2001 to April 2002.

6.3.3 Seasonal Changes in Crude Protein

The CP content of forage samples of *D. pentaphyllum* displayed an overall decrease between September 2001 and December 2002 (see Figure 6.4). The CP content increased in January to a similar level as observed in September and then decreased through April. The highest and lowest percentage CP was 15.7 and 8.4 % respectively for *D. pentaphyllum*. The CP values remained above or equal to a ewes requirements throughout the sampling period.

The CP content of *D. hirsutum* was relatively constant during September and October and then declined in November and remained at a relatively low level through April. The highest recorded CP for *D. hirsutum* was 12.9 %, with the lowest 5.0 %. The CP content of *D. hirsutum* was greater or equal to a ewes requirement throughout the sampling period.

The CP content of the <30 cm forage samples of *D. rectum* increased significantly between September and October, remained relatively constant until January and then decreased slightly over the next three months until the last sampling in April. The highest and lowest CP levels for *D. rectum* <30 cm was 18.1 and 6.3 % respectively. The CP levels for *D. rectum* <30 cm were above a ewes requirements throughout the sampling period. The CP content of *D. rectum* >30 cm was relatively low and declined after the December sampling, with levels falling below a ewes requirements in April (2002). Crude protein levels of 8.8 and 4.1 % were the highest and lowest CP levels for *D. rectum* >30 cm.

Dorycnium rectum <30 cm samples had the highest CP levels of *Dorycnium* spp. samples analysed, whereas, >30 cm samples had the lowest CP levels.

Lucerne had consistently higher CP levels throughout the sampling period than *Dorycnium* spp. Overall the CP levels of lucerne decreased throughout the sampling period, except for samples taken in December and March, which showed an increase in CP levels.

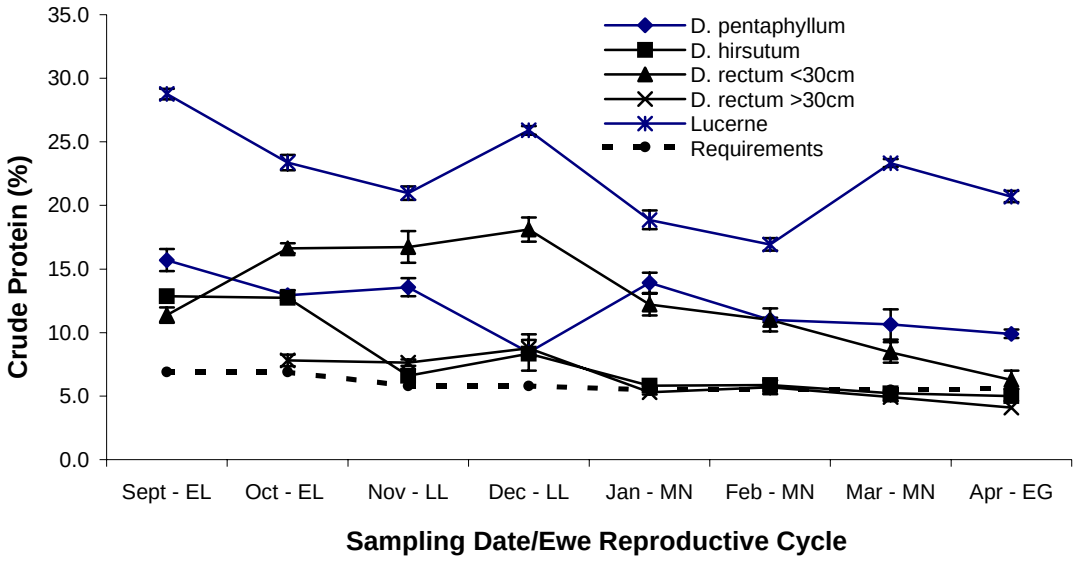

Figure 6.4 Crude protein content of *Dorycnium* spp. and lucerne throughout the 2001/2002 sampling period. Vertical bars are standard deviations. **Key:** EG – Early Gestation, EL – Early Lactation, LL – Late Lactation, MN – Maintenance. Crude protein requirements were obtained from Anonymous (1975).

There was a significant probability ($P<0.05$) that there was a linear relationship between stage of development and CP content for *D. hirsutum* and *D. rectum* >30 cm

(see Table 6.2). The R^2 values for *D. hirsutum* and *D. rectum* >30 cm were 0.79 and 0.63 respectively. There was not a linear relationship between stage of development and CP for *D. pentaphyllum, D. rectum* <30 cm and lucerne.

Table 6.2 Regression analysis of stage of growth and crude protein. Probability values are given where P<0.05. R^2 values and the regression equation are given where the analysis displays a significant probability (P<0.05) of a linear relationship.

Sample	P value	R^2	Equation
D. pentaphyllum	NS		
D. hirsutum	0.003	0.79	Y = -1.97X + 21.9
***D. rectum* <30 cm**	NS		
***D. rectum* >30 cm**	0.033	0.63	Y = -0.52X + 9.59
Lucerne	NS		

6.3.4 Seasonal Changes in Neutral Detergent Fibre

The neutral detergent fibre (NDF) content of forage samples from the three species of *Dorycnium* and lucerne was found to increase during the course of the sampling period in 2001/2002 (see Figure 6.5). The trends in NDF shown by all species was basically the opposite of that shown by CP (see Figure 6.4) i.e an increase in NDF corresponded to a decrease in CP.

Dorycnium pentaphyllum forage displayed a gradual increase in NDF from September to December, when NDF levels peaked at 47.5 %. The NDF content declined in January and remained relatively constant at about 42 % through April. The lowest NDF value of 32 % for *D. pentaphyllum* forage was in September.

The NDF values for *D. hirsutum* forage were relatively constant during September and October 2001. There was an increase in NDF in November before decreasing again in December. Neutral detergent fibre levels steadily increased from December through to March, when NDF content peaked at 57.8 %. There was a slight decline in NDF in April relative to the March sampling. The lowest NDF value was recorded for *D. hirsutum* in October with 37.9 %.

Dorycnium rectum <30 cm forage decreased in NDF from September to October 2001. There was a gradual increase in NDF from October through April 2002, when NDF peaked at 48.9 %. The lowest NDF value for *D. rectum* <30 cm forage was

27.6 % in October 2001. *Dorycnium rectum* >30 cm forage had much higher NDF values than *D. rectum* <30 cm throughout the sampling period. The NDF content of *D. rectum* >30 cm forage increased from October to November, after which, the NDF content decreased in December, when the NDF content for *D. rectum* >30 cm was the lowest at 52.9 %. *Dorycnium rectum* >30 cm NDF content increased in January, where it peaked at 68.5 %. The NDF decreased slightly in February, and then increased slightly through to April.

Overall, *D. rectum* <30 cm forage had the lowest NDF values throughout the trial period relative to the other *Dorycnium* spp., whereas, *D. rectum* >30 cm forage had the highest NDF values.

Lucerne displayed similar levels and seasonal trends in NDF to that of *D. hirsutum* and *D. pentaphyllum* in 2001/2002. All of the species sampled met the model ewes fibre requirements throughout the sampling period.

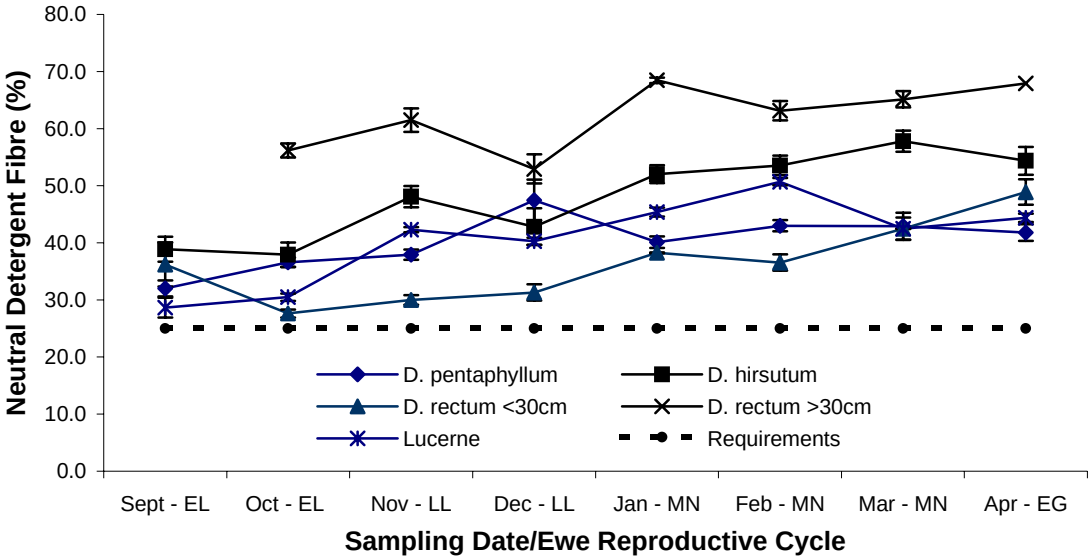

Figure 6.5 Neutral detergent fibre of *Dorycnium* spp. and lucerne throughout the 2001/2002 sampling period. Vertical bars are standard deviations. **Key:** EG – Early Gestation, EL – Early Lactation, LL – Late Lactation, MN – Maintenance. Fibre requirements were obtained from Anonymous (1975).

6.3.5 Seasonal Changes in Dry Matter Digestibility and Metabolisable Energy

The determination of ME from the DMD (see Materials and Methods Equation 6.1) meant the trends in results are identical and therefore results will be described

together in this section (see Figures 6.6 and 6.7). *Dorycnium* spp. and lucerne displayed an overall decline in DMD and ME throughout the sampling period.

The DMD and ME of *D. pentaphyllum* forage remained relatively constant throughout the 2001/2002 sampling period, however, there was a slight peak in December of 51.9 % and 7.3 MJ/kgDM for DMD and ME respectively. The lowest DMD and ME values for *D. pentaphyllum* were recorded from January to April 2002.

There was an overall decrease in DMD and ME for *D. hirsutum* forage throughout the 2001/2002 sampling period. During September and October 2001 the DMD and ME remained relatively constant. There was a small peak in DMD and ME in November, with values 57.6 % and 8.2 MJ/kgDM recorded respectively. The DMD and ME of *D. hirsutum* gradually declined from November to April, where DMD and ME values were the lowest with 49.1 % and 6.8 MJ/kgDM respectively.

The DMD and ME for *D. rectum* forage remained relatively constant in September and October 2001. There was an increase in DMD and ME in November, with peak values of 66.5 % and 9.6 MJ/kgDM respectively. The DMD and ME then declined gradually through April where the lowest values for *D. rectum* were recorded, with 47.9 % and 6.7 MJ/kgDM for DMD and ME respectively. The DMD and ME for *D. rectum* >30 cm forage remained relatively constant in October and November 2001. The DMD and ME peaked in December with values of 47.5 % and 6.6 MJ/kgDM respectively, before declining to low levels of 35.9 % and 4.7 MJ/kgDM for DMD and ME respectively in January. The DMD and ME remained relatively constant from January through to April 2002. Overall, *D. rectum* <30 cm had much higher DMD and ME values than *D. rectum* >30 cm throughout the sampling period.

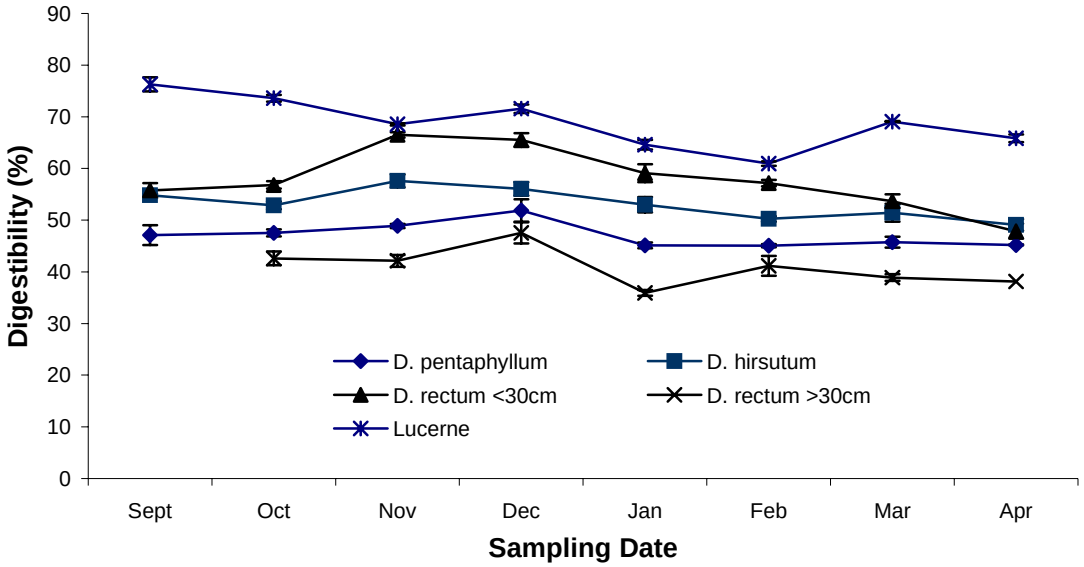

Figure 6.6 Dry matter digestibility of *Dorycnium* spp. and lucerne throughout the 2001/2002 sampling period. Vertical bars are standard deviations.

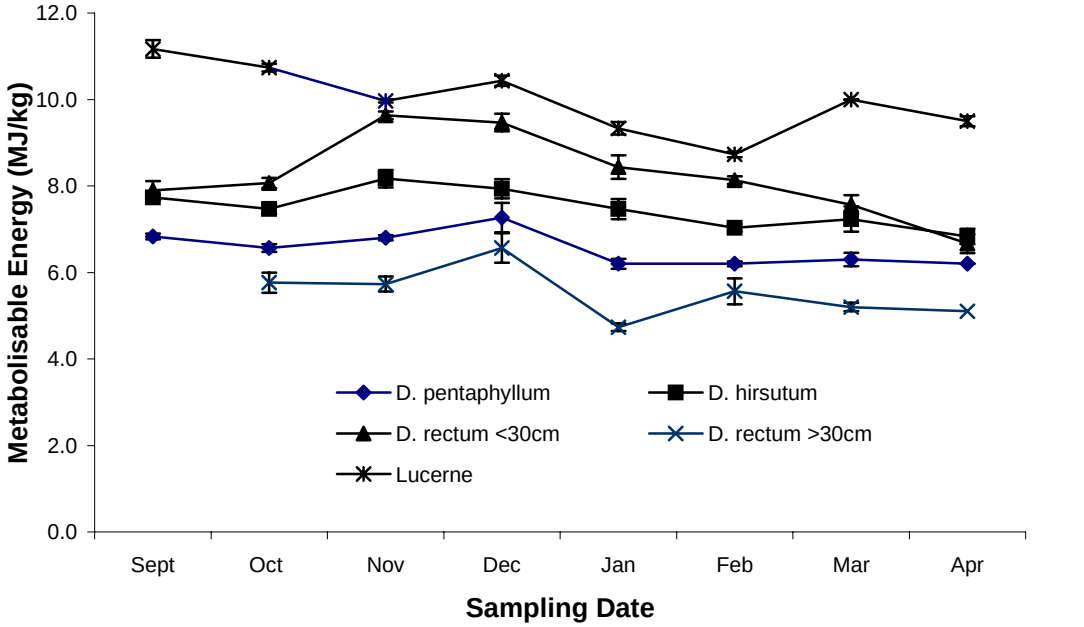

Figure 6.7 Metabolisable energy of *Dorycnium* spp. and lucerne throughout the 2001/2002 sampling period. Vertical bars are standard deviations.

Lucerne had higher DMD and ME than *Dorycnium* spp. (see Figures 6.6 and 6.7) throughout the 2001/2002 sampling period. Lucerne, *D. hirsutum* and *D. rectum* <30 cm met the model ewes requirements for ME throughout the sampling period (see Figure 6.8). The ME provided by *D. pentaphyllum* was similar to the required levels throughout the sampling period, however, early in the season the ME intake was

below the requirements. The ME provided by *D. rectum* >30 cm was below the daily requirements throughout the sampling period.

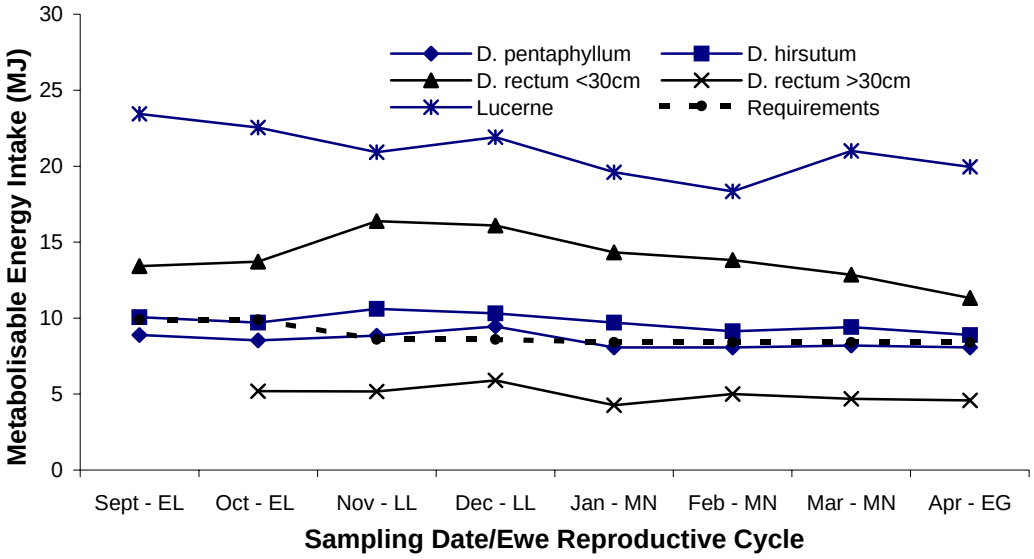

Figure 6.8: Metabolisable energy provided by *Dorycnium* spp. and lucerne throughout the 2001/2002 sampling period based on daily voluntary feed intake. **Key:** EG – Early Gestation, EL – Early Lactation, LL – Late Lactation, MN – Maintenance. Metabolisable energy requirements were obtained from Anonymous (1975), and daily intake predictions were determined from Anonymous (1990). Average seasonal DMD (%) and voluntary intakes (kg/day) were determined for *Dorycnium* spp. and lucerne as follows, *D. pentaphyllum* 47 and 1.3, *D. hirsutum* 53 and 1.3, *D. rectum* <30 cm 58 and 1.7, *D. rectum* >30 cm 36 and 0.9, and lucerne 69 and 2.1.

There was a significant probability ($P<0.05$) that there was a linear relationship between stage of development and ME content for lucerne (see Table 6.3). The R^2 value for lucerne was 0.62. There was not a linear relationship between stage of development and ME for *D. pentaphyllum, D. hirsutum, D. rectum* <30 cm and >30 cm.

Table 6.3 Regression analysis of stage of growth and metabolisable energy. Probability values are given where $P<0.05$. R^2 values and the equation of a line are given where analysis displays a significant probability and hence a linear relationship.

Sample	P value	R^2	Equation
D. pentaphyllum	NS		
D. hirsutum	NS		
***D. rectum* <30 cm**	NS		
***D. rectum* >30 cm**	NS		
Lucerne	0.021	0.62	Y = -0.38X + 11.8

6.3.6 Stem and Leaf Influence on Forage Quality

Forage samples collected in September, December and March were separated into leaf and stem fractions to examine more closely how these individual components contributed to the overall feed quality of *Dorycnium* spp.

The CP content of the leaf and stem material of *D. hirsutum* remained relatively constant throughout the sampling period, however, the leaf was found to have a much higher CP content at each sampling date (see Table 6.4). The CP content of the whole samples of *D. hirsutum* was found to decrease across the three sampling dates which was not reflected in the individual leaf and stem components.

The NDF content of the leaf fraction of *D. hirsutum* was approximately the same at each sampling date. The NDF content of the stem fraction of *D. hirsutum* increased from September through to March with values about twice that of the leaf fraction. The NDF content of the whole plant samples was intermediate between the leaf and stem fractions.

The DMD, and hence ME values (see Materials and Methods Equation 6.1) for *D. hirsutum* leaf and whole plant samples were found to be similar and to decrease slightly over the three sampling dates. The stem material had similar DMD and ME values to that of the whole and leaf samples in September 2001, but were significantly less in December, and March 2002.

Table 6.4 Crude protein, neutral detergent fibre, dry matter digestibility and metabolisable energy of *D. hirsutum* leaf and stem, and whole samples from three harvest dates in 2001/2002. Standard deviations were determined from three replicates at each harvest date.

***D. hirsutum* Leaf**	**September**	**December**	**March**
CP (% DM)	14.3 ±1.5	12.4 ±1.5	13.9 ±3.0
NDF (% DM)	32.0 ±2.7	28.6 ±3.2	32.0 ±3.8
DMD (% DM)	61.3 ±1.9	53.9 ±3.4	54.4 ±8.0
ME (MJ/kg DM)	8.8 ±0.3	7.6 ±0.6	7.7 ±1.3

***D. hirsutum* Stem**	**September**	**December**	**March**
CP (% DM)	8.0 ±0.7	6.9 ±1.0	6.9 ±0.5
NDF (% DM)	55.8 ±0.4	66.7 ±2.9	69.1 ±1.6
DMD (% DM)	54.3 ±0.4	45.9 ±2.9	41.3 ±1.2
ME (MJ/kg DM)	7.6 ±0.1	6.3 ±0.5	5.6 ±0.2

D. hirsutum	**September**	**December**	**March**
CP (% DM)	12.9 ±1.7	8.3 ±3.8	5.2 ±0.6
NDF (% DM)	38.9 ±3.8	42.8 ±5.6	57.8 ±3.2
DMD (% DM)	54.8 ±0.5	56.1 ±2.3	51.4 ±3.0
ME (MJ/kg DM)	7.7 ±0.1	7.9 ±0.4	7.2 ±0.5

A random sample of *D. hirsutum* shoots at the time of flowering found that the relative contribution of leaf and stem to the total DM yield was approximately 2.3:1

The CP content of the leaf fraction of *D. rectum* <30 cm was similar at each sampling (see Table 6.5). The CP of the stem fraction was similar in September 2001 and March 2002, with the December 2001 values significantly higher and similar to that of the leaf fraction. The whole plant sample displayed a similar trend to that of the stem fraction, however, the September and March CP values were higher in the whole plant sample. Overall, the *D. rectum* <30 cm whole plant sample displayed intermediate CP values to that of the leaf and stem fractions, however, CP values were similar in all three samples in December.

The NDF content of the leaf fraction of *D. rectum* <30 cm was approximately the same at each sampling date. The NDF content of the stem fraction was similar in September and March with a significantly lower value in December. The NDF content of the stem fraction in September and March was approximately treble that of the leaf fraction. The NDF content of the whole plant sample was intermediate between leaf and stem fractions.

The DMD, and hence ME of the *D. rectum* <30 cm leaf samples remained relatively constant over the three sample dates. The December 2001 *D. rectum* <30 cm leaf sample had the highest ME and DMD value, however, the whole and stem samples had similarly high ME and DMD values in December. The *D. rectum* <30 cm stem ME and DMD increased in December relative to September 2001, and then declined in March 2002 to a level similar to that recorded in September. The DMD and ME of *D. rectum* <30 cm forage increased in December relative to September 2001, and then declined in March 2002 to levels similar to those found in September 2001. The DMD and ME values of the whole plant samples were intermediate to those of the leaf and stem fractions.

Table 6.5 Crude protein, neutral detergent fibre, dry matter digestibility and metabolisable energy of *D. rectum* <30 cm leaf and stem, and whole samples from three harvest dates in 2001/2002. Standard deviations were determined from three replicates at each harvest date.

***D. rectum* <30 cm Leaf**	**September**	**December**	**March**
CP (% DM)	18.8 ±1.5	21.0 ±2.0	17.1 ±1.4
NDF (% DM)	20.6 ±2.2	23.3 ±0.5	21.1 ±1.9
DMD (% DM)	64.4 ±1.3	71.1 ±1.3	67.9 ±1.4
ME (MJ/kg DM)	9.3 ±0.2	10.4 ±0.2	9.8 ±0.3

***D. rectum* <30 cm Stem**	**September**	**December**	**March**
CP (% DM)	6.6 ±0.9	18.9 ±1.0	5.6 ±0.2
NDF (% DM)	58.4 ±5.2	23.6 ±1.0	63.3 ±0.2
DMD (% DM)	46.1 ±3.5	67.4 ±1.4	40.3 ±0.7
ME (MJ/kg DM)	6.4 ±0.6	9.7 ±0.2	5.4 ±0.1

***D. rectum* <30 cm**	**September**	**December**	**March**
CP (% DM)	11.4 ±1.0	18.1 ±1.6	8.4 ±1.4
NDF (% DM)	36.2 ±6.7	31.3 ±2.5	42.5 ±3.4
DMD (% DM)	55.7 ±2.5	65.5 ±2.3	53.7 ±2.3
ME (MJ/kg DM)	7.9 ±0.4	9.5 ±0.4	7.6 ±0.4

A random sample of *D. rectum* <30 cm shoots at the time of flowering found that the DM ratio of leaf:stem was approximately 2:1.

The CP content of the *D. rectum* >30 cm leaf fraction increased significantly from December 2001 to March 2002 (see Table 6.6). Conversely, the stem fraction declined slightly from December to March. The CP content of the stem fraction was lower than that of the leaf material. The whole plant sample displayed a similar trend

to that of the stem fraction, however, the CP values were higher in September 2001. Overall, the CP content of the whole plant sample was intermediate to that of the leaf and stem fractions.

The NDF content of the *D. rectum* >30 cm leaf fraction decreased significantly from December 2001 to March 2002. The NDF content of the stem fraction was higher than that of the leaf material and remained relatively constant over the two sampling dates. The NDF content of the whole plant sample was similar to that of the leaf fraction in December, with an increase in March.

The DMD, and hence ME of the *D. rectum* >30 cm leaf fraction increased significantly in March 2002 relative to the December 2001 sampling. The DMD and ME of the stem fraction remained relatively constant in December and March, and were lower values than those recorded for the leaf fraction. The DMD and ME of the whole plant sample was similar to that of the leaf fraction in December, however, decreased to levels similar to the stem fraction in March 2002.

Table 6.6 Crude protein, neutral detergent fibre, dry matter digestibility and metabolisable energy of *D. rectum* >30 cm leaf and stem, and whole samples from two harvest dates in 2001/2002. Standard deviations were determined from three replicates at each harvest date.

D. rectum **>30 cm Leaf**	**December**	**March**
CP (% DM)	10.6 ±1.1	18.2 ±1.4
NDF (% DM)	53.3 ±1.8	19.9 ±0.6
DMD (% DM)	49.3 ±1.7	68.3 ±1.3
ME (MJ/kg DM)	6.9 ±0.3	9.9 ±0.2

D. rectum **>30 cm Stem**	**December**	**March**
CP (% DM)	5.2 ±0.8	3.9 ±0.3
NDF (% DM)	71.9 ±3.3	71.5 ±2.0
DMD (% DM)	38.0 ±2.3	36.9 ±2.5
ME (MJ/kg DM)	5.0 ±0.4	4.9 ±0.4

D. rectum **>30 cm**	**December**	**March**
CP (% DM)	8.8 ±0.5	4.9 ±1.0
NDF (% DM)	52.9 ±3.6	65.1 ±2.9
DMD (% DM)	47.5 ±2.0	38.9 ±3.3
ME (MJ/kg DM)	6.6 ±0.3	5.2 ±0.5

A random sample of *D. rectum* >30 cm shoots taken at the time of flowering found that the DM ratio of leaf:stem was 1:1

6.4 Discussion

6.4.1 Crude Protein

The CP content of *Dorycnium* spp. and lucerne displayed an overall decrease throughout the sampling period as the plant material matured. Minson (1990) and McDonald *et al.* (1995) stated that the maturation of forage leads to a decrease in CP. The changes in CP seen in *Dorycnium* spp. were potentially due to a number of factors associated with environmental conditions and plant stage of growth.

Environmental factors such as, temperature, light and moisture accelerate the maturation process, which leads to a decline in nutritive value (Van Soest, 1994). The increase in day length, light intensity and average daily temperature associated with the progression from spring through to summer is likely to have influenced and facilitated the maturation of the *Dorycnium* spp. forage. Minson (1990) stated that there is a reduction in CP associated with high light intensity due to the dilution of CP with DM production. The unseasonal rainfall observed in October and November would have also contributed to the maturation of the plant material, as described by Van Soest (1994), who stated moisture promotes plant development and lowers forage quality. The early increase in CP observed in *D. rectum* <30 cm is possibly due to the production of fresh growth and removal of more lignified material as represented by the >30 cm fraction.

Dorycnium spp. displayed a decrease in CP content following the onset of flowering. The CP content of *D. hirsutum* and *D. rectum* continued to decrease following flowering, which was attributed to the change in plant morphology and dry matter constituents. The initiation of flowering is known to cause a decrease in CP as protein is used in the reproductive parts of the plant. Minson (1990) stated that with maturing forage there is a decrease in the proportion of leaf to flowering stem and along with the maturation process there is a decrease in CP content of the plant. McDonald *et al.* (1995) found that the CP of lucerne decreased from 25.3 % in the pre-bud stage to 17.1 % in early flower, whereas, white clover was found to have a

mean CP content of 29.8 % (Anonymous, 1992), and with a reduction to 23.7 % at the early flowering stage (McDonald *et al.,* 1995). Similar CP contents and subsequent decreases as those described by McDonald *et al.* (1995) were observed in lucerne during this experiment between December 2001 and February 2002, where bud formation and early flowering occurred.

The regression analysis of stage of development and CP data suggested that there were linear relationships for *D. rectum* >30 cm and *D. hirsutum*. This finding is supported by McDonald *et al.* (1995) who suggested that there may be a linear relationship between stage of growth and CP. However, there was not a linear relationship for lucerne or the other *Dorycnium* samples, which may be due to seasonal and growth condition differences between the two trials. More frequent samplings may be required in order to effectively determine any relationships between CP and stage of development.

6.4.2 Neutral Detergent Fibre

The seasonal changes in NDF essentially displayed the opposite trend to the CP content during the sampling period. McDonald *et al.* (1995) stated that the maturation of a plant leads to a decrease in protein content and an increase in fibre content, and therefore the two components have essentially an inverse relationship. Increasing plant maturity means that there is a greater need for structural plant material and therefore, cellulose, hemicellulose and lignin content increases (McDonald *et al.,* 1995). This was evident with the formation of woody stem with increasing DM production, and suggested the importance of forage management in terms of optimising forage quality.

6.4.3 Dry Matter Digestibility and Metabolisable Energy

The DMD and ME of *Dorycnium* spp. and lucerne displayed an overall decrease throughout the sampling period, however, this decrease was relatively minor and there were peaks in ME and DMD in the late spring/early summer period. The overall decrease in ME and DMD during the sampling period was associated with environmental factors and plant maturation. Van Soest (1994) and Beever *et al.* (2000) stated that temperature, light and moisture accelerate the maturation process, with ME and DMD decreasing with plant maturity (McDonald *et al.,* 1995). The

increasing average daily temperatures combined with relatively high spring rains would have promoted the maturation process. Lower digestibilities and ME at higher temperatures are due to increased lignification of the plant cell wall and the use of cell contents through metabolic processes (Van Soest, 1994). The degree of lignification of the DM is a primary factor in defining the digestibility, and therefore the nutritive value of a forage (McDonald *et al.,* 1995; Reynolds, 2000). Reynolds (2000) stated that the digestibility of forage may be affected by the concentration of CP in the diet, and this may also account for the overall decrease in ME and DMD with the decrease in CP.

The peaks in ME and DMD and subsequent decreases displayed by *Dorycnium* spp., in general coincided with the initiation of flowering as displayed in Figures 6.3, 6.6 and 6.7. The onset of flowering and subsequent seed production appeared to have a negative effect on the ME and DMD of all four species. Plants that are flowering will generally be more lignified than plants in the vegetative phase under the same environmental conditions (Van Soest, 1994), and hence the DMD will be decreased. In addition, a decrease in the relative proportions of leaf fractions would have an effect on DMD of the forage as the plants become more mature, as described by Minson (1990) in relation to flowering with grasses.

McDonald *et al.* (1995) found that the ME of lucerne decreased from 10.2 MJ/kgDM in the pre-bud stage to 8.2 MJ/kgDM in early flower. Similar decreases in ME were observed with lucerne and *Dorycnium* spp. in this experiment during these developmental stages. McDonald *et al.* (1995) suggested that lucerne should be harvested at the early bud stage when there is acceptable digestibility. This also appears to be the case with *Dorycnium* spp. where forage quality can be optimised with the harvest of material prior to flowering, as was shown with the CP content. The relative stability of DMD and ME displayed during changes in *Dorycnium* spp. lifecycle and environmental conditions allows the grazier greater flexibility in terms of plant management, however, other factors such as, protein and fibre must be considered.

The mean ME and DMD of white clover is reported to be 11.6 MJ/kgDM and 69.9 % respectively (Anonymous, 1992). The lucerne sampled during this experiment

displayed similar average levels to white clover. These two pasture species are regarded as high quality forage species, and when compared to the ME and DMD values observed with *Dorycnium* spp., their forage quality is greater. However, the ability of *Dorycnium* spp. to produce this forage under 'arid' conditions is the basis for considering the potential use of this genus as a source of forage.

6.4.4 Stem and Leaf Influence on *D. rectum* and *D. hirsutum* Forage Quality

Beever *et al.* (2000) stated that the chemical and physical characteristics of forage species change due to changes in the leaf:stem ratio as influenced by the environment and developmental stage. The leaf:stem ratio is highly likely to be dynamic with changes in plant morphology with the vegetative/reproductive cycle, such as, the shedding of older leaves that was observed in *D. rectum* during summer and early autumn. Van Soest (1994) stated that forage typically shows a decrease in leafiness and an increase in the stem to leaf ratio with age, which is responsible for the change in plant composition (McDonald *et al.,* 1995).

The separation of leaf and stem components highlighted the relative influence of each component on the overall quality of the forage. In most common green forages the leaves are richer than stem material in protein (McDonald *et al.,* 1995), which was clearly displayed by *D. rectum* and *D. hirsutum*. Stems are often of lower quality than leaves in mature forage, which is associated with the lignification of structural tissue (Van Soest, 1994), and exerts greater influence on the digestibility of the forage as the plants mature (McDonald *et al.,* 1995). The differences in observed forage qualities between the leaf and stem fractions, and the whole plant samples highlighted the influence of each component as a proportion of the total DM. The inclusion of flowers and seed pods in the whole plant sample would have also influenced forage quality relative to the leaf and stem fractions. The influence of leaf and stem on the overall quality of the forage demonstrated the importance of ensuring plants are managed in such a manner to optimise the leaf DM fraction and the quality of this fraction.

The ratio of leaf:stem was 2.3:1, 2:1 and 1:1 for *D. hirsutum, D. rectum* <30 cm and *D. rectum* >30 cm respectively at the time of flowering. If it was assumed that the average leaf:stem ratio of *D. rectum* was 1.5:1, then the relative contribution of leaf

material to the DM based on the DM production figures of Douglas and Foote (1994) would be 2.60 t DM/ha (i.e 2.3/3.3 x 3.73 (t/ha of *D. hirsutum* forage)) for *D. hirsutum.* Similarly for *D. rectum*, assuming the stem:leaf ratio was 1.5:1, then the relative contribution of leaf material would be 2.30 t DM/ha (i.e 1.5/2.5 x 3.83 (t/ha of *D. rectum* forage)). These estimated DM production figures for *D. rectum* and *D. hirsutum* leaf material are more than double that of the lucerne whole plant sample (assuming *Dorycnium* spp. leaf:stem ratio does not change throughout the year). In addition to this, the nutritional value of the *D. rectum* and *D. hirsutum* leaf material is greater than the whole plant sample, and similar to that of lucerne. The *D. rectum* and *D. hirsutum* leaf data suggests that maintenance requirements of the model ewe during the December-March period would be met for CP, NDF and ME.

Otsyina *et al.* (1997) stated that in the dry season the leaves of browse shrubs and trees often have higher CP content than grasses. The results of this experiment support this notion based on the nutritional levels displayed by *D. rectum* and *D. hirsutum* leaves during the spring-summer period. Therefore, the provision of nutritional forage may be possible when there is little or no production from the primary pasture species.

The observed grazing behaviour of livestock (sheep and cattle) has shown that there is a tendency for selective grazing, and hence optimum nutritional value is obtained from the DM presented. Nelson and Moser (1994) suggested that animals tend to graze young tissue on the upright stems of legumes. Therefore, the management of plants is crucial for optimising fresh growth and limiting 'woody' stem.

6.4.5 General Discussion

It is proposed that forage produced by *Dorycnium* spp. be utilised during summer and autumn when feed shortages are likely to occur, and therefore the *Dorycnium* spp. forage characteristics between December and March were considered in relation to the maintenance requirements of the model ewe (see Figures 6.4, 6.5, and 6.8). Maintenance is defined as an animals minimum requirement for nutrients to maintain essential processes and prevent loss of body weight (Beever *et al.,* 2000). All *Dorycnium* spp. met the requirements for CP and NDF and ME as specified by Anonymous (1975) for a 60 kg ewe based on estimated voluntary feed intake

(Anonymous, 1990). However, forage samples of *D. rectum* >30 cm did not meet the ME requirements at any stage throughout the sampling period. Although *D. pentaphyllum* and *D. hirsutum* theoretically met the ME requirements throughout this period, the seasonal fluctuations in digestibility and presence of condensed tannins (see Chapter 7) may limit voluntary intake and provision of ME. This may also be the case with *D. rectum,* as non selective grazing of either the <30 or >30 cm fraction may lower the overall quality.

The provision of energy or protein supplements to animals is a means of overcoming the shortfall of a particular forage (Minson, 1990; Van Soest, 1994). The inability to meet ME requirements may be overcome by supplementing the diet of livestock with a high energy concentrate, such as a grain. The required supplementation of the diet with a concentrate needs to be done with a relatively high degree of accuracy to ensure requirements are met.

There may be potential for *Dorycnium* spp. to be harvested and stored for later feeding to livestock, which means that consideration of the form in which the forage is presented may influence digestibility. The digestibility of the forage could be improved by increasing the surface area:volume ratio by chopping or grinding, and hence, improving the ability of the animal to attain nutrients. However, if the forage is cut too finely this may decrease the digestibility due to an increased rate of passage through the digestive system (Minson, 1990)

The inclusion of a model animal was done as a means of evaluating the *Dorycnium* spp. as a source of forage, however, this will vary greatly depending on the characteristics of the livestock and environment of each individual grazing system.

Douglas and Foote (1994) found that moisture stressed lucerne, *D. hirsutum, D. pentaphyllum* and *D. rectum* produced, 1.02, 3.73, 0.31 and 3.83 t DM/ha respectively. These plants were approximately one year old at the time of harvest. If you were to consider lucerne as an example of a high quality source of forage with a relatively high production, *D. hirsutum* and *D. rectum* produce almost four times as much DM when subjected to the same moisture stress conditions. The relatively low DM production of the prostrate *D. pentaphyllum,* suggests that the potential of this

species to meet maintenance requirements in terms of quality and quantity are low for a range of grazing stock types. Nelson and Moser (1994) stated that legumes with upright stems are generally more productive than those with prostrate stems. *D. hirsutum* and *D. rectum* have excellent potential as a source of forage due to their ability to produce a relatively large amount of DM under moisture stress conditions.

The broad comparison of lucerne with *Dorycnium* spp., suggested that overall *Dorycnium* spp. displayed lower levels of nutrition in terms of CP, DMD and ME than lucerne. However, the data collected suggested that *Dorycnium* spp. can provide a valuable source of nutrition to grazing animals. Although the quality of the feed produced is lower than some other forage crops, the inherent characteristics of *Dorycnium* spp. means that this production can occur under adverse conditions i.e low rainfall periods. This is where *Dorycnium* spp. have the potential to be included in grazing systems as a late season or alternative source of forage when feed gaps occur and help maintain stock and/or reduce losses in animal production.

6.5 Conclusions

Whilst the nutrition levels of the plants analysed were lower than that of the lucerne sampled during the same growing season, the inherent ability of *Dorycnium* spp. to grow during periods of low rainfall suggests that these plants are potentially valuable sources of feed and nutrition in low rainfall areas and reduce losses in animal production. Changes in feed characteristics appeared to be related to plant development and environmental factors. The grazing management of these plants is critical in optimising forage quality and quantity. *Dorycnium rectum* (despite the lower quality of the >30 cm fraction) and *D. hirsutum* forage appeared to be the most promising of the *Dorycnium* spp. examined.

6.6 Acknowledgements

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Chapter 7. Seasonal Changes in Condensed Tannins

7.1 Introduction

The presence of condensed tannins (CT) in plant material is believed to have evolved as a means of plant defence (Barry, 1989). Condensed tannins in forage can affect the value of the diet due to a number of factors, including, voluntary feed intake, digestive processes and the metabolism of nutrients (Barry and McNabb, 1999). Concentrations of CT considered to be high (>4 % DM) in plant material are believed to reduce voluntary intake and the digestion of fibre, whereas, lower condensed tannin concentrations (<2-4 % DM) are believed to be beneficial by increasing the amount of protein available for digestion and absorption (Barry, 1989).

Literature pertaining to the quality of forage produced by *Dorycnium* spp. revealed that the species under investigation are known to contain condensed tannins. Various authors have described a range of tannin contents, however, no data has been presented on the tannin content of plants grown under Tasmanian conditions, or the change in tannin content throughout the predominant spring/summer growing season. To compliment the description of changes in forage qualities during the main growing season, the seasonal changes in condensed tannins were examined to determine any detrimental or beneficial effects.

7.2 Materials and Methods

Dorycnium spp. plants that were well established were located at three sites selected for the purpose of describing the seasonal changes in CT. The sites were located at Mt. Pleasant Laboratories Launceston (E511500, N5409000, GDA 94), Elliott Research Station (E395000, N5451000, GDA 94), and Swansea (E587000, N5333000, GDA 94). Soil and climatic data were collected at the three experimental sites.

The same three *Dorycnium* spp. accessions were selected for sampling as used in the seasonal change in forage quality research (see Chapter 6, Materials and Methods). The species/accessions were, *D. hirsutum* TAS1002, *D. rectum* TAS1274, and *D.*

pentaphyllum TAS1273, where three representative plants were selected and tagged for each species. All three species/accessions were available at Mt. Pleasant and Swansea, however, *D. pentaphyllum* was not present at Elliott.

Four samplings were conducted at approximately six week intervals, on the 15/11/2002, 24/12/2002, 12/2/2003 and 28/3/2003. The final sampling at Elliott was not able to be undertaken as site security was breached by dairy cattle, and consequently consumed. The top 30cm of growth (all plant material, including leaf, stem, flowers, pods), was removed using hand shears, 'snap' frozen using dry ice, and subsequently stored at –18 ^{o}C. The growth stage of each species was described using the summary of development of lucerne quoted in Frame *et al.* (1998) (see Table 6.1, Chapter 6: Materials and Methods for description).

Frozen samples were sent to the University of Adelaide, where they were freeze dried and ground to <1 mm. Condensed tannin assays were performed using a modified butanol-HCl method and standardised against condensed tannins extracted and purified from the same plant samples (Brooker pers. comm., 2003).

7.3 Results

7.3.1 Climatic Data

The total monthly rainfall and average monthly temperatures are presented for the period October 2002 through to March 2003 (see Table 7.1). The average monthly temperature increased from October through to January/February after which it declined for Mt Pleasant, Swansea and Elliott.

The monthly rainfall between the three sites was similar in October and November 2002, with a decrease in rainfall in November relative to October. Rainfall at Elliott was significantly higher in December than for the other two sites, whereas, in January 2003, rainfall was significantly greater at Mt Pleasant. Rainfall for February and March was minimal at all three sites except Swansea for March when 104 mm of rain was recorded.

Table 7.1 Total monthly rainfall and average monthly temperatures for Mt Pleasant, Swansea and Elliott from October 2002 through to March 2003.

	Mt Pleasant		Swansea		Elliott	
Month	**Temperature (^oC)**	**Rainfall (mm)**	**Temperature (^oC)**	**Rainfall (mm)**	**Temperature (^oC)**	**Rainfall (mm)**
October	12.3	69	12.5	64	11.4	84
November	14.8	29	15.4	17	13.6	26
December	16.9	15	17.2	10	15.1	42
January	18.6	78	18.6	37	17.1	47
February	19.1	0	17.6	7	17.4	6
March	15.7	0	16.1	104	13.9	2

7.3.2 Sample Sites Soil Characteristics

The soil data from the three sites displays the different characteristics of the sites used as part of this experiment (see Table 7.2). The pH was slightly acidic at Elliott and Mt. Pleasant. The phosphorus and potassium levels may be considered to be mildly deficient at all three sites. The Elliott soil is known as the 'Lapoinya Clay Loam slump complex.' Swansea, known as 'Kelvedon,' is a heavy clay soil. Mt Pleasant is described as a gravelly clay loam over heavy clay (Hall pers. comm., 2003).

Table 7.2 Soil characteristics for Swansea, Elliott and Mt Pleasant topsoils.

	Depth (cm)	**pH (1:5H_2O)**	**EC (ds/m)**	**P (Colwell mg/kg)**	**K (Colwell mg/kg)**
Swansea	0 - 7.5	6.2	0.14	24	365
Elliott	0 - 10	5.9	0.12	81	265
Mt. Pleasant	0 - 7.5	5.3	0.24	66	178

7.3.3 Seasonal Changes in Developmental Stage

Dorycnium pentaphyllum plants at Mt Pleasant and Swansea displayed the same stage of development at each sampling date in 2002/2003 (see Figure 7.1). *Dorycnium penatphyllum* was in late flower stage in November, and early seed pod stage in December 2002. *Dorycnium pentaphyllum* resumed vegetative growth in February and March 2003.

Dorycnium rectum displayed similar trends in developmental stages at Mt Pleasant, Swansea and Elliott throughout the sampling period. *Dorycnium rectum* plants progressed from late bud stage/early flower in November 2002, to ripe seed pod

stage in March 2003. Flowering occurred in December for *D. rectum* plants at Mt Pleasant and Swansea, whereas, flowering at Elliott occurred between the December and February samplings.

Dorycnium hirsutum displayed similar changes in developmental stages at Mt Pleasant, Swansea and Elliott throughout the 2002/2003 sampling period. All *D. hirsutum* plants were in the early flower stage in November and progressed to ripe seed pod stage by February/March 2003. *Dorycnium hirsutum* flowered between the November and December 2002 samplings at Mt Pleasant and Swansea, whereas, *D. hirsutum* was flowering at Elliott at the time of the December sampling.

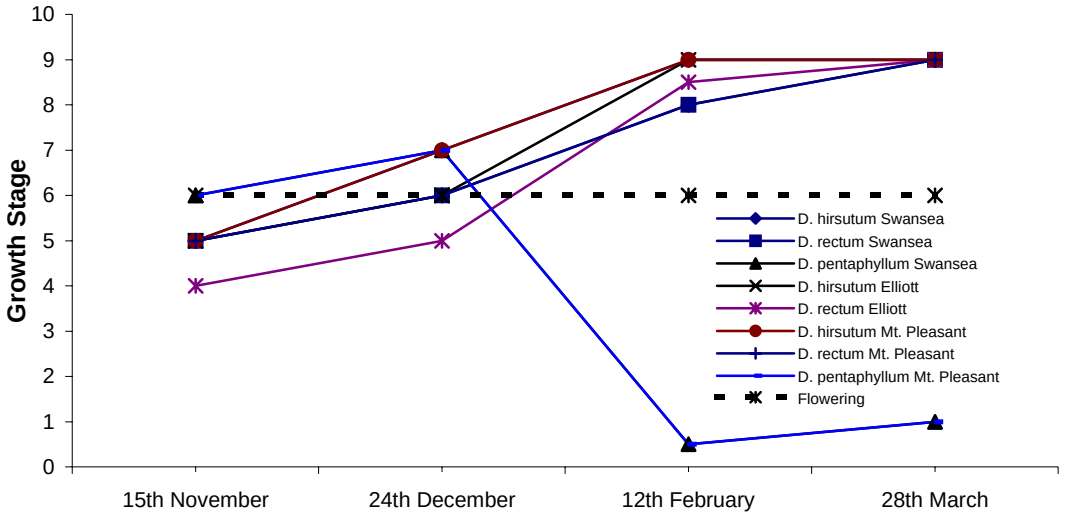

Figure 7.1 Seasonal changes in *Dorycnium* spp. stage of development at Mt Pleasant, Swansea and Elliott from November 2002 to March 2003.

7.3.4 Seasonal Changes in *Dorycnium* spp. Condensed Tannin Content

The CT data obtained for *D. hirsutum* were consistent between plants within sites at all sampling dates in 2002 and 2003. *Dorycnium hirsutum* displayed quite dramatic changes in CT through the sampling period (see Figure 7.2). Plants sampled from Swansea in November increased in CT content from an average of 3.2 % to an average of 16.2 % in December 2002. The CT content then decreased from December 2002 through March 2003. *Dorycnium hirsutum* plants at Mt Pleasant displayed a similar trend to Swansea, however the changes in CT content were not as great. The CT content of *D. hirsutum* plants at Mt Pleasant was higher than Swansea

and Elliott at the November sampling. Following the decrease between December and February, there was a slight increase in CT content for the Mt Pleasant plants. *Dorycnium hirsutum* at Elliott increased from an average of 3 % CT in November to an average of 12.9 % CT in December 2002 and then remained essentially constant through February 2003.

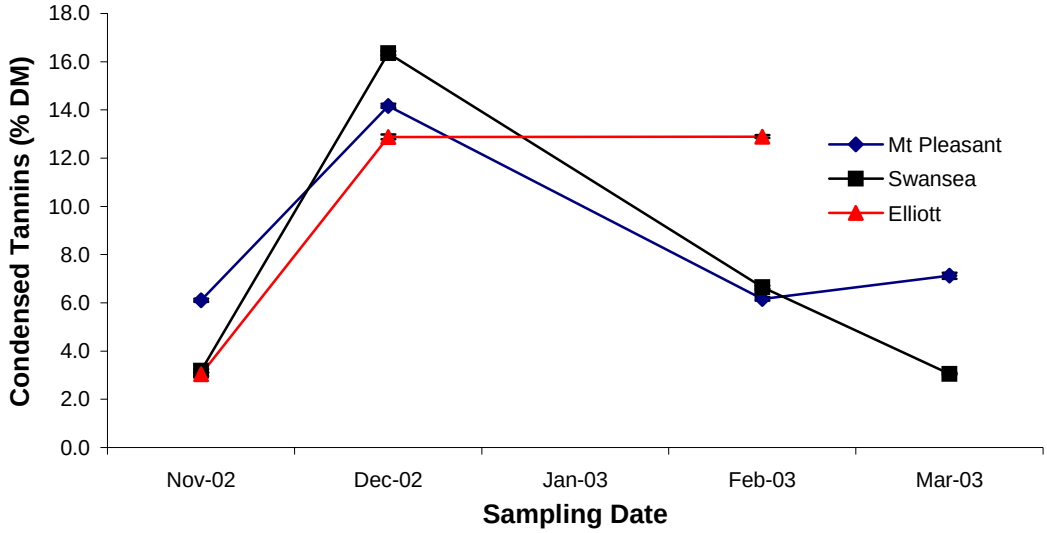

Figure 7.2 Seasonal changes in condensed tannins for *D. hirsutum* at three sites from November 2002 to March 2003. Vertical bars are standard deviations.

The CT content was consistent between plants within sites for *D. rectum* throughout the 2002/2003 sampling period. *Dorycnium rectum* contained consistently high levels of CT throughout the sampling period (see Figure 7.3). The lowest CT value observed was 7.7 % in March 2003 at Swansea, with the highest 16.6 % at Mt Pleasant in February 2003. The CT content of *D. rectum* at Swansea remained relatively constant throughout the sampling period with a slight decrease from February to March 2003. The CT content of *D. rectum* at Elliott increased from 12.8 % in November 2002 through to 14 % in February 2003. *Dorycnium rectum* at Mt. Pleasant increased in CT content from 12.2 % in November 2002 to 16.6 % in February 2003, and then the CT content decreased to less than 14 % in March 2003.

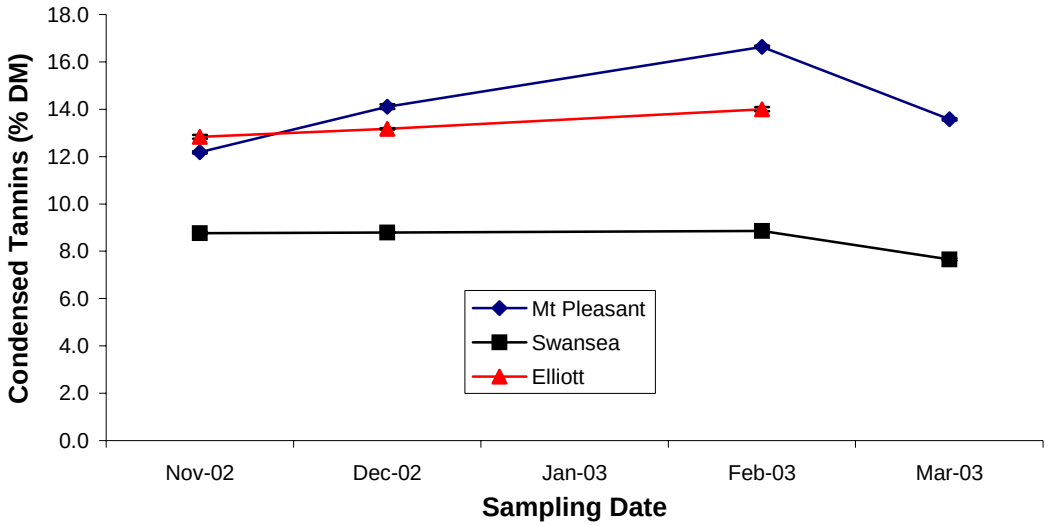

Figure 7.3 Seasonal changes in condensed tannins for *D. rectum* at three sites from November 2002 to March 2003. Vertical bars are standard deviations.

The CT content of *D. pentaphyllum* sampled at Mt Pleasant and Swansea were consistent between plants within sites throughout the 2002/2003 sampling period. *Dorycnium pentaphyllum* sampled at Mt. Pleasant displayed a similar trend in CT content to *D. pentaphyllum* at Swansea throughout the sampling period (see Figure 7.4). The CT content of *D. pentaphyllum* increased from about 8 % in November to about 15 % in December 2002, after which there was a decrease to levels similar to those observed in November 2002. The CT content of *D. pentaphyllum* at Swansea increased slightly in March 2003, which was also the case with the Mt Pleasant plants, however, CT content increased to levels similar to those in December 2002.

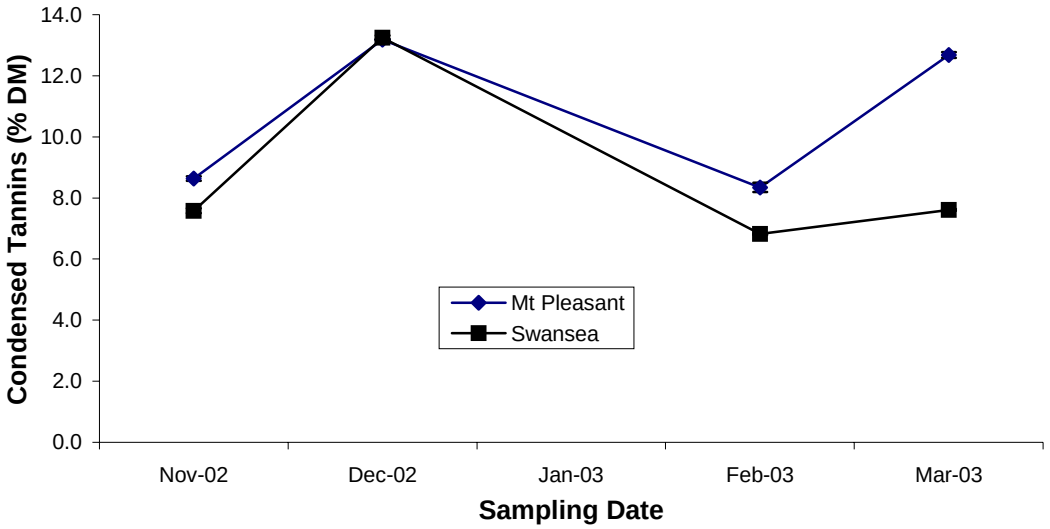

Figure 7.4 Seasonal changes in condensed tannins for *D. pentaphyllum* at two sites from November 2002 to March 2003. Vertical bars are standard deviations.

7.4 Discussion

Dorycnium spp. displayed varying CT contents depending on the species, time of sampling and location. The results revealed that *Dorycnium* spp. contain high levels of CT throughout the main growing season. The data obtained provided an excellent overall indication of the seasonal changes in CT, however, more data points would have provided a more accurate description. More frequent samplings were unable to be taken due to the inherent cost of CT analysis, which were prohibitive.

7.4.1 Condensed Tannins in *D. hirsutum*

Dorycnium hirsutum displayed the greatest variation in CT of all three species with a range of 3 - 16.4 %. The dramatic change in CT content was attributed to a combination of factors relating to both the plant and environment. The overriding influence appears to be the change in plant developmental stages. The onset of flowering and subsequent pod production led to a dramatic increase in CT content, in particular at Swansea where a change from 3.2 % to 16.4 % was observed during flowering. *Dorycnium hirsutum* at all three sites displayed a peak with the December sampling with all plants flowering. Following flowering, the CT levels dramatically decreased. The results support the notion by Barry (1989) that CT are a mechanism for defence i.e the protection of the reproductive organs. Both the Mt. Pleasant and Swansea *D. hirsutum* CT contents returned to their original levels by March.

The similarity in developmental changes over time combined with different CT contents of *D. hirsutum* at any point in time between sites suggested an interaction between the environment and plant genotype. Swansea displayed the greatest change in CT of the three sites. This site also had the lowest rainfall, except for March 2003, and lowest soil phosphorus level.

The CT content of *D. hirsutum* at Swansea and Mt Pleasant in November and at Swansea in March were all approximately 3 %, which may be considered to be beneficial. Barry (1989) stated that <2-4 % CT are believed to be beneficial by increasing the amount of protein available for digestion and absorption. Condensed tannin content greater than 4% may significantly reduce palatability and digestion processes. *Dorycnium hirsutum* plants contained >4 % CT for most of the growing season. The CT observed in *D. hirsutum* were not as high or low as those described by Terrill *et al.* (1992) and Barry (1989) respectively, however, they were within the general range given by Terrill *et al.* (1992). This experiment only examined a particular phase of *D. hirsutum* life cycle, and is in no way a complete investigation of this topic.

7.4.2 Condensed Tannins in *D. rectum*

Dorycnium rectum displayed less dramatic changes in CT during the sampling period when compared to *D. hirsutum*. There was very little change observed at Swansea

throughout the sampling period, approximately 1 %. The values of CT observed in *D. rectum* were consistent with 14.3 % as described by Terrill *et al*. (1992). The CT contents are considered to be high with a minimum value of 7.7 % and maximum of 16.6 % observed. Such CT contents would suggest a decrease in plant palatability and reduced digestive function.

There was a distinctive environment/species interaction as typified by the differences between Swansea and the other two sites. Swansea was on average in the order of >3 % lower than the other two sites, with the soil and climatic data suggesting a more favourable environment than the other two sites, and hence there was a lower production of CT. The CT trends for the three sites may be loosely correlated with the average daily temperatures. The slight increases in CT between November and December as observed at Elliott and Mt. Pleasant coincided with flowering and pod maturation timing observed in *D. rectum*.

7.4.3 Condensed Tannins in *D. pentaphyllum*

Dorycnium pentaphyllum material was only available in sufficient quantities at Mt. Pleasant and Swansea. Plants sampled from these two sites displayed similar patterns of CT fluctuations, however, the March sampling varied greatly. The CT contents observed were high throughout the sampling period, ranging from 7.6 % to 13.3 %. The CT values observed for *D. pentaphyllum* were consistent with those described by Terrill *et al.* (1992) for this species. The high levels of CT observed in *D. pentaphyllum* would suggest problems associated with palatability and digestion.

The peak in CT content, where levels increase from approximately 8 to 13 % in December coincided with the flowering and pod initiation of the plants and also decreases in the monthly rainfall at both sites. The decrease in CT levels between December and February followed the completion of flowering and resumption of the vegetative phase. There was a dramatic increase in rainfall during this period. The first sampling of *D. pentaphyllum* in November classified the plants as developmental stage 6 i.e flowering, and therefore the CT levels may actually be lower prior to flowering. An earlier sampling may have provided another perspective on the CT levels leading up to flowering and under less harsh environmental conditions. Another sampling would have also been useful between the December

and February sampling to characterise the change from reproductive to vegetative cycles more clearly.

The decrease in CT between December and February in *D. pentaphyllum* coincided with an increase in the rainfall at both sites. Swansea experienced high rainfall in March, and CT levels remained relatively constant in relation to levels observed in February. Mt. Pleasant received essentially no rainfall in February and March and there was an increase in CT content in *D. pentaphyllum.*

7.4.4 General Discussion

Dorycnium spp. examined in this experiment generally displayed an increase in CT levels during flowering. The CT content of *D. hirsutum* dramatically increased during this reproductive phase, with five fold increases observed at Swansea. The apparent relationship between CT levels and flowering supports the notion that the production of these compounds is an evolutionary defence mechanism.

The management of *D. hirsutum* and *D. pentaphyllum*, and to some extent *D. rectum* appears to be important in relation to ensuring CT levels are kept to a minimum. The harvesting of material prior to flowering appears to be a critical factor in terms of providing the grazing animal with forage with CT levels that are as low as possible, and even beneficial as observed with *D. hirsutum* at certain locations and harvests.

Differences in CT levels observed between sites for a particular *Dorycnium* spp. revealed an environment/species interaction. This study was unable to identify the critical factors associated with increases/decreases in CT levels. However, trends displayed by the plants sampled suggest that factors such as, rainfall, temperature and soil fertility all may influence CT levels. The changes in plant CT content provide support to the notion that there is an inverse relationship between plant CT levels and rainfall, temperature or soil fertility. The type of environments in which *Dorycnium* spp. will be widely introduced will undoubtedly present such conditions. This poses the question does *Dorycnium* spp. forage consumed under such conditions possess any nutritional value for the grazing animal if they contain CT, and therefore, animal feeding trials need to be undertaken to determine this.

7.5 Conclusion

Dorycnium spp. were found to contain CT levels that ranged from 3 to 16.6 %. Levels less than 4 % were considered to be low and may have beneficial properties for grazing animals. In general CT levels were high and possibly would result in reduced palatability and digestive function when consumed. Increases in CT levels were observed during flowering with subsequent decreases thereafter. Interactions between species and the environment were observed with a number of factors believed to influence CT levels. An inverse relationship between, rainfall, temperature and soil fertility and CT was proposed.

7.6 Acknowledgements

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Chapter 8. Rhizobia Literature Review

8.1 Rhizobia

Bacteria that are able to elicit nodule formation on legumes are called rhizobia (Dénarié *et al.,* 1996). Rhizobia are a genetically diverse and physiologically heterogenous group of bacteria (Somasegaran and Hoben, 1994). Rhizobia comprises of the genera *Rhizobium, Bradyrhizobium* and *Azorhizobium* (Dénarié *et al.,* 1992) and *Sinorhizobium* (Dénarié *et al.,* 1996).

Rhizobia are a ubiquitous part of the soil micro-flora in a free-living state in the rhizosphere of legumes (Allen and Allen, 1981; Somasegaran and Hoben, 1994) until the point where nodulation becomes possible (Rendig and Taylor, 1989). The nature and properties of soil allows billions of organisms to coexist (Pepper and Upchurch, 1991).

The ability to form symbiotic relationships with members of the plant family Fabaceae is a unique feature associated with bacteria belonging to the family *Rhizobiaceae* (Pepper and Upchurch, 1991). The number of symbiotic relationships that can form between rhizobia and hosts is restricted, and vice versa (Dénarié *et al.,* 1992). Rhizobia elicit on their host the formation of nodules in which they fix nitrogen (Dénarié *et al.,* 1992; Dénarié *et al.,* 1996; Prescott *et al.,* 1996) and provide the plant with ammonia for growth (Rendig and Taylor, 1989).

Despite the widespread distribution of leguminous crops, many soils remain void of rhizobial strains (Brockwell *et al.,* 1995). Grobbelaar and Clarke (1974) concluded that under local conditions nodulation may not occur with introduced plants due to the lack of suitable *Rhizobium* strains.

8.1.1 Rhizobial Characteristics

Rhizobia are bacteria that selectively infect the roots of some legumes and have the following characteristics; gram negative, motile rod-shaped (approximately 0.5-0.9 μm in width and 1.2-3.0 μm in length) and heterotrophic (Pepper and Upchurch, 1991; Somasegaran and Hoben, 1994; Prescott *et al.,* 1996).

Root nodule bacteria generally grow under the following conditions 25-30 ^{o}C (optimum) in the pH range of 6-7 (Vincent, 1970; Somasegaran and Hoben, 1994). *Rhizobium* growth normally occurs under aerobic conditions. However, when fixing nitrogen, low levels of oxygen are required to protect the enzyme nitrogenase (Rendig and Taylor, 1989), and hence, *Rhizobium* are able to grow in microaerophilic conditions (Somasegaran and Hoben, 1994).

8.2 Nodule Formation

The presence of appropriate rhizobia in the soil is the first critical factor for nodule formation (Pepper, 1991; Pepper and Upchurch, 1991), with the relationship between certain bacteria and legumes being highly selective (Rendig and Taylor, 1989). In some cases, only one *Rhizobium* spp. is effective on a particular species of legume (Salisbury and Ross, 1992).

8.2.1 Signalling Between Host and Root Nodule Bacteria

Rhizobial *nod* genes are important in the determination of host specificity, infection and nodulation, and are involved in the exchange of signals between the plant and bacteria (Dénarié *et al.,* 1992). Legumes release several flavonoids, some of which may be specific to a particular *Rhizobium*. Both *Bradyrhizobium* and *Rhizobium* spp. are attracted by flavonoids (Dénarié *et al.,* 1992).

8.2.2 Linkage of Host and Root Nodule Bacteria

The attachment of the bacteria to the root hairs is the start of the infection process (Rendig and Taylor, 1989; Prescott *et al.,* 1996). The infection process involves a complex series of interactions, before the rhizobia enter the root hairs of the host (Somasegaran and Hoben, 1994).

Rhizobium spp. produce *nod* genes (nodulation genes) that promote the binding between bacteria and root hairs (Pepper and Upchurch, 1991; Prescott *et al.,* 1996). Attraction between the rhizobia and root surface occurs due to van der Waals forces and leads to the linking of free carboxyl groups in the peptidoglycan of the rhizobial cell to organic acids found on the root surface (Howieson, 1995). Specialised

proteins, known as lectins, found on the surface of the roots are believed to act as recognition sites (Allen and Allen, 1981; Raven *et al.,* 1992). Rhizobial multiplication occurs in the rhizosphere and on the root surface (Dénarié *et al.,* 1992).

8.2.3 Rhizobial Entry into the Root

The method by which root infection occurs includes simple 'crack entry' or through the root hairs (Dénarié *et al.,* 1992 and Dénarié *et al.,* 1996). 'Crack entry' involves the entry of rhizobia through intercellular spaces in the epidermis or in the middle lamella, as seen in *Arachis* L. (peanut) (Dénarié *et al.,* 1992 and Dénarié *et al.,* 1996) and *Sesbania rostrate* Bremek & Oberm. (Webster *et al.,* 1997). Alfalfa and vetch both display infection of the roots by rhizobia through the root hairs (Dénarié *et al.,* 1992 and Dénarié *et al.,* 1996). Infection by rhizobia through the root hairs is described in greater detail below.

8.2.4 Rhizobial Entry through Root Hairs

The nodulation gene *nodD,* is a regulatory proteins that is activated by plant flavonoids, which in turn control the transcription of the *nod* operons (Dénarié *et al.,* 1992). Structural *nod* genes are involved in the synthesis of specific lipo-oligosaccharides (Nod factors) (Dénarié *et al.,* 1996), which signal back to the plant to elicit root hair deformations, cortical cell divisions and nodule-meristem formation (Dénarié *et al.,* 1992).

Once the rhizobia are attached to the root hairs they are entrapped as the root hairs curl tightly during extension (Rendig and Taylor, 1989; Raven *et al.,* 1992; Salisbury and Ross, 1992; Somasegaran and Hoben, 1994). The cell wall of the root hairs is then degraded with the release of specialised enzymes by the rhizobia, which allows for entry of the rhizobia into the cell itself (Salisbury and Ross, 1992).

8.2.5 Infection Thread Formation

Tubular structures known as infection threads allow the invasion of the root hairs and the underlying cortical cells by the rhizobia (Allen and Allen, 1981; Rendig and Taylor, 1989; Dénarié *et al.,* 1992; Raven *et al.,* 1992; Salisbury and Ross, 1992; Somasegaran and Hoben, 1994; Prescott *et al.,* 1996). The infection thread (see

Figures 8.1 and 8.2) is important to the rhizobia as it provides a means of avoiding the plant defence mechanisms (Pepper and Upchurch, 1991). Rhizobia are released from the infection thread into the cytoplasm of the host cell, where bacterial cell multiplication takes place (Somasegaran and Hoben, 1994).

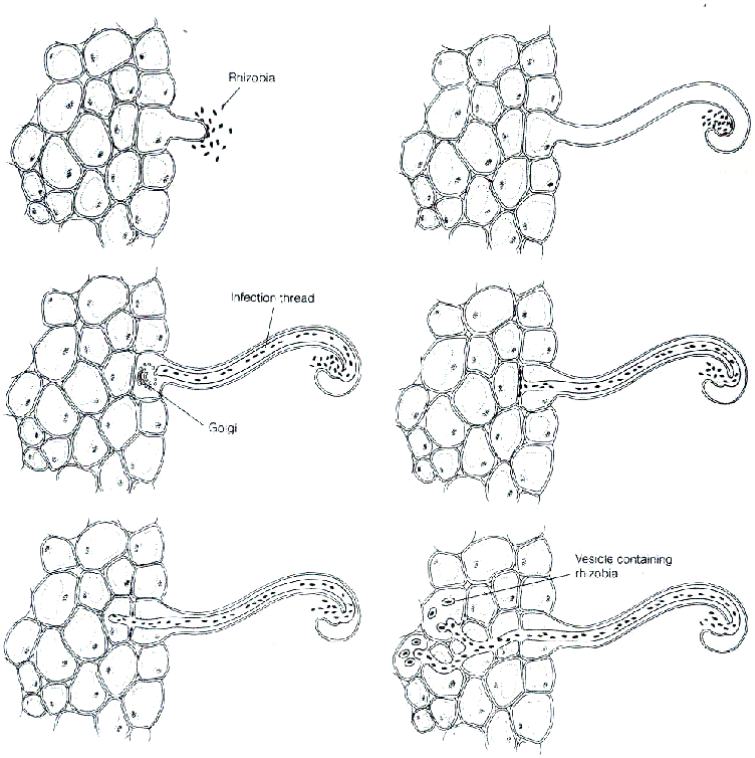

Figure 8.1 Infection Thread Formation

Figure 8.1 displays the infection process taken from Taiz and Zeiger (1991). The above diagram describes the binding of rhizobia to the root hair, root hair extension and curling and the formation of an infection thread through which the rhizobia cells enter the root cytosol.

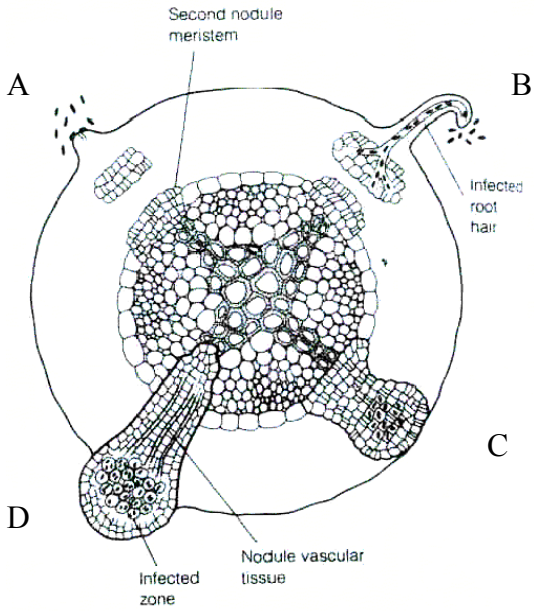

Figure 8.2 *Rhizobial* Infection and Nodule Formation

Figure 8.2 displays a cross section of a soybean root and four stages (A - rhizobia attachment to root hair, through to D – formation of root nodule) of the process of nodule formation (Taiz and Zeiger, 1991).

8.2.6 Bacteroid Development

The movement of rhizobia from the infection thread into the host cell results in the rhizobia being surrounded by a membrane known as the peribacteroid membrane (Prescott *et al.,* 1996). This is the formation of nodules and leads to the proliferation of the enlarged rhizobia and cortical cells (Allen and Allen, 1981; Dénarié *et al.,* 1992; Raven *et al.,* 1992; Salisbury and Ross, 1992). The enlarged rhizobia are often referred to as bacteroids in which the fixation of nitrogen by legumes occurs (Dénarié *et al.,* 1992; Raven *et al.,* 1992; Salisbury and Ross, 1992; Somasegaran and Hoben, 1994).

The further differentiation of the bacteroid results in the nitrogen fixing structure known as a symbiosome (Prescott *et al.,* 1996). The cytosol of bacteroids is the site of synthesis of nitrogenase, the enzyme responsible for the reduction of atmospheric nitrogen to ammonium (Somasegaran and Hoben, 1994). *Rhizobium* spp. are unable to reproduce once a functioning nodule is formed (Prescott *et al.,* 1996). Root

nodules are genuine organs, not mere tumors (Dénarié *et al.,* 1992 and Dénarié *et al.,* 1996).

The formation of nodules allows for the control of oxygen concentration and the conditions for nitrogen gas to be fixed into ammonia. The rate of nitrogen fixation is dependent on the size of the nodules and the activity of nitrogen fixation (Rendig and Taylor, 1989). According to Rendig and Taylor (1989), the time taken for the host-rhizobia relationship to be formed and be fully functional is dependent on four main factors: presence of *Rhizobium,* inoculants that are competitive with background populations, provision of nutrients by the host to the rhizobia, and the influence of the environment and soil factors. Nitrogen fixed from the atmosphere is eventually stored within the legume or used for the assimilation of proteins (Allen and Allen, 1981).

8.3 Nitrogen Fixation

Nitrogen fixation is the biological process by which atmospheric N_2 gas (nitrogen diatom) is reduced to NH_4^+ (Raven *et al.,* 1992; Salisbury and Ross, 1992). Nitrogen can thus be added to ecosystems (Boddey *et al.,* 2000). Biological nitrogen fixation (BNF) is of great importance in a number of environments, such as, terrestrial, freshwater, marine, and arctic (Salisbury and Ross, 1992).

The fixation of nitrogen is a process upon which all living organisms are dependent. Biological nitrogen fixation is estimated to be approximately 150 to 200 million tonnes annually on the earth's surface (Raven *et al.,* 1992). The symbiotic relationships between specific soil micro-organisms and plants are the most significant contributor of BNF in most terrestrial ecosystems (Boddey *et al.,* 2000).

8.3.1 Symbiotic Nitrogen Fixation

Symbiotic nitrogen fixation is a process carried out by root nodule bacteria in association with legumes (Raven *et al.,* 1992). The fixation of nitrogen provides the plant with available ammonium, whilst the plant provides the rhizobia with simple sugars (Pepper, 1991; Pepper and Upchurch, 1991). The increase of rhizobia

numbers in the rhizosphere is a response to the release of nutrients by the host legume (Somasegaran and Hoben, 1994).

8.4 Importance of Host-Rhizobia Interactions to Agriculture

The symbiotic fixation of nitrogen is crucial for the provision of nitrogen in the plant world (Allen and Allen, 1981). Nitrogen fixing associations are of significant ecological and agricultural importance (Dénarié *et al.,* 1996). Nutrients are lost through natural processes, such as, leaching or volatilisation, and therefore, in sustainable ecosystems these nutrients must be replaced either by fertilisers or through natural processes (Boddey *et al.,* 2000). The long-term effect of poor nodulation and nitrogen fixation is a decrease in soil nitrogen reserves and a reduction in production potential (Peoples and Herridge, 1990). Brockwell *et al.* (1995) stated that legumes account for approximately 40 % of total nitrogen fixation. According to Pepper and Upchurch (1991) the ability of legumes to fix nitrogen makes them an obvious choice for use in agricultural areas considered to be semiarid or arid.

The commercial use of rhizobial strains within Australian agriculture is largely based around the ability to fix nitrogen optimally across a broad range of species. This is driven by the economics of commercial inoculant production. Australian farmers have a greater need for optimal performance of their legumes due to the return they receive on their goods and the relative cost of inputs (Howieson *et al.,* 2000a).

8.5 Legumes in Australian Agriculture

There has been a rapid change in the range of legumes found within Southern Australia's agricultural systems during the last decade (Howieson *et al.,* 2000a). The origin of many of these legumes and their associated root-nodule bacteria are outside Australia, which provides serious challenges for adaptation in terms of soil conditions. The development of new commercial pasture legumes particularly on acid sandy soils is hindered by the lack of well adapted root-nodule bacteria (Parker 1962 cited in Howieson *et al.,* 1995).

The selection of suitable rhizobia is vitally important for the successful introduction of new legumes onto soils substantially different from where they originated (Howieson *et al.,* 2000a). A number of legumes and associated root-nodule bacteria have been selected for use in the Mediterranean areas of Australia due to their acid tolerance (Howieson, 1995). Howieson *et al.* (2000c) concluded that Mediterranean legumes do not always fix nitrogen optimally and that it should not be assumed that rhizobia and legume are ideally matched. There is a great potential for the improvement of nitrogen fixation by Mediterranean legumes in their natural and introduced habitats by matching symbionts.

8.6 Rhizobia and *Dorycnium* spp.

Prior to planting, *Dorycnium* spp. seed must be inoculated with an appropriate nitrogen fixing bacterium (Wills and Douglas, 1984). Trials in New Zealand have shown that *Lotus corniculatus* rhizobia (SU343) are effective inoculants of *Dorycnium* spp. (Sheppard and Douglas, 1986; Wills *et al.,* 1989a). Allen and Allen (1981) also found rhizobia for *Lotus corniculatus* were effective symbionts for *Dorycnium* spp.

Brockwell and Neal-Smith (1966) found that the member of the tribe Loteae, *D. hirsutum* entered into a nitrogen fixing symbiotic relationship with the rhizobia of nine members of the tribe Loteae and one species of the tribe Ononideae. The diverse symbiotic range exhibited by *D. hirsutum* is unique within the tribe Loteae (Brockwell and Neal-Smith, 1966). *D. hirsutum* inoculated with *Rhizobium* isolated from *Anthyllis vulneria* was found to fix sufficient nitrogen for this bacterium to be considered a useful inoculant (Brockwell and Neal-Smith, 1966). Brockwell and Neal-Smith (1966) found the only ineffective *Rhizobium* strain for the inoculation of *D. hirsutum* was isolated from *Lotus cruentus*, a species, which is found in inland Australia.

8.6.1 *Dorycnium* spp. Nodulation in the Field

Nodulation of *D. hirsutum* in the field has been highly variable, where low soil fertility is believed to be limiting nodulation, and hence the sucessful establishment of *D. hirsutum* (Douglas *et al.,* 1996b). New Zealand trials have found that individual

D. hirsutum and *D. pentaphyllum* plants have spread quite some distance from the parent plants with the roots being nodulated and healthy with the rhizobia most likely associated with *Trifolium arvense* L. (Wills *et al.,* 1989a). The suggestion that rhizobia associated with *Trifolium* spp. L. would nodulate *D. pentaphyllum* is questionable due to the general inability of *Rhizobium* spp. to cross inoculate two genera.

8.7 Rhizobial Studies

Rhizobia occurring naturally in soils may be able to infect legume roots and form nodules, however the effectiveness in terms of nitrogen fixation may be highly variable (Pepper, 1991). Somasegaran and Hoben (1994) stated that nodules with a pink colour infer an effective nodule, hence active leghaemaglobin, whereas white or greenish nodules infer ineffective symbiosis. The inoculation of seeds or legume roots prior to planting with effective and competitive rhizobia may help improve host/rhizobia interactions and nitrogen fixation. The development of effective inoculants for particular legumes is very important, however, the rhizobia need to be suited physiologically to the inherent soil conditions in which they will be expected to survive and persist (Pepper, 1991). Attention to the *Rhizobiuml*legume association can improve the productivity of symbiosis in semiarid regions (Pepper and Upchurch, 1991).

Brockwell *et al.* (1982), and Somasegaran and Hoben (1994) described some of the various methods that may be used for the selection of rhizobia as inoculants, from the use of growth cabinets to field trials. Wilson *et al.* (1995) stated that in glasshouse experiments the differences between inoculated and un-inoculated plants could be clearly seen due to differences in nitrogen availability. However, the repetition of these findings in the field is difficult due to two main factors, the inability to remove all nitrogen from the soil and the interactions with other introduced and background rhizobia.

8.7.1 Considerations for Rhizobial Studies

The investigation of populations of rhizobia has been driven by ecological and agricultural considerations, where the symbiotic functions of legumes are often

overlooked by agronomists when investigating aspects of legume growth in the field (Brockwell *et al.*, 1995). Howieson *et al.* (2000c) stated that in order to effectively harness biological nitrogen fixation (BNF), the interaction between plant and bacterial genotypes, and soil conditions needs to be understood. This is further complicated as the genotypes of both the symbiont and host need to be considered and therefore, the many interactions that can occur need to be understood. Howieson *et al.* (2000c) described the notion of a G^2 (genotype) X E (environment) interaction for legumes and rhizobia introduced to new environments. This interaction considers the three way interaction between host genotype, bacterial genotype and the environmental conditions.

To gain a greater understanding of the ecology of rhizobia in soil colonisation studies are essential. The complexity of the relationship between plant and *Rhizobium* with environmental factors (both soil and climatic) often means that each individual situation needs to be examined (Chatel and Greenwood, 1973). The determination of the best method of inoculation is necessary prior to planting, as poor inoculation may be reflected in poor seedling establishment (Wright, 1985). The successful nodulation and subsequent fixation of nitrogen following sowing is reliant on the inoculation of legumes with the appropriate strain of *Rhizobium* (Wills, 1986), where an effective symbiosis generally confers a beneficial plant response (Allen and Allen, 1981). Brockwell *et al.* (1982) stated that when selecting rhizobial strains for testing, strains associated with the host should be selected, as, in a mixed culture situation there will be preferential selection for more effective strains.

8.7.2 Rhizobia Strain Selection

Knowledge of the hosts preferences for rhizobia might help in the selection of superior inoculum in the field and assist in avoiding competition between inoculum and background rhizobial populations. Strains could be selected so that nodules formed by legumes are dominated by effective rhizobia (Vincent and Waters, 1953). Howieson *et al.* (2000a) described a four stage strain selection process that is used for the selection of new strains;

Stage 1: Strain Isolation

The first stage of strain selection involves the excavation of plants in their natural environment and removal of nodules. Specialised media is then used to culture bacteria isolated from the nodules. Colonies displaying the typical characteristics of rhizobia i.e. slightly raised, opaque and entire, are then selected for purification.

Stage 2: Strain Nitrogen Fixing Ability

The second stage of strain selection as described by Howieson *et al.* (2000a) involves the determination of host/symbiont compatibility via the examination of nitrogen fixation. The undertaking of this screening process makes three assumptions; nitrogen is the only limiting factor in the screening environment, host/rhizobia interactions are expected and elite strains under examination must not compromise existing agricultural legumes. The detailed methodology is described by Howieson *et al.* (1995 and 2000a and b).

Stage 3: Field Evaluation

The third stage of strain selection involves the examination of strain survival, persistence and migration in soils of interest. This type of trial is commonly referred to as a cross row experiment. Inoculated plants are established in accessions with buffer rows and are supplied with all macro and micro-nutrients required, except nitrogen. Plants are grown through the winter and are removed in the following summer. Seeds are then planted the following autumn across the original rows running perpendicular (see Chapter 10, Figure 10.1). After 10-12 weeks, plant shoots and roots are excavated, with shoot DM determined and root nodulation patterns observed.

Stage 4: Rhizobial Survival

The final stage of strain selection is the *in situ* assessment of nitrogen fixation, nodule formation and plant production. Following stage 3, a break crop is planted in order to reduce the soils nitrogen status and expose the rhizobia to the stresses of living as a saprophyte.

8.7.3 Desirable Strain Characteristics

Howieson *et al.* (2000c) stated that the following characteristics are required for quality rhizobial strains; 1: Host/rhizobia compatibility examined via nitrogen fixation studies as described by Howieson *et al.* (1995 and 2000a and b). 2: The ability to produce consistent nodulation patterns in field soils using methods as described by (Howieson *et al.,* 2000c). 3: Rhizobial strain genetic stability is a very important and desirable characteristic. Genetic drift via subculturing using rich medium is highly undesirable, and therefore the production of genetically stable strains is desirable. 4: The survival of rhizobia during commercial production and the exposure to physiological stresses during culturing, such as acidity, can help improve acid tolerance in the field. 5: The ability to compete effectively with the indigenous soil micro-flora is also highly desirable.

8.8 Factors Affecting Host-Rhizobia Interactions

Howieson (1995) stated that the long term survival and persistence of a legume is dependent on the adaptation of the legume and bacterial symbiont to soil conditions. The rhizobial populations in the soil can be affected by a number of factors including other plants, competition, temperature, moisture, soil acidity and alkalinity (Somasegaran and Hoben, 1994), all of which will influence the effectiveness of nitrogen fixation in the soil by the legume/*Rhizobium* interaction (Howieson, 1995; Peoples *et al.,* 1996). Some soil micro-organisms are believed to be responsible for producing inhibitors that have an adverse effect on *Rhizobium* bacteria (Rendig and Taylor, 1989).

The success of any *Rhizobium* spp. introduced to the soil as an inoculant or as part of the existing microflora is dependent on competitiveness and/or effectiveness (Pepper, 1991). The ability of root-nodule bacteria to persist in the soil between legume rotations will determine the ability of the legume to be nodulated upon regeneration (Howieson, 1995).

Legumes have a higher requirement for calcium during infection and subsequent nodule formation than is required by the legume for growth. Liming can improve pasture establishment via the provision of calcium and immobilisation of manganese

and aluminium (Brockwell *et al.,* 1995). The ineffective nodulation of roots or shedding of roots and nodules may be due to a number of factors, such as drought, removal of plant material, unfavourable soil conditions, temperature extremes, or the absence of suitable effective rhizobia in the soil (Allen and Allen, 1981).

8.8.1 Rhizobial Competition/Interactions

Rhizobium strains growing within the rhizosphere compete with each other for potential infection sites on their host (Lim and Burton, 1982). Competition between effective and ineffective rhizobia will determine nitrogen fixation by a legume, whether or not effective strains are part of the microflora or are introduced as inoculum (Vincent and Waters, 1953). The success of an introduced inoculum is subject to competition with the background rhizobia, which often form ineffective nodules on the host (Lim and Burton, 1982).

The complex relationship between rhizobial strains in the soil cannot be clearly defined, as a number of factors associated with the host and rhizobia need to be considered. These factors include, growth rate of rhizobia, the physiological state of rhizobia, nutrient levels, temperature, moisture, oxygen, inoculation timing, and most importantly the relative numbers of rhizobia present (Lim and Burton, 1982).

Differing levels of effectiveness are displayed by the various components of a mixed rhizobial population (Brockwell *et al.,* 1995). Vincent and Waters (1953) found that in a mixed rhizobia culture in the rhizosphere, unequal growth was displayed. Individual rhizobial strains can display comparative advantages, such as, increased ability to use nutrients, or produce inhibitory substances (Lim and Burton, 1982). In a mixed population the more effective strains are generally more competitive (Vincent and Waters, 1953) and the host legume appears to exhibit some selective preference (Brockwell *et al.,* 1995).

8.9 Introduction of Rhizobial Populations

The successful introduction of a new strain of rhizobia in the environment requires a host to be present and edaphic conditions to be favourable. As long as the bacteria are able to survive to such time that the rhizosphere is available for colonisation,

nodulation should occur as a matter of course. In situations where soils are relatively void of background rhizobia, the introduction of new effective strains may be achieved relatively easily, however, the introduction of such strains should not impact on desirable inoculant strains i.e. other introduced inoculants. Where there is a significant background population, successful inoculation requires a heavy rate of persistent effective inoculum to be applied close to the roots of the legume concerned (Brockwell *et al.,* 1995).

Chapter 9. Rhizobia Selection

9.1 Introduction

Rhizobia used to inoculate *L. corniculatus* have previously been found to be an effective inoculant group for *Dorycnium* spp. (Allen and Allen, 1981; Wills *et al.,* 1989a+b). Brockwell and Neal-Smith (1966) reported that *D. hirsutum* entered into a nitrogen fixing symbiosis with the rhizobia isolated from nine members of the tribe Loteae and one species of the tribe Ononideae. This diverse symbiotic range exhibited by *D. hirsutum* was considered unique within the tribe Loteae (Brockwell and Neal-Smith, 1966).

There has been a rapid change in the range of legumes utilised in southern Australia's agricultural systems during the last decade (Howieson *et al.,* 2000a) and *Dorycnium* spp. are further candidates for exploitation. As the origin of many of these legumes is outside Australia, there have often been serious challenges for adaptation to soil conditions for both the legume and associated root nodule bacteria as they are introduced into Australia (Howieson *et al.,* 2000b).

Howieson *et al.* (2000c) concluded that Mediterranean legumes do not always fix nitrogen optimally *in situ* and it should not be assumed that rhizobia and legume are ideally matched. Inoculation of *Dorycnium* spp. in Australia is generally with a commercial *Lotus* spp. inoculum (SU343) that has been widely evaluated for neither effectiveness nor adaptation to difficult soils. Therefore, this research was undertaken to evaluate inoculant strains for *Dorycnium* spp., and to compare these strains with the commercial *L. corniculatus* strain currently available in Australia. Further, SU343 and a range of selected strains were evaluated for positive and negative interactions with a number of important and symbiotically related agricultural legumes. Selected strains were finally evaluated for adaptation to Tasmanian field conditions as described in Chapter 10.

9.2 Materials and Methods

9.2.1 Rhizobia Selection Experiment

A pot experiment in sterile sand culture was undertaken at the Centre for *Rhizobium* Studies (CRS), Murdoch University (Western Australia). The methods used were a modified version of 'Experiment 2' as described by Howieson *et al*. (1995). This experiment was the basis for strain selection and evaluation, and subsequent field evaluation (see Chapter 10) for *Dorycnium* spp.

9.2.2 Pot Preparation

Pots 15cm in diameter were sterilized in hypochlorite solution (4 %) and then rinsed thoroughly in sterile distilled water. A sterilised paper towel was placed in the base of the pot and steam sterilized sand (1 river:2 yellow) mixture was then packed into the pots to a level of two cm from the top of the pots. The pots were then flushed twice with hot autoclaved water (~100 ^{o}C) to ensure sand sterility and that any free nitrogen was removed.

9.2.3 Strain Selection and Preparation

Strain selection was based on preliminary work undertaken by the CRS, which identified those strains believed to have the potential for improving nitrogen fixation under field conditions. The selected strains were SU343, WSM805, WSM1284, WSM1293, WSM2323, WSM2337, WSM166, CC856, CC801, and WSM2338 (see Table 9.1 for rhizobial origin). Rhizobial strains from the WSM (Western Soil Media) rhizobia collection were recovered from ampoules using glycerol and 7% sucrose solution. Strains were plated out on ½LA (lupin agar) media (see Appendix 2) and incubated at 28 ^{o}C for 72 hours until opaque colonies were produced prior to inoculation.

Table 9.1 Origins of rhizobial strains used for inoculation of pasture legumes.

Strain	Plant Source	Collection Site (source)
CC801	*L. edulis* L.	St Charles Algeria
CC829	*L. pedunculatus* Cav.	Australia Group D commercial
CC856	*Anthyllis vulneraria* L.	
SU343	*L. corniculatus*	USA commercial
WSM1284	*B. pelecinus* L.	Sardinia Italy
WSM1293	*Lotus spp.*	Serifos Greece
WSM2323	*D. hirsutum*	Mt Pleasant (Tasmania)
WSM2337	*D. rectum*	Swansea (Tasmania)
WSM2338	*D. rectum*	Swansea (Tasmania)
WSM805	*Lotus spp.*	Andros Island Greece

9.2.4 Seed Preparation

Seeds of the selected legumes, three *Dorycnium* spp., five *Lotus* spp. and *Biserrulla pelecinus,* were scarified using a drum scarifier. The seed surface was then sterilized with 90 % ethanol (one minute), and 4 % hypochlorite solution (three minutes), then rinsed thoroughly with distilled sterile water and spread onto water agar 1.5 % where imbibition and germination took place.

9.2.5 Experimental Design

The design was a split-plot factorial experiment in which main treatments were imposed in triplicate within a completely randomised design. Ten rhizobial strains (and two uninoculated controls, +/- nitrogen solution, see Appendix 1) as the main treatments were inoculated separately onto six *Dorycnium* spp., five *Lotus* spp. or *Biserrulla pelecinus*, which were the sub-treatments (three plants of three species per pot). The *Dorycnium* genotypes were; *D. pentaphyllum* TAS2309 and TAS1272, *D. rectum* TAS135 and TAS1274, and *D. hirsutum* TAS1002 and TAS2001. The *Lotus* spp. were *Lotus purshianus* Clem & E.G. Clem., *L. tenuis* Waldst. and Kit. Ex Willd., *L. corniculatus*, *L. pedunculatus* and *L. ornithopodioides* L.. *Biserrulla pelecinus* was included because of its value in Australian agriculture and its capacity to cross-nodulate with *Lotus* root nodule bacteria (Loi *et al*., 2001; Nandasena *et al.,* 2001). *Dorycnium* spp. seed accessions were supplied by the Tasmanian Institute of Agricultural Research (TIAR). *Lotus* spp. and *Biserrulla pelecinus* seed was supplied by the CRS.

9.2.6 Planting and inoculation

As previously described, three species/accessions were planted in each pot, with five seeds per species/accession. A solution of 1 % sucrose (~10 ml) was used to wash rhizobial cultures off the ½ LA plates into a stock solution of 1 % sucrose solution (~50 ml). Approximately 3 ml of this solution was applied to seeds in each pot using a sterile syringe. The seeds were then covered lightly with sand. The sand surface in all pots was covered with a thin layer of alkathene beads to reduce the potential for airborne contamination and evaporation. All plants (excluding nitrogen free controls) received a 'pulse' of nitrogen (20 mls of a 5 mM solution of KNO_3) at the start of the experiment (see Appendix 1, +nitrogen solution).

9.2.7 Maintenance of Experiment

The plants were grown in a temperature controlled (22 ^{o}C) glasshouse. All pots were watered every second day with a nitrogen free (-nitrogen) nutrient solution (see Appendix 1) and then daily as the plant requirements increased. Each received the same nitrogen free solution apart from the +nitrogen controls, which had potassium nitrate and ammonium nitrate included in the solution. Plant numbers were reduced to three per pot after five weeks, where, three plants with similar vigour were selected.

9.2.8 Harvesting and Analysis

Two of the three remaining plants (the third was discarded) were harvested after ten weeks of growth. The whole plants were carefully excavated and the roots were scored for nodulation, nodule size, position of nodules and nodule colour. Dissected nodules were rated for their colour and effectiveness: B – Black (Ineffective), G – Green, LG – Light Green, P – Pink, PR – Pink/Red (Effective). Ps – Pseudo nodules (Yates pers. comm., 2001). The shoots were dried at 70 ^{o}C for two days and relative yields determined.

Four rhizobial strains and two *Dorycnium* spp./accessions were selected from this experiment to undergo evaluation in the field in the form of a 'cross-row' experiment as described by Howieson *et al.* (2000b). The DM (dry matter) yield of replicates from this experiment with these rhizobia/species combinations were bulked (due to very small quantities) and were sent to Feedtest Laboratories (Hamilton, VIC) for

total nitrogen analysis. This was undertaken using the rapid nitrogen combustion method.

The DM data was analysed using analysis of variance (ANOVA) to determine least significant difference (LSD at 5 %) with SAS (SAS Institute, 1991).

9.3 Results:

The experimental arrangement for evaluating the effectiveness of various strains on the selected *Dorycnium* spp. host legumes is shown in Figure 9.1. The differences in shoot growth of the plants provides an indication of the effect of the inoculants on DM production.

Figure 9.1 *Dorycnium* spp. with rhizobia treatments after 10 weeks of growth.

Although no inoculant was uniformly outstanding in fixing nitrogen with all plant genotypes of *Dorycnium,* WSM2338 was found to have the highest DM production per plant for 5 of the 6 *Dorycnium* spp. host accessions (see Table 9.2) (assuming all of the *D. pentaphyllum*/inoculant combinations had the highest and lowest DM production). The commercial *L. corniculatus* inoculant SU343 produced the highest DM yield for 4 of the 6 *Dorycnium* spp., however it appeared poorly effective on *D. rectum* genotype TAS135. Inoculant CC856 originally isolated from *Anthyllis vulneraria* nodulated and fixed nitrogen with all representatives of the *Dorycnium* genus. The rhizobial strains used to inoculate *D. pentaphyllum* did not have a significant (P<0.05) affect on DM production when compared to the control plants (see Table 9.2).

Table 9.2 *Dorycnium* spp. and rhizobial strain interactions grouped according to DM production (LSD P<0.05).

Treatment	***D. pentaphyllum* TAS2309**	***D. pentaphyllum* TAS1272**	***D. rectum* TAS135**	***D. rectum* TAS1274**	***D. hirsutum* TAS1002**	***D. hirsutum* TAS2001**
Control	NS	NS	D	B	DE	C
Nitrogen	NS	NS	AB	B	A	C
CC801	NS	NS	D	B	E	C
CC829	NS	NS	D	A	DE	C
CC856	NS	NS	BCD	AB	ABC	B
SU343	NS	NS	D	AB	BCD	A
WSM1284	NS	NS	ABC	AB	BCDE	B
WSM1293	NS	NS	BCD	B	CDE	B
WSM2323	NS	NS	CD	AB	AB	B
WSM2337	NS	NS	ABCD	AB	BCD	B
WSM2338	NS	NS	A	AB	AB	B
WSM805	NS	NS	BCD	B	DE	C

Key: Treatments with the same letter are not significantly different (P<0.05). NS – Not significantly different (P>0.05).

The ranking of strains based on LSD values revealed that the performance of strains varied greatly with the host. WSM2338 had the best overall performance of the treatments examined with CC801 having the lowest (see Table 9.3). The ranking of strains from highest to lowest was as follows, WSM2338, WSM1284=WSM2323=WSM2337=CC856, WSM1293, SU343=WSM805=CC829, control, and CC801. There were a number of strains that appeared to be equally effective when an overall view was taken.

Table 9.3 Rhizobial strain ranking based on LSD values for DM production.

Treatment/ Strain	***D. rectum* TAS135**	***D. rectum* TAS1274**	***D. hirsutum* TAS1002**	***D. hirsutum* TAS2001**	**Average Rank**	**Overall Rank**
Control	7	3	7	3	5	6
Nitrogen	2	3	1	3	2.25	2
CC801	7	3	8	3	5.25	7
CC829	7	1	7	3	4.5	5
CC856	5	2	3	2	3	3
SU343	7	2	4	1	4.5	5
WSM1284	3	2	5	2	3	3
WSM1293	5	3	6	2	4	4
WSM2323	6	2	2	2	3	3
WSM2337	4	2	4	2	3	3
WSM2338	1	2	2	2	1.75	1
WSM805	5	3	7	3	4.5	5

Note: 1 = highest ranked, higher the number, the lower the ranking. The overall ranking was based on the average rankings.

All inoculants achieved nodulation (see Table 9.4), although the symbiotic combination of *D. pentaphyllum* TAS1272/CC829 appeared to form pseudo nodules, which were defined as slightly raised opaque bumps on the root surface. Nodulation was abundant on all inoculated treatments, although the colour of nodules was frequently green rather than pink. For example, *D. hirsutum* TAS2001 formed predominately green nodules with all inoculants whilst *D. pentaphyllum* formed only pink nodules with WSM2323 and WSM2337. The low level of nodulation in the nitrogen free and plus nitrogen controls revealed a very low incidence of contamination.

Table 9.4 Average nodule number and colour for *Dorycnium* spp./inoculant combinations.

Treatment	***D. pentaphyllum* TAS2309**	***D. pentaphyllum* TAS1272**	***D. rectum* TAS135**	***D. rectum* TAS1274**	***D. hirsutum* TAS1002**	***D. hirsutum* TAS2001**
Control	2 LG	4 B	1 LG	7 G	1 G	1 G
Nitrogen	5 G	1 B	1 LG	5 LG	1 B	2 LG
CC801	6 G	21 G	10 P	16 LG	14 G	10 LG
CC829	17 G	Ps	8 P	28 P	21 LG	6 P
CC856	18 LG	23 LG	14 P	42 G	16 G	8 P
SU343	18 LG	22 G	6 P	50+ P	13 LG	6 LG
WSM1284	19 G	30 G	31 LG	46 LG	26 G	15 LG
WSM1293	9 LG	18 LG	26 P	34 LG	14 LG	14 LG
WSM2323	26 P	31 G	11 P	46 P	28 LG	10 P
WSM2337	21 P	22 G	15 P	33 P	20 LG	11 P
WSM2338	28 LG	18 LG	18 PR	26 P	14 LG	7 P
WSM805	15 G	29 G	22 LG	27 LG	11 G	11 LG

Key: Effectiveness scale: B – Black (Ineffective), G – Green, LG – Light Green, P – Pink, PR – Pink/Red (Effective). Ps – Pseudo nodules, e.g 5 G = 5 green nodules.

Where the agricultural legumes were paired with their commercial inoculant (marked *), the symbiosis appeared effective (see Table 9.5). *Biserrula pelecinus* was also found to accumulate a high level of DM production with *Lotus* inoculants WSM805 and WSM1293. Importantly, the commercial lotus inoculants SU343 and CC829 were sub-optimal in N-fixation with the annual legumes *L. ornithopodioides* and *B. pelecinus.* A number of strains produced significantly higher DM production over the control plants for all *Lotus* spp. Where non-commercial species were evaluated (e.g. *L. ornithopodioides*) excellent inoculant strains were identified, such as WSM1293 and WSM1284. CC856, the isolate from *Anthyllis vulneraria,* fixed nitrogen with all the species of *Lotus* as well as with *B. pelecinus.*

Table 9.5 *Lotus* and *Biserrula* spp. and rhizobial strain interactions grouped according to DM production analysed using LSD.

Treatment	*L. purs.*	*L. tenu.*	*L. corn.*	*L. pedu.*	*L. orni.*	*B. peli*
Control	D	B/C	D/E	C	C	B/C
Nitrogen	C	B/C	D	B/C	C	B
CC801	D	C	E	C	A	B/C
CC829	A	C	E	A*	C	C
CC856	D	A/B/C	B/C	C	C	B/C
SU343	D	A/B/C	A*	C	C	B/C
WSM1284	D	A/B/C	B/C	C	A	A**
WSM1293	D	A/B/C	B/C/D	C	A	A
WSM2323	D	A/B	A/B	C	C	C
WSM2337	D	A	B/C	B/C	C	B/C
WSM2338	B	A/B/C	B/C/D	B	C	B/C
WSM805	D	B/C	C/D	C	B	A

Key: *L. purs = Lotus purshianus, L. tenu = L. tenuis, L. corn = L. corniculatus, L. pedu = L. pedunculatus, L. orni. = L. ornithopodioides, B. peli = B. pelicinus.*
* Indicates commercial inoculant for that species ** Indicates experimental inoculant
Treatments with the same letter are not significantly different (P>0.05).

Lotus ornithopodioides formed pseudo nodules with the commercial lotus inoculant SU343 and also with WSM2338 whilst *Lotus tenuis* formed psuedo nodules with WSM1284 and WSM2323 (see Table 9.6). There was a substantial level of nodulation in the control treatments for *L. purshianus. Lotus corniculatus* formed effective nodules with SU343 and WSM2323. *Lotus pedunculatus* formed effective nodules with WSM2338, and *B. pelecinus* with WSM1284. All *Lotus* spp. and *B.*

pelecinus formed nodules with at least one of the selected strains, however, the effectiveness appeared to vary greatly between host/rhizobia combinations.

Table 9.6 Average nodule number and colour for *Lotus* and *Biserrula* spp. with selected rhizobial strains.

Treatment	*L. purs.*	*L. tenu.*	*L. corn.*	*L. pedu.*	*L. orni.*	*B. peli*
Control	24 P	NNF	7 LG	NNF	NNF	2 G
Nitrogen	25 LG	1 G	4 G	NNF	1 B	5 LG
SU343	50+ P	12 G	24 P*	4 G	Ps	3 G
WSM1284	33 LG	Ps	16 LG	Ps	39 LG	33 P**
WSM2323	50+ P	Ps	41 P	1 G	NNF	NNF
WSM2338	26 LG	50+ LG	19 LG	29 PR	Ps	12 G

Key: *L. purs = Lotus purshianus, L. tenu = L. tenuis, L. corn = L. corniculatus, L. pedu = L. pedunculatus, L. orni. = L. ornithopodioides, B. peli = B. pelicinus.* Effectiveness scale: B – Black (Ineffective), G – Green, LG – Light Green, P – Pink, PR – Pink/Red (Effective). Ps – Pseudo nodules, NNF – Nodule not formed, e.g 4 G = 4 green nodules. * Indicates commercial inoculant for that species, ** Indicates experimental inoculant for that species

9.4 Discussion

9.4.1 *Dorycnium* spp./Rhizobia Interactions

The non-significant (P>0.05) differences in plant DM production between control plants and plants provided with +nitrogen nutrient solutions suggests that provision of nitrogen and/or nutrients was at suboptimal levels for *D. rectum* and *D. hirsutum* (see Table 9.2). This was also observed on other *Dorycnium* spp. accessions. The non-significant (P>0.05) DM production differences in *D. pentaphyllum* with the range of inoculants used suggest that the strains were not suitable inoculants or there were not any superior strains. Another possible reason for this result is the slower growth rate of *D. pentaphyllum* relative to the other species. Therefore, there may not have been sufficient time (10 weeks) to exhibit DM yield differences in response to the different inoculants.

All of the control plants for the three *Dorycnium* spp. were observed to be subject to low level contamination. However, the relatively low number of nodules formed appeared to be ineffective due to their colouration and should not have influenced DM production of these plants to any great extent.

Dry matter production for *D. rectum* TAS135 was significantly (P<0.05) greater with the inoculants WSM1284, WSM2337 and WSM2338. The DM production was significantly (P<0.05) greater than that observed with SU343, which suggests that these strains are potentially superior inoculants. The +nitrogen controls also performed as well as these strains suggesting that the provision of nitrogen to these plants was sufficient and the nodulated plants were fixing effectively. Nodule colour for these host/rhizobia combinations, also suggested effective nitrogen fixation was occurring.

Dorycnium rectum TAS1274 was effectively nodulated with a range of inoculants and produced significantly (P<0.05) greater amounts of DM with SU343, WSM1284, WSM2323, WSM2337, CC829, CC856 and WSM2338. Nodule colours suggest that these were effective interactions, in particular that with SU343. SU343 is a suitable inoculant for *D. rectum* TAS1274, however, a number of alternatives are also suitable.

Dorycnium hirsutum TAS1002 had greater (P<0.05) DM production when inoculated with WSM2323, CC856, and WSM2338. These results suggest that these strains are superior alternatives to SU343. The +nitrogen controls also performed as well as these strains which suggested that nitrogen supply was optimal by both the provision via nutrient solution and nitrogen fixation. However, the nodule colours observed for the inoculated plants revealed that the host/rhizobia interactions were not as effective as they potentially could be. The effective interaction with CC856 supports the findings by Brockwell and Neal-Smith (1966) that *D. hirsutum* inoculated with *Rhizobium* isolated from *Anthyllis vulneria* was found to fix sufficient nitrogen.

Dorycnium hirsutum TAS2001 had significantly (P<0.05) greater DM production with SU343 than any other treatment. However, the colour and number of the nodules produced suggested that the interaction was not as effective as some of the other inoculants.

The significant (P<0.05) differences observed in DM production for the host/rhizobia combinations examined suggested that alternative strains to SU343 may be more suitable inoculants. Therefore, a range of alternative strains could be used to

inoculate *Dorycnium* spp. for optimum DM production and nitrogen fixation. However, these inoculants need to be assessed under field conditions to ascertain suitability when exposed to inherent environmental conditions.

9.4.2 Validation of Experimental Methods

This experiment included three host/rhizobia combinations where a commercial or experimental inoculant was associated with its intended host, *L. corniculatus*/SU343, *L. pedunculatus*/WSM166 and *B. pelicinus*/WSM1284. Effective interactions were observed with all three combinations with highest DM productions observed. These observations demonstrated the suitability of strains selected for these legumes and that the experimental method was effective in analysing nitrogen fixation potential of host/rhizobia interactions.

9.4.3 Rhizobial Promiscuity

The nodulation data as shown in Table 9.4 revealed that *Dorycnium* spp. are susceptible to nodulation from a broad range of root nodule bacteria. All host/rhizobia combinations resulted in nodule formation, however, the effectiveness of the interactions varied, demonstrating the importance of rhizobia selection for optimising nitrogen fixation.

The formation of nodules with all host/rhizobia combinations also demonstrated the promiscuity of the root nodule bacteria examined. The interactions of *Dorycnium* spp. with root nodule bacteria associated with *Lotus* spp. is not unexpected due to the plants similarities taxonomically, however, interactions with the root nodule bacteria associated with *Anthyllis vulneria* and *B. pelicinus* was unusual. This displays the promiscuity of the root nodule bacteria in question, and the susceptibility of *Dorycnium* spp. to infection via root nodule bacteria, in particular *D. rectum* TAS1274.

9.4.4 Cross Genus Rhizobial Interactions

This experiment has provided further information on the cross-nodulation characteristics of rhizobia from *Dorycnium, Lotus, Biserrula and Anthyllis* genera. They complement previous studies by incorporating representatives of the above genera that have contemporary agricultural relevance and for which effective

commercial inocula must be developed. The nodulation of *Dorycnium* by rhizobia from *B. pelecinus*, which is in the tribe Galegeae, extends the findings of Brockwell and Neal-Smith (1966) who commented on the unusual nodulation of this genus by rhizobia isolated from a taxonomically diverse group of legumes.

9.4.5 Selection of Rhizobia for Field Evaluation

The results of this experiment combined with statistical analysis allowed for the ranking of inoculants using LSD values (see Tables 9.2 and 9.3). This provided a means of ranking the overall performance of inoculants and a means for selection for further field based evaluation. The selection of strains was for the establishment of a 'cross row' field experiment, which would compare SU343 along side three alternative inoculants (WSM2338, WSM1284 and WSM2323) and a control.

Based on previous field experiments examining the agronomic characteristics of *Dorycnium* spp. and accessions, *D. rectum* TAS1274 and *D. hirsutum* TAS1002 had been identified as possessing the greatest potential. Therefore strain selection was not only based upon ranking, but interactions with these *Dorycnium* spp. accessions. Inoculant WSM2338 was selected due to the relative DM production (see Table 9.3). Inoculants WSM1284 and WSM2323 were selected over WSM2337 due to their interactions with the selected *Dorycnium* spp. accessions.

The overall performance of inoculants WSM1284, WSM2338, SU343 and WSM2323 with the six *Dorycnium* spp. hosts formed the basis for selection to undergo further evaluation in the field.

9.4.6 Negative Host/Rhizobia Interactions

Stastical analysis of DM production data (see Table 9.2) and nodulation data (see Table 9.4), revealed that there are a number of possible detrimental interactions that could occur between the pasture legumes examined and the strains selected for field examination (SU343, WSM1284, WSM2323, and WSM2338) if introduced into agricultural systems. All of the interactions where nodules were formed and the DM production groupings were not 'A' could be considered to be negative interactions and could possibly compromise production from the legumes concerned.

In consideration of negative interactions between hosts and non-target root nodule bacteria in the soil system, factors such as host selectivity and competitiveness of the introduced strain need to be considered. Brockwell *et al.* (1995) stated that in a mixed population the more effective strains are generally more competitive and the host legume appears to exhibit some selective preference. This assertion requires some validation at the host genus level.

9.4.7 Effectiveness of Nodules

A number of interactions of the pasture legumes with selected strains produced pink, seemingly effective nodules, however this was not manifested in increased plant DM production (e.g *L. purshianus*/SU343 combination). Somasegaran and Hoben (1994) suggested that nodules with a pink colour infers an effective nodule, whereas white or greenish nodules infer ineffective symbiosis. The results suggested that interactions that are considered to be 'effective' may not necessarily confer a beneficial plant response although it is possible that the growing environment in the glasshouse was not optimal for all the plants evaluated in this experiment. For example, the non-significant ($P>0.05$) DM production differences between the inoculants on *D. pentaphyllum* suggest that the provision of nitrogen and/or nutrients was at suboptimal levels or not delivered effectively to this species, hence, growth was potentially impaired.

9.4.8 Evaluation of rhizobial strains for nitrogen fixation

Based upon the glasshouse results one of the Australian commercial *Lotus* spp. inoculants (SU343) would be an effective strain for the inoculation of some accessions of *Dorycnium* spp. However, SU343 fixes nitrogen poorly with several annual legumes of agricultural relevance, such as *B. pelecinus*. This may present a problem for inoculant usage on soils where these legumes are likely to be used together. Planting a field with *L. corniculatus* or *Dorycniuym* spp. inoculated with SU343 may preclude future effective nodulation of either *L. ornithopodioides* or *B. pelecinus*. The performance of alternative strains such as WSM1293, WSM1284 and WSM2338 across *Dorycnium* spp. hosts and more broadly across the other agricultural legumes examined suggests they would be useful commercial alternatives to SU343. In this context, the formation of psuedo nodules by both WSM2338 and SU343 on *L. ornithopodioides,* which is a species with considerable

agronomic potential, is notable. The broad host-range of WSM1284 on legumes such us *D. hirsutum* has been previously reported (Nandasena *et al*., 2001) and suggests this strain could be a candidate for future genetic and physiological studies of nodulation and nitrogen fixation. The ability of isolates from *Anthyllus vulneraria* to nodulate *D. hirsutum* has previously been reported (Brockwell and Neal-Smith 1966) and we extend these observations to include the additional two *Dorycnium* spp., *D. rectum* and *D. pentaphyllum*.

In commercial terms, the use of a single broad-spectrum rhizobial strain is more economically viable than the development of a number of specific inoculants. However, this requires the selection of elite strains that combine the desired host infection and nitrogen fixation characteristics with adaptation to the target soils of the multiple hosts. This is not a menial task. The further development of the broad-host range strains identified in this research as potential inoculants for *Lotus, Dorycnium* and *Biserrula* would require further ecological investigation of their behaviour in soil.

9.5 Conclusions

The results of this experiment suggested that SU343 is a suitable inoculant for some *Dorycnium* spp. The Tasmanian isolates WSM2338, WSM2323 and *Biserrula* experimental inoculum WSM1284 were identified as complementary inoculant for the broad-spectrum inoculation of *Dorycnium* spp. However, further evaluation is required under field conditions to further support or negate these findings. This experiment has highlighted the importance of the selection and evaluation process in terms of identifying superior strains, negative host/rhizobia interactions and the interaction between host, rhizobia and soil environment.

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Chapter 10. Field Evaluation of Rhizobia

10.1 Introduction:

The completion of the rhizobia selection and evaluation experiment in the glasshouse described in Chapter 9 led to the next stage of the evaluation process which involved field evaluation via a 'cross row' experiment (Howieson *et al.,* 2000a).

Four of the ten rhizobial strains used in the glasshouse experiment were selected to undergo evaluation in the field. The selection of these four strains was based on the overall performance of these strains on all three *Dorycnium* spp. examined. The low DM production of *D. pentaphyllum* and the non-significant (P>0.05) effect of rhizobial inoculants on DM production in the glasshouse experiment led to this species not being evaluated in the field.

The overall aim of this experimental work was to continue the rhizobial selection and evaluation process to identify suitable rhizobial strains for the inoculation of *Dorycnium* spp in the field. Rhizobial strains identified in the glasshouse trial as suitable or potentially effective inoculants were used in a cross row field trial. The cross row trial aimed to examine the effectiveness of inoculants under Tasmanian field conditions in relation to the persistence of inoculants and the potential for nitrogen fixation. Molecular techniques were employed in conjunction with the cross row trial which aimed to identify background populations of rhizobia and the survival and spread of introduced inoculants.

10.2 Materials and Methods

10.2.1 Cross Row Experiment - 1st Planting (Inoculated Rows)

The experiment was designed so that treatments were imposed in quadruplicate in a randomised complete block design with four rhizobial strains (and an un-inoculated control) and two *Dorycnium* spp. hosts. The hosts were *D. hirsutum* TAS1002 and *D. rectum* TAS1274. Rhizobial strains were SU343, WSM1284, WSM2323 and WSM2338.

A sandy loam site (13 m x 15 m) was selected on the University of Tasmania Farm, Cambridge (E534500, N5260500, GDA 94) for a 'cross row' field experiment as described by Howieson *et al.* (2000b). The trial site prior to establishment was in its second rotation of oats, which were removed by hand. The surface was lightly cultivated and rolled, followed by an application of 100 kg/ha of 0,7,11,9 Impact Fertiliser (6.9 % phosphorus, 11.1 % potassium chloride, 8.6 % sulphate W/W). Seed beds were established to a depth of approximately 1.5 cm and were 1.5 m long.

Seeds of the selected hosts were surface-sterilised by soaking in 70 % Ethanol for 2 minutes, and then coated with a mixture of methocel, lime and inoculated peat containing a specific rhizobial strain. Plate counts (from serial dilutions of broths) on the peat/inoculants revealed that rhizobial numbers were in the order of 1 x 10^{10} cfu/ml. Seeds of *D. rectum* and *D. hirsutum* were sown at 0.8 and 1.0 g per 1.5 m respectively. All seed preparation and planting was undertaken using strict sterile methods to reduce the chance of external contamination. Planting occurred in December (2001).

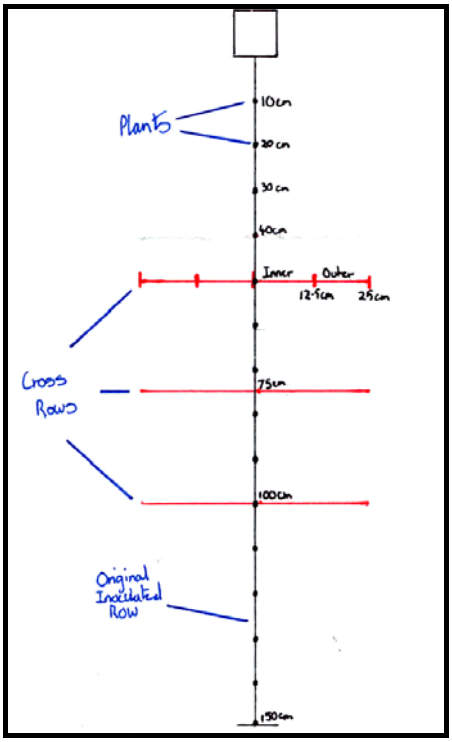

Figure 10.1 Diagram of the design of the cross row experiment. The original inoculated row is cross planted by the uninoculated 'cross' rows.

Figure 10.1 shows the basic structure of an individual treatment row with cross rows located at 50, 75 and 100 cm. Figure 10.2 below displays the layout of the cross row experiment in relation to species, inoculant and replicates.

Replicate 1	**Replicate 2**	**Replicate 3**	**Replicate 4**
1 DR WSM1284	2 DR SU343	3 DR SU343	4 DR WSM1284
1 DR WSM2338	2 DR WSM2338	3 DR WSM1284	4 DR WSM2338
1 DR CONTROL	2 DR WSM2323	3 DR WSM2338	4 DR SU343
1 DR SU343	2 DR CONTROL	3 DR WSM2323	4 DR WSM2323
1 DR WSM2323	2 DR WSM1284	1 DR CONTROL	4 DR CONTROL
Reference Plants	**Reference Plants**	**Reference Plants**	**Reference Plants**
1 DH WSM2323	2 DH SU343	3 DH WSM1284	4 DH SU343
1 DH CONTROL	2 DH CONTROL	3 DH WSM2338	4 DH WSM2323
1 DH WSM1284	2 DH WSM2338	3 DH SU343	4 DH WSM1284
1 DH WSM2338	2 DH WSM2323	3 DH WSM2323	4 DH WSM2338
1 DH SU343	2 DH WSM1284	3 DH CONTROL	4 DH CONTROL

Figure 10.2 Design of cross experiment – 1st planting, with species and inoculation treatments indicated. **Key:** 1 DR WSM1284 = replicate **1,** *D. rectum* **DR,** inoculant **WSM1284.**

The trial site was generally watered twice (depending on rainfall) a week and weeded on a regular basis. Plants were thinned to one plant per 10cm eight weeks after sowing. Care was taken to limit movement within the site and any soil disturbance, however it was endeavoured to keep the site clean of weeds for the majority of the trial period.

Five months after planting, the plant shoots were harvested at ground level and dried at 60 ^{o}C for two days and relative yields determined on a dry matter (DM) basis per plant. Representative samples of the DM produced (~10 grams) were ground to <1 mm for total nitrogen analysis using NIRS (Feedtest, Hamilton Victoria) and for ^{15}N using the ^{15}N natural abundance method (Murdoch University, Western Australia). Samples of two non-nitrogen fixing non-leguminous reference plants were also sampled. These were a grass (*Phalaris minor* Retz.) and a broadleaf weed (*Chenopodium album* L.) replicated four times. The reference plants were grown in a 1 metre wide strip between the *D. rectum* and *D. hirsutum* plots i.e where replicates 1-4 are written in Figure 10.2.

The percentage nitrogen fixed and amount of nitrogen fixed were calculated using the formulas described in Equations 10.1 and 10.2 (Peoples, pers.comm., 2003):

Equation 10.1: $$\% \text{ Nitrogen Fixed} = \frac{100 \times (^{15}\text{N control} - {}^{15}\text{N legume})}{(^{15}\text{N control} - {}^{15}\text{N fully fixing legume})}$$

Note: A reference value for the fully fixing *Dorycnium* spp. plant was not available and so a notional value of –1.0 was used. The ^{15}N control value was based upon an average of the ^{15}N grass and broadleaf averages, which was 6.26.

Equation 10.2: Amount of Nitrogen Fixed (g/pl.) = DM/pl. x (%N/100) x (%N fixed/100)

Data presented as percentages were converted to arcsin values for analysis. The results were analysed using SAS with analysis of variance (ANOVA) and least significant differences (LSD) at 5% (SAS Institute, 1991).

10.2.2 Cross Row Experiment - 2nd Planting (Cross Rows)

Following a ley period of approximately three months, two cross rows were planted across each original planted row in late August (2002). Surface sterilised seed was planted at right angles to the original planted rows (see Figure 10.1). The cross rows were 50 cm in length and therefore were planted 25 cm either side of the original row. The rows crossed the original row at 50 cm and 100 cm along the length. A number of these cross rows failed to establish, so a second planting was undertaken late in October (2002).

The experimental site was maintained as described for the 1st planting, however, a broad spectrum herbicide (glyphosate) was applied via spot spraying (and shielding *Dorycnium* spp. plants) to control weeds. The experimental site was generally watered twice a week (depending on rainfall).

The cross rows were harvested in February 2003. Cross rows were divided into inner (0 – 12.5 cm) and outer (12.5 – 25 cm) sections for analysis (see Figure 10.1). The plants were cut off at ground level and dried at 70 ^{o}C for two days and then weighed

for DM yield determination. The DM yield was determined on a per plant per day degrees basis due to the secondary cross row planting and sampling occurring from both early and late plantings.

Following the removal of the above ground plant material the roots were carefully washed free of soil and examined for nodulation. Nodules found on the roots were scored for approximate number, size, position and colour. Nodules were removed, desiccated and stored at room temperature for future identification.

The results were analysed using SAS and analysis of variance (ANOVA) with least significant differences (LSD) at 5% (SAS Institute, 1991).

10.2.3 Nodule Isolate Identification

Nodules harvested from *D. hirsutum* and *D. rectum*, 1st and 2nd replicates of the cross row experiment were selected to undergo strain identification using PCR and gel band matching. Desiccated nodules were rehydrated by immersion in sterile distilled water for four hours. The nodules were surfaced sterilised with 70 %(v/v) ethanol for 30 seconds followed by 4 %(w/v) $NaHClO_4$ for 60 seconds, and then washed six times in sterile distilled water. Nodules were crushed individually into one drop of sterile water, and then plated out onto ½ LA agar (see Appendix 2) and incubated at 28 ^{o}C. Single colonies slightly raised and opaque were selected for identification. The four inoculants used in the cross row experiment were also cultured as type strains, WSM2338, SU343, WSM1284 and WSM2323.

Prior to undertaking the main PCR analysis, primers ERIC (De Buijun, 1992) and RPO1 (Richardson *et al.,* 1995), were examined for their effectiveness on the type strains. Four culture preparations of each were also examined with the two primers, with optical densities at 600 nm (OD_{600}) of one, two and four along with pure cells. An OD_{600} of two was selected for the main analysis along with the primer RPO1 (see Results and Discussion for selection of OD and primer). Isolates were prepared by diluting cells in saline (0.89% NaCl), centrifuging at 14,000 RPM for 2 minutes and removing the supernatant. The cells were then resuspended in saline, absorbances read (600 nm), and samples diluted (saline) further to an OD_{600} of two.

Richardson *et al.* (1995) designed the primer RPO1 (5'-AATTTTCAAGCGTCGTGCCA-3'). A reaction mixture was prepared for the isolates to undergo the PCR. The reaction mixture consisted of 11.1µL Miliq (sterile filtered) water, 4 µL 5x PCR polymerisation buffer, 2.4 µL 25 mM $MgCl_2$, 1 µL of 50 mM RPO1 primer, 0.5 µL Taq DNA (*Tth* plus DNA polymerase), and 1 µL of cell suspension (or loop full of cells for pure cells methods). 20 µL of reaction mixture was subject to the following PCR cycle conditions, 94 ^oC for 5 min, 5 cycles of (94 ^oC for 0.3 min, 50 ^oC for 0.2min, 72 ^oC for 1.3min), 35 cycles of (94 ^oC for 0.05 min, 55 ^oC for 0.24 min, 72 ^oC for 1.3 min), 5 cycles of (94 ^oC for 0.1 min, 55 ^oC for 0.2 min, 72 ^oC for 5 min), and hold at 15 ^oC.

De Buijun (1992) designed the primers ERICF (5'-AAGTAAGTGACTGGGGTGAGCG-3') and ERIC R (5'-ATGTAAGCTCCTGGGGATTCAC-3'). A reaction mixture was prepared for the isolates to undergo the PCR. The reaction mixture consisted of, 14.5 µL Miliq water, 5 µL 5x PCR polymerisation buffer, 2 µL 25 mM $MgCl_2$, 1 µL 50 mM ERICF primer, 1 µL 50 mM ERICR primer, 0.5 µL Taq DNA (*Tth* plus DNA polymerase), and 1 µL cell suspension (or loop full of cells for pure cells method). 25 µL of the reaction mixture was subject to the following PCR cycle conditions, 94 ^oC for 5 min, 35 cycles of (94 ^oC for 1 min, 52 ^oC for 1 min, 65 ^oC for 5 min), 65 ^oC for 5 min, and hold at 15 ^oC.

Agarose (1%) (Sigma) gels were prepared and buffered with Tris-acetate-EDTA (TAE). Samples were dyed with Blue/Orange 6X loading dye (Promega) and placed in the gel wells, along with four reference strains in each, and a Promega 1kb DNA ladder at each end of the gels. The gels were run at 100 volts for 4-5 hours, then stained for 0.5 hour in ethidium bromide (10 µg/ml) solution, after which, gels were de-stained in DDi H_2O for 20 min. DNA bands were photographed using the Geldocumentation system (BIORAD Gel Doc 2000) under UV light.

Photographed gels were analysed using two software packages, Phoretics™ Advance and Phoretics™ Database, and Gene Profiler™ 1-D Gel Analysis. Gels were analysed with an RF value of 150 and NR of 3 using Phoretics™ Advance and a

Tolerance of 390 with Phoretics™ Database. A confidence tolerance of 95 % was used in gel analysis with Gene Profiler™.

10.3 Results:

10.3.1 Soil Characteristics – Brown Kurosol

The soil at the University farm experimental site is known locally as the Radar loamy sand (Brown Kurosol) and is a strong texture contrast soil. This soil type is strongly duplex featuring light grey-brown deep sand to sandy loam surface above a medium clay at 30-70 cm depth. This soil type is acidic with a pH (1:5 0.01M $CaCl_2$) of 5.6. The cation exchange capacity (CEC) is 4.1 Cmol $^{(+)}$/Kg. The percentage organic carbon (OC) and clay content was 2.1 and 4.9 % respectively.

10.3.2 Cross Row Experiment - 1st Planting (Inoculated Rows)

Plant growth was rapid throughout the summer and early autumn. Unusually regular summer rain and irrigation ensured an excellent establishment of plants and subsequent growth. Plant growth slowed towards the end of autumn, at which time, the plants were harvested.

Figure 10.3 is a photograph taken of replicates three and four of the inoculated and control *D. rectum* plants after three months of growth. The difference in DM production can be seen between rows and the effect of inoculation. The inoculated rows had noticeably greater DM production than uninoculated plants.

Figure 10.3 *Dorycnium rectum* inoculated rows after three months growth.

There were significant (P<0.05) differences in DM production for *D. rectum* with the selected inoculants, whereas, there was no significant (P>0.05) difference for *D. hirsutum* (Figure 10.4 and Table 10.1). *Dorycnium rectum* inoculated with WSM1284, SU343 and WSM2338 had the highest DM production. The control plants for *D. rectum* had the lowest average DM per plant.

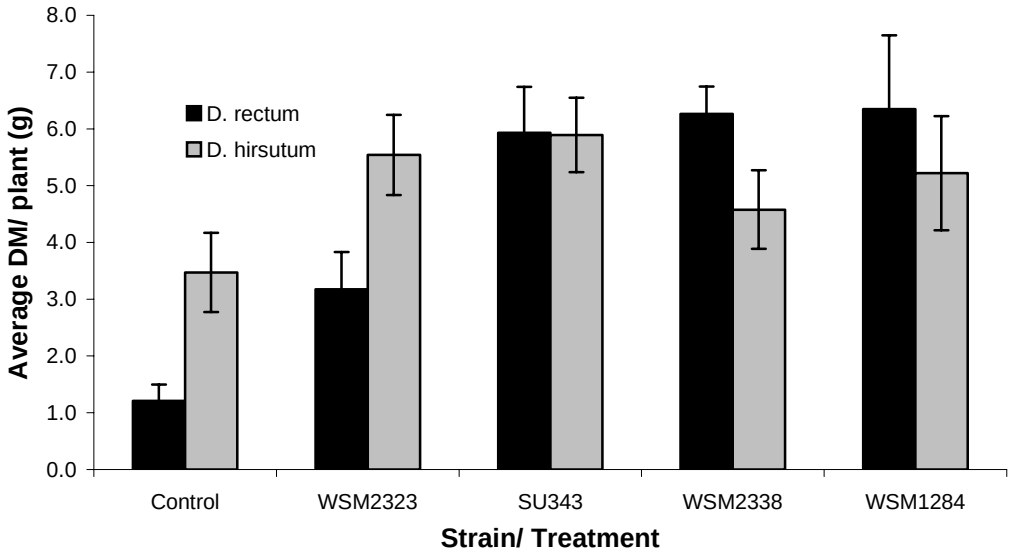

Figure 10.4 Dry matter production of *Dorycnium* spp. inoculated with various rhizobial strains under field conditions. Vertical bars are standard deviations.

The average DM production per plant for *D. rectum* and *D. hirsutum* varied greatly within experiments, and between the glasshouse and field experiments (see Table 10.1). Under glasshouse conditions the DM produced by *D. hirsutum* was far greater than that of *D. rectum*, however, the average DM produced per plant under field conditions was relatively similar between the two species. The average DM per plant for *D. rectum* with the various rhizobial treatments was not significantly different ($P>0.05$) in the glasshouse, whereas, in the field there were significant ($P<0.05$) differences. *Dorycnium rectum* inoculated with SU343, WSM1284 and WSM2338 had significantly ($P<0.05$) higher DM per plant under field conditions. In the glasshouse there was no significant ($P>0.05$) difference between inoculants on *D. hirsutum*, however, there was a significant ($P<0.05$) difference between inoculants and the control. In the field there was no significant ($P>0.05$) difference between all treatments for *D. hirsutum*.

Table 10.1 Average DM production for *Dorycnium* spp. inoculated with various rhizobial strains, under glasshouse and field conditions.

		Glasshouse		Field	
Species	**Treatment**	**DM/plant (g)**	**T**	**DM/plant (g)**	**T**
D. rectum	Control	0.03	NS	1.2	C
D. rectum	SU343	0.04	NS	5.9	A
D. rectum	WSM1284	0.06	NS	6.3	A
D. rectum	WSM2323	0.04	NS	3.2	B
D. rectum	WSM2338	0.05	NS	6.3	A
D. hirsutum	Control	0.18	B	3.5	NS
D. hirsutum	SU343	0.10	AB	5.9	NS
D. hirsutum	WSM1284	0.09	AB	5.2	NS
D. hirsutum	WSM2323	0.16	A	5.5	NS
D. hirsutum	WSM2338	0.16	A	4.6	NS

Key: T – T groupings based on LSD values. Treatments with the same letter within species are not significantly different ($P>0.05$). NS – Not significantly different ($P>0.05$).

The four selected rhizobial strains evaluated under glasshouse and field conditions with *D. hirsutum* and *D. rectum* were analysed for their total nitrogen content (see Table 10.2). The glasshouse samples could not be analysed statistically as the sample replicates (1-3) were bulked due to the relatively small sample weights. The total nitrogen content of *D. rectum* was similar for the glasshouse and field samples, whereas, the total nitrogen content of *D. hirsutum* in the field was much lower than

the glasshouse samples. In general it appeared that *D. rectum* contained a higher percentage of total nitrogen than *D. hirsutum*.

The highest (P<0.05) total nitrogen content for *D. rectum* was found with the inoculants SU343, WSM1284 and WSM2338 under field conditions. *Dorycnium rectum* inoculated with WSM1284 had the highest total nitrogen content in the glasshouse. *Dorycnium hirsutum* inoculated with WSM2323 had a significantly (P<0.05) higher total nitrogen content than any of the other inoculants under field conditions, but there was no significant (P>0.05) difference in total nitrogen content for any of the inoculant strains in the glasshouse.

The grass and broadleaf non-nitrogen fixing controls had the lowest total nitrogen contents of all of the samples analysed when compared with *D. rectum* and *D. hirsutum*. The total nitrogen content of the broadleaf plant samples was significantly (P<0.05) greater than that of the grass samples.

Table 10.2 Total nitrogen contents for *Dorycnium* spp./rhizobia combinations.

		Glasshouse		Field	
Species	**Treatment**	**Total N (%DM)**	**T**	**Total N (%DM)**	**T**
D. rectum	Control	1.95 (+N)	NA	2.4	C
D. rectum	SU343	3.0	NA	3.1	A
D. rectum	WSM1284	3.4	NA	2.8	AB
D. rectum	WSM2323	3.1	NA	2.5	BC
D. rectum	WSM2338	2.7	NA	3.0	A
D. hirsutum	Control	2.51 (+N)	NA	1.6	B
D. hirsutum	SU343	3.2	NA	1.6	B
D. hirsutum	WSM1284	2.8	NA	1.7	B
D. hirsutum	WSM2323	2.8	NA	1.9	A
D. hirsutum	WSM2338	2.8	NA	1.7	B
Grass	Control	NA		0.7	B
Broadleaf	Control	NA		1.4	A

Key: T – T groupings based on LSD values. Treatments with the same letter within species/groups are not significantly different (P>0.05). NA – Not applicable.

The percentage and amount of nitrogen fixed for *Dorycnium* spp. with rhizobial treatments varied greatly (see Table 10.3). There was no significant (P>0.05) difference in percentage nitrogen fixed for *D. rectum*, whereas, the amount of nitrogen fixed by SU343, WSM1284 and WSM2323 was significantly (P<0.05) greater than the other treatments. The percentage nitrogen fixed with *D. hirsutum* was significantly (P<0.05) greater with WSM2323 than SU343 and WSM1284. The controls of both *D. rectum* and *D. hirsutum* displayed evidence of nitrogen fixation.

Table 10.3 Percentage and amount of nitrogen fixed under field conditions by *Dorycnium* spp./rhizobia combinations.

Species	Treatment	Percentage (%) N Fixed	T	N Fixed mgN/plant	T
D. rectum	Control	12.6	NS	3.2	B
D. rectum	SU343	46.8	NS	75.5	A
D. rectum	WSM1284	29.4	NS	38.7	AB
D. rectum	WSM2323	17.2	NS	7.7	B
D. rectum	WSM2338	27.9	NS	45.6	A
D. hirsutum	Control	11.6	AB	6.6	B
D. hirsutum	SU343	6.7	B	9.5	B
D. hirsutum	WSM1284	0.1	B	0.1	B
D. hirsutum	WSM2323	27.3	A	31.5	A
D. hirsutum	WSM2338	11.5	AB	10.6	B

Key: T – T groupings based on LSD values. Treatments with the same letter within species are not significantly different (P>0.05). NS – Not significantly different (P>0.05). Statistical analysis of percentage nitrogen fixed are based on data converted to arcsin values.

The estimations of annual nitrogen fixation per hectare (see Table 10.4) varied greatly between treatments and species. *D. rectum* inoculated with SU343 displayed a nitrogen fixing potential almost eight times greater than *D. hirsutum* inoculated with the same strain. The highest estimation of nitrogen fixation for *D. rectum* was with the SU343 inoculant, and this was the highest estimation for nitrogen fixation for all of the rhizobia/host combinations examined. The highest estimate of nitrogen fixation for *D. hirsutum* was with the inoculant WSM2323. *Dorycnium hirsutum* inoculated with WSM1284 appeared to be an ineffective combination.

Table 10.4 Estimations of the annual amount (per hectare) of atmospheric nitrogen fixed by *Dorycnium* spp. inoculated with various rhizobial inoculants.

Species	Treatment	N Fixed kg/ha/yr
D. rectum	Control	7.9
D. rectum	SU343	184.4
D. rectum	WSM1284	94.5
D. rectum	WSM2323	18.8
D. rectum	WSM2338	111.5
D. hirsutum	Control	16.2
D. hirsutum	SU343	23.3
D. hirsutum	WSM1284	0.3
D. hirsutum	WSM2323	77.0
D. hirsutum	WSM2338	26.0

Note: Estimations of the amounts of nitrogen fixed per hectare, per annum are based on extrapolated values of nitrogen fixed from the cross row experiment (see Table 10.3). The amount of nitrogen fixed occurred over a five month period in 2002 (2415 day degrees), so estimates of nitrogen fixed are based on the total amount of day degrees (4875) that were observed during 2002 at the experimental site and assumes there was adequate soil moisture. Day degrees refers to the cumulative daily maximum temperatures for a specified growth period. Plant density was based on the experimental setup of one plant per 10 cm i.e 121 plants/m^2.

10.3.3 Cross Row Experiment - 2nd Planting (Cross Rows)

For the majority of the rhizobia/*D. hirsutum* combinations the outer sections were observed to have greater average DM production per plant than the inner sections (see Table 10.5). The highest average DM was observed with the outer section of the WSM2323 rows, however, the outer sections of the control rows were very similar. All of the nodules examined appeared to be partially effective to almost fully effective based on cross section colour. The controls were nodulated and appeared to be at least partially effective.

Table 10.5 Average DM production and nodule colours of *D. hirsutum* with rhizobial treatments for inner and outer sections of cross-rows.

	DM/Day Degree/plant (mg)		Average Nodule Colour	
Treatment	**Inner**	**Outer**	**Inner**	**Outer**
Control	1.27	3.26	4.25	3.75
SU343	0.90	1.58	4.00	4.50
WSM1284	0.85	0.76	3.75	3.50
WSM2323	1.46	3.32	4.50	4.50
WSM2338	1.84	2.24	4.00	4.00

Key: Inner cross row represents 0 – 12.5 cm from original row, outer cross row represents 12.5 – 25 cm section from the original row. Nodule colours: 1 – Black (Ineffective), 2 – Green, 3– Light Green, 4 – Pink, 5 – Pink/Red (Effective). **Note:** The DM production of the host/rhizobia combinations is expressed as DM/Day degree due to the fact that cross rows were different ages. Where nodule colours are given as a decimal, this was an average colour.

The cross rows for *D. rectum* displayed a wide range of average DM production per plant, with some of the inner sections greater than outer sections and vice versa (see Table 10.6). The highest observed DM production per plant was with the inner sections of the WSM1284 cross rows. The nodules were observed to be partially effective to effective, based on nodule colour. The control plants were nodulated and appeared to be at least partially effective.

Table 10.6 Average DM production per plant and nodule colours of *D. rectum* with rhizobial treatments for inner and outer sections of cross-rows.

	DM/Day Degree/plant (mg)		Average Nodule Colour	
Treatment	**Inner**	**Outer**	**Inner**	**Outer**
Control	0.60	0.72	3.75	4.00
SU343	2.71	0.37	4.25	4.00
WSM1284	6.12	0.61	3.33	4.33
WSM2323	1.01	0.57	4.25	4.00
WSM2338	0.64	1.54	4.00	4.33

Key: Inner cross row represents 0 – 12.5 cm from original row, outer cross row represents 12.5 – 25 cm section from the original row. Nodule colours: 1 – Black (Ineffective), 2 – Green, 3– Light Green, 4 – Pink, 5 – Pink/Red (Effective). **Note:** the DM production of the host/rhizobia combinations are expressed is DM/Day degree due to the fact that cross rows were different ages. Where nodule colours are given as a decimal, this was an average colour.

The average DM production from combining both inner and outer cross row samples, varied greatly (see Table 10.7), however, there were no significant (P>0.05) differences in DM production per plant between treatments for either species.

Table 10.7 Combined inner and outer sections average DM production per plant for cross rows.

Species	Treatment	DM/Day Degree/plant (mg)	T
D. hirsutum	Control	2.27	NS
D. hirsutum	SU343	1.24	NS
D. hirsutum	WSM1284	0.81	NS
D. hirsutum	WSM2323	2.39	NS
D. hirsutum	WSM2338	2.04	NS
D. rectum	Control	0.66	NS
D. rectum	SU343	1.54	NS
D. rectum	WSM1284	3.37	NS
D. rectum	WSM2323	0.79	NS
D. rectum	WSM2338	1.09	NS

Key: Inner cross row represents 0 – 12.5 cm from original row, outer cross row represents 12.5 – 25 cm section from the original row. **Note:** the DM production of the host/rhizobia combinations are expressed as DM/Day degree due to the fact that cross rows were different ages.

10.3.4 Nodule Isolate Identification

Isolates from recovered nodules were analysed using PCR techniques and software to match gel bands. The isolates were cross matched and compared with reference strains.

Figure 10.5 displays the band separation of the four reference strains using the ERIC primer. The Promega 1kb DNA ladders separated well at either end of the gel, however, there was little or no separation of the reference strain bands.

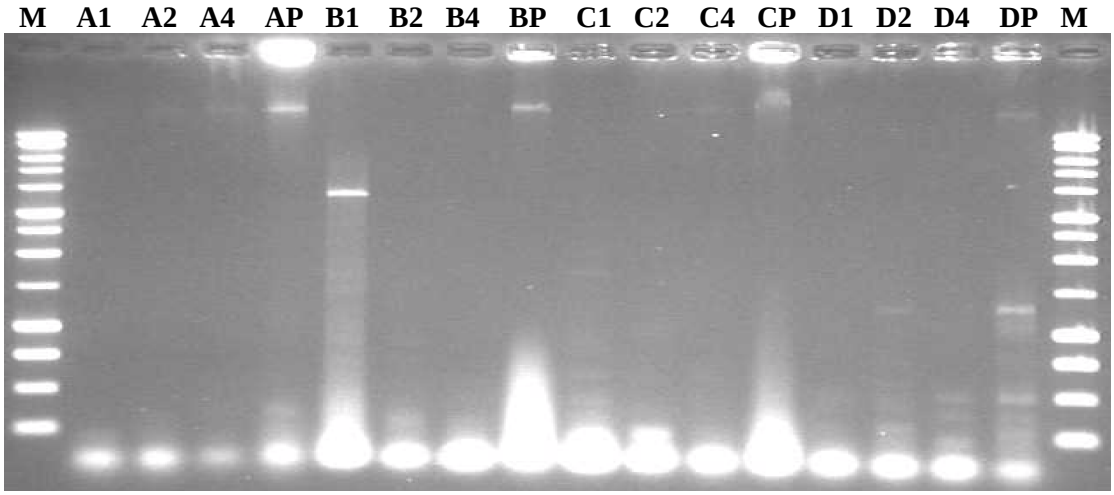

Figure 10.5 Gel band separation of reference strains with ERIC primer.
Key: M – 1kb Promega DNA ladder marker, A – WSM2338, B – SU343, C – WSM1284 and D – WSM2323. 1 = OD_{600} 1, 2 = OD_{600} 2, 4 = OD_{600} 4, P – pure cells.

Figure 10.6 displays the band separation of the four reference strains using the RPO1 primer. The Promega 1kb DNA ladders separated well at either end of the gel, as have most of the reference strain/treatment combinations. Isolates prepared at an OD_{600} of 2 and 4 consistently separated well. The pure cell preparation of SU343 and WSM2323 separated poorly with the RPO1 primer.

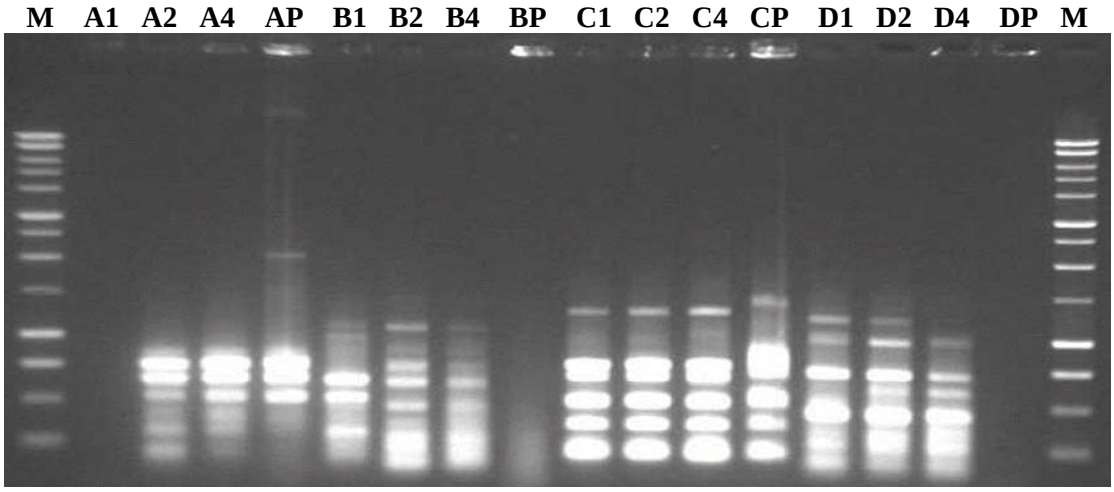

Figure 10.6 Gel band separation of reference strains with RPO1 primer.
Key: M – 1kb Promega DNA ladder marker, A – WSM2338, B – SU343, C – WSM1284 and D – WSM2323. 1 = OD_{600} 1, 2 = OD_{600} 2, 4 = OD_{600} 4, P – pure cells.

The dendrogram displayed that the reference strains used on each gel produced different banding patterns (see Figure 10.7). Phoretics™ Database has matched WSM2323-1 and WSM1284-1 as being 90 % similar. Most reference strains were classified as having a low similarity index, even those of the same origin, but on separate gels.

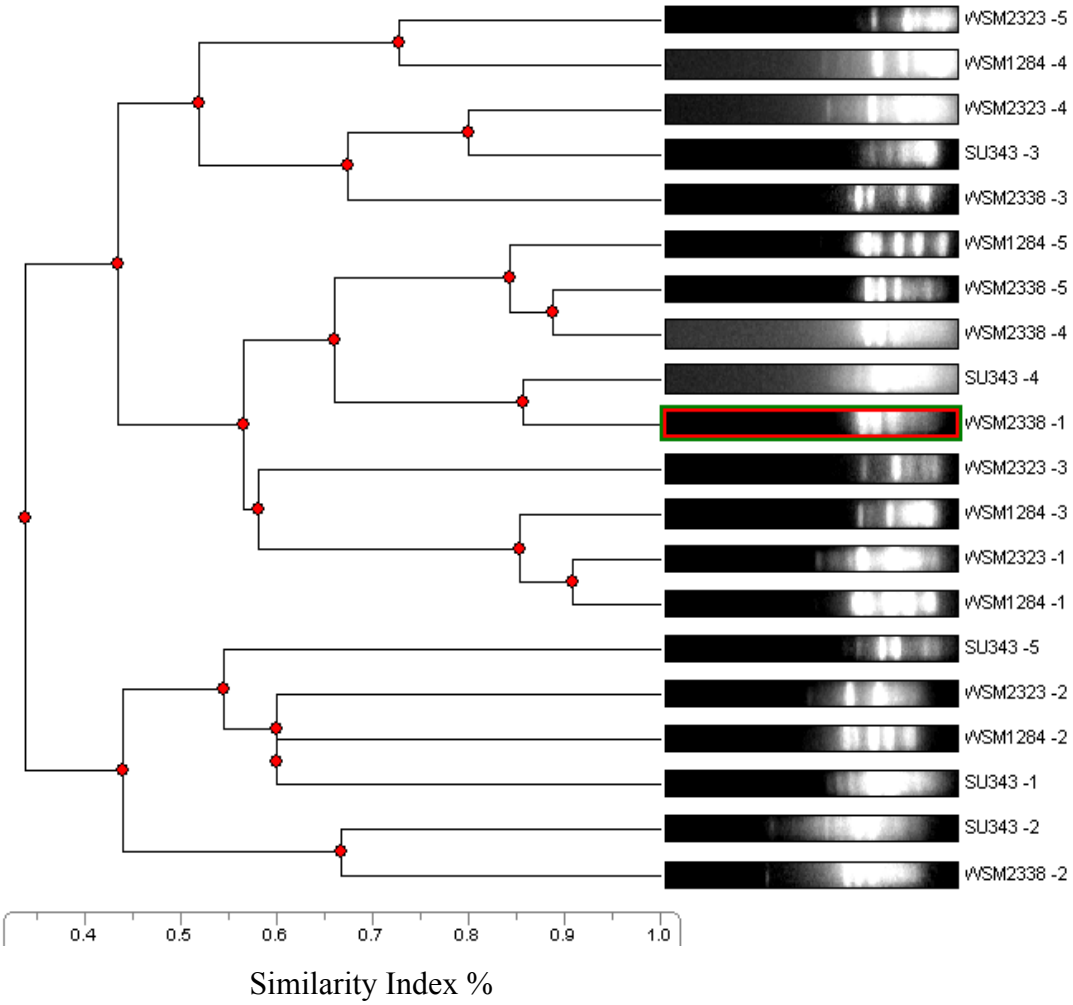

Figure 10.7 UPGMA cluster analysis dendrogram (Phoretics™ Database) for reference strains, WSM2338, SU343, WSM1284 and WSM2323 used as references on the five gels. **Note:** The annotations describe the reference strains and the gel number from which they were captured. The reference strains were from a single source and applied to each gel as a reference source.

The Phoretics™ Advance software matched the Promega 1kb DNA ladders from each gel together with a similarity index of >80 % (see Table 10.8). Two separate isolates cultured from nodules that were removed from replicate 2 *D. hirsutum* WSM2338 Inner were matched as identical (100 %). There were a number of lanes

matched where the strains were neighbouring in the field cross row experiment. A number of lanes were not matched.

Table 10.8 *D. hirsutum* lane matching using Phoretics™ Advance and a threshold of 80% similarity or greater.

Lane	Matched Lane	Similarity Index (%)
2 DH WSM2338 Inner 1	2 DH WSM2338 Inner 2	100*
Promega 1kb-3	Promega 1kb-4	100*
Promega 1kb-5	Promega 1kb-6	100*
Promega 1kb-1	Promega 1kb-2	92*
1 DH Control Outer 5	**WSM1284**	90***
1 DH Control Outer 3	**WSM2323**	88***
2 DH WSM1284 Inner 1	2 DH Control Outer 2	83
2 DH SU343 Outer 2	1 DH Control Outer 5	80
2 DH SU343 Outer 2	**WSM1284**	80
2 DH WSM1284 Inner 1	**WSM2323**	80***
2 DH WSM2338 Inner 3	**WSM1284**	80
2 DH WSM2338 Outer 1	2 DH WSM2323 Outer 4	80***

Key: 1 DH SU343 Outer 3 = Replicate **1**, ***D****orycnium* ***h****irsutum*, Inoculant **SU343**, **Outer** 12.5cm of cross row, isolate **3**. * indicate matches of isolates of the same origin, *** denotes lanes matched where reference strains/treatments were neighbouring in cross row experiment (see Figure 10.2). Bold lanes indicate reference strains.

A number of lanes including the Promega 1kb DNA ladders were matched with isolates of the same origin (see Table 10.9). The isolate denoted replicate 1 *D. hirsutum* WSM2323 Outer 2 was matched with the reference strain WSM2323. There were five pairs of isolates that were matched, where reference strains were neighbouring in the field.

Table 10.9 *Dorycnium hirsutum* lane matching using Gene Profiler™ 1D Gel Analysis and a threshold of 80% similarity or greater.

Lane	Matched Lane	Similarity Index (%)
Promega 1kb-3	Promega 1kb-4	100*
Promega 1kb-1	Promega 1kb-2	92*
Promega 1kb-5	Promega 1kb-6	92*
2 DH WSM2338 Outer 2	2 DH Control Outer 1	89***
2 DH Control Outer 1	2 DH Control Outer 2	86*
WSM2323	1 DH WSM2323 Outer 2	83**
1 DH WSM2323 Outer 2	2 DH Control Outer 1	80
2 DH Control Outer 1	2 DH WSM1284 Inner 1	80
2 DH WSM1284 Inner 4	2 DH WSM1284 Inner 1	80*
2 DH WSM2323 Outer 4	2 DH Control Outer 1	80
2 DH WSM2338 Inner 3	2 DH Control Outer 1	80***
WSM1284	2 DH WSM2338 Inner 2	80
WSM2323	2 DH Control Outer 1	80
WSM2323	2 DH WSM1284 Inner 4	80***
WSM2323	2 DH WSM1284 Inner 1	80***
WSM2338	2 DH Control Outer 1	80***

Key: 1 DH SU343 Outer 3 = Replicate **1**, ***D****orycnium* ***h****irsutum*, Inoculant **SU343**, **Outer** 12.5cm of cross row, isolate **3**. * indicate matches of isolates of the same origin, ** indicates matches between isolates and original inoculants (reference strains), *** denotes lanes matched where reference strains/treatments were neighbouring in cross row experiment (see Figure 10.2). Bold lanes display reference strains.

A number of lanes including the Promega 1kb DNA ladders were matched with isolates of the same origin (see Table 10.10). The isolate denoted replicate 2 *D. rectum* WSM2323 Outer 2 was matched with the reference strain WSM2338, which were neighbouring inoculants in the field experiment. There were a number of

isolates/reference strains, which were matched that did not have a physical 'relationship' in the field and were not the original inoculants.

Table 10.10 *Dorycnium rectum* lane matching using Phoretics™ Advance and a threshold of 80% similarity or greater.

Lane	Matched Lane	Similarity Index (%)
1 DR WSM1284 Outer 4	1 DR WSM1284 Outer 3	100*
2 DR WSM1284 Inner 2	1 DR SU343 Outer 7	100
Promega 1kb-5	Promega 1kb-6	100*
Promega 1kb-7	Promega 1kb-8	100*
1 DR WSM1284 Outer 5	1 DR WSM1284 Outer 2	92*
1 DR SU343 Outer 7	2 DR WSM1284 Inner 1	90
2 DR WSM1284 Inner 2	2 DR WSM1284 Inner 1	90*
2 DR Control Outer 3	**WSM2338**	89
2 DR WSM2323 Outer 2	WSM2338	88***
1 DR SU343 Outer 3	1 DR SU343 Outer 4	87*
1 DR WSM2323 Inner 4	1 DR WSM2323 Inner 1	85*
2 DR WSM2323 Outer 2	2 DR WSM1284 Inner 2	82
2 DR WSM2323 Outer 2	1 DR SU343 Outer 7	82
2 DR WSM2323 Outer 2	2 DR WSM1284 Inner 1	82
WSM2338	2 DR WSM1284 Inner 2	82
WSM2338	1 DR SU343 Outer 7	82
WSM2338	2 DR WSM1284 Inner 1	82
1 DR WSM2338 Outer 4	1 DR WSM2338 Outer 2	81*
1 DR WSM2338 Inner 4	1 DR WSM2338 Inner 3	80*

Key: 1 DH SU343 Outer 3 = Replicate **1**, ***D**orycnium **h**irsutum*, Inoculant **SU343**, **Outer** 12.5cm of cross row, isolate **3**. *indicate matches of isolates of the same origin, ***denotes lanes matched where reference strains/treatments were neighbouring in cross row experiment (see Figure 10.2). Bold lanes display reference strains.

Reference strains WSM2323 and WSM2338 were matched with isolates that were from cross row plants originally inoculated with these reference strains (see Table 10.11). A number of isolates of the same origin were matched, including the Promega 1 kb DNA ladders. A number of isolates/reference strains were matched where the original inoculants were neighbouring in the field cross row experiment. A number of isolates/reference strains were matched, that were not physically related in the field.

Table 10.11 *Dorycnium rectum* lane matching using Gene Profiler™ 1D Gel Analysis and a threshold of 80% similarity or greater.

Lane	Matched Lane	Similarity Index (%)
Promega 1kb-7	Promega 1kb-8	100*
Promega 1kb-9	Promega 1kb-10	96*
Promega 1kb-5	Promega 1kb-6	92*
1 DR SU343 Outer 7	2 DR WSM1284 Inner 1	91
1 DR SU343 Outer 7	2 DR WSM1284 Inner 2	91
1 DR Control Inner 2	1 DR SU343 Inner 1	89***
1 DR SU343 Outer 3	1 DR SU343 Outer 4	89*
1 DR SU343 Outer 7	1 DR WSM1284 Outer 1	89
2 DR Control Inner 2	1 DR SU343 Inner 1	89
WSM2323	1 DR SU343 Inner 1	89***
1 DR WSM2323 Inner 4	2 DR WSM2323 Outer 1	88
1 DR SU343 Inner 1	1 DR SU343 Outer 2	86*
1 DR WSM1284 Outer 2	1 DR WSM2323 Inner 4	86
1 DR WSM1284 Outer 2	2 DR WSM2323 Outer 1	86
WSM2323	1 DR WSM2323 Inner 1	86**
WSM2323	1 DR WSM2323 Inner 4	86**
WSM2323	2 DR WSM2323 Outer 1	86**
1 DR WSM2338 Outer 2	1 DR WSM2338 Outer 3	83*
1 DR WSM2338 Outer 2	1 DR WSM2338 Outer 5	83*
2 DR Control Outer 1	2 DR Control Outer 3	83*
2 DR WSM1284 Inner 1	2 DR WSM1284 Inner 2	83*
2 DR WSM1284 Inner 1	2 DR WSM2323 Outer 2	83
WSM2323	1 DR WSM1284 Outer 2	83
WSM2323	1 DR WSM2338 Inner 3	83
WSM2338	1 DR WSM2338 Outer 2	83**
WSM2338	1 DR WSM2338 Outer 3	83**
1 DR SU343 Outer 3	1 DR SU343 Outer 5	82*
1 DR SU343 Outer 4	1 DR SU343 Outer 5	82*
1 DR SU343 Outer 4	1 DR SU343 Outer 6	82*
1 DR Control Inner 2	2 DR Control Inner 2	80
2 DR WSM1284 Inner 1	1 DR WSM1284 Outer 1	80
2 DR WSM1284 Inner 2	1 DR WSM1284 Outer 1	80
SU343	1 DR Control Inner 1	80***
WSM2323	1 DR Control Inner 2	80
WSM2323	2 DR Control Inner 2	80***

Key: 1 DH SU343 Outer 3 = Replicate **1**, ***D****orycnium* ***h****irsutum*, Inoculant **SU343**, **Outer** 12.5cm of cross row, isolate **3**. * indicate matches of isolates of the same origin, ** indicates matches between isolates and original inoculants (reference strains), *** denotes lanes matched where reference strains/treatments were neighbouring in cross row experiment (see Figure 10.2). Bold lanes display reference strains.

10.4 Discussion

10.4.1 Glasshouse and Field Performance Compared

The results of this experiment suggested that the interaction between host and inoculant rhizobial strain appeared to be dependent on the inherent environmental conditions. For example, strains could not be statistically separated in the glasshouse for their effectiveness on *D. rectum* TAS1274 yet growth of this genotype in the field was significantly ($P<0.05$) improved by inoculation with WSM1284, WSM2338 and SU343.

The use of appropriate inoculants may be even more critical under lower soil fertility conditions than at this field site and fully mature plants may demand optimal nitrogen fixation, especially where competition is greater for soil nitrogen than observed in these experiments. The difference in ranking of the performance of inoculants between the glasshouse and field trial support the contention of Howieson *et al.* (2000b,c) that the complex three way interaction of host and rhizobial genotypes with the environment (G^2 x E, as discussed in Chapter 9) must be embraced in rhizobial strain selection.

The performance of WSM1284 and WSM2338 on *D. rectum* in the field suggests these are inoculants complement SU343. In commercial terms the use of a single broad-spectrum rhizobial strain is more viable than development of a number of inoculants. However, Vincent and Waters (1953) stated that in a mixed culture more effective strains are generally more competitive, with host legumes exhibiting some selective preference (Brockwell *et al.,* 1995). Therefore, a mixed population may be successful for the inoculation of a stand of *D. rectum* or *D. hirsutum.* The development of WSM2338 and WSM1284 as additional inoculants for *Dorycnium* spp. would first require the negative interactions with pasture legumes to be considered and further investigated along with the commercial viability. Howieson *et al.* (2000a) highlighted the importance of the screening process, not only for nitrogen fixing capacity, but for the impact on non-target legumes.

Dorycnium hirsutum growth in the field was not significantly ($P>0.05$) improved by the use of inoculants, whereas this was not the case in the glasshouse. Possible

explanations for these observations were that the strains were not suitable for the species and/or environmental conditions. As well, plant variation within and between replicates may have masked treatment effects. The observed differences in responses to inoculants between the glasshouse and field suggested that *D. hirsutum* may be an efficient scavenger of mineral nitrogen in the soil. Hence, there is less reliance on nitrogen fixation when growing in a medium containing moderate levels of nitrogen.

10.4.2 Total Nitrogen Values

The total nitrogen values for the rhizobia/*Dorycnium* spp. combinations in the glasshouse appeared to be improved by inoculation over the control (+nitrogen uninoculated) plants, however, the results could not be statistically analysed. In the field the growth of inoculated plants was significantly ($P<0.05$) improved suggesting nitrogen supply was increased via fixation. WSM1284, SU343 and WSM2338 were the superior strains in terms of improving total nitrogen content of *D. rectum,* whereas, SU343 was the superior strain on *D. hirsutum*.

10.4.3 Nitrogen Fixation

The nitrogen fixation values (percentage nitrogen fixed and nitrogen fixed per plant) for inoculated *Dorycnium* spp. should be treated with caution due to the design of the experiment. Spatial differences between reference plants and *Dorycnium* spp. meant that localised soil differences may have influenced ^{15}N values. Reference plants and *Dorycnium* spp. were not planted 'side by side' due to the inability to disturb soil or create competition and the reference plants may have had a different rooting depth to the *Dorycnium* spp. However, these values provide a source of reference for assessing the effectiveness of host/rhizobia symbiosis.

For *D. rectum*, there was no difference between treatment strains and the control plants in the percentage nitrogen fixed. The values obtained were generally low (<50 %) and variable. Overall this suggested the control plants were infected by a background strain or had been contaminated by the treatment strains of rhizobia. As well there may have been adequate levels of soil nitrogen for growth of the *Dorycnium* plants, despite the growth of two cereal crops prior to this experiment.

Although there were statistically significant differences in results for *D. hirsutum* for percentage nitrogen fixed for the various host/strain combinations, no clear conclusions could be made for the same reasons as stated for *D. rectum*. The percentage nitrogen fixed values for *D. hirsutum* were much less (<30 %), which suggested a low level of dependence on nitrogen fixation by these plants.

Dorycnium rectum inoculated with SU343, WSM2338 and WSM1284 fixed nitrogen in quantities greater ($P<0.05$) than the control and plants inoculated with WSM2323. Significantly ($P<0.05$) improved performance was also observed for these host/rhizobia combinations in terms of DM production and total nitrogen content. The results revealed that WSM2338 and WSM1284 are suitable complementary inoculants (to SU343) for *D. rectum*. These results suggest WSM2338, WSM1284 and SU343 effectively fix nitrogen with *D. rectum*.

Dorycnium hirsutum fixed significantly ($P<0.05$) greater amounts of nitrogen with WSM2323 than any other treatment in the field. This effective host/rhizobia interaction was displayed in the glasshouse experiment in terms of DM production and total nitrogen content. Therefore, WSM2323 was the most effective inoculant for *D. hirsutum* in the field.

Previously negative interactions between inoculants and non-target pasture species were examined (see glasshouse experiment Chapter 9). The ^{15}N nitrogen fixation results demonstrated that negative interactions between *D. hirsutum* and *D. rectum* and their respective inoculants, may be an issue to be considered. Superior strains for *D. hirsutum* (WSM2323) and *D. rectum* (SU343, WSM2338 and WSM1284) significantly ($P<0.05$) reduced nitrogen fixation in the other species. As discussed previously Vincent and Waters (1953) and Brockwell *et al.* (1995) stated that in a mixed rhizobia population the most effective strains are generally the most competitive. In a mixed sward of *Dorycnium* spp. inoculation with a range of strains may result in effective strains on one host species being out competed, therefore leading to negative interactions. Brockwell *et al.* (1995) also suggested that in a mixed rhizobia population host legumes exhibit some selective preference.

The evidence of nitrogen fixation displayed by the control plants, suggested effective nodulation had occurred. However, the DM production and amount of nitrogen fixed would suggest that nitrogen fixation with these plants differed from the inoculated plants. This implies the nodulation process was delayed and was possibly due to a relatively small background population of rhizobia.

10.4.4 Annual Nitrogen Fixation

The estimations of the amount of nitrogen fixed per plant during the experimental period were extrapolated to an annual nitrogen fixation value (kg/ha) based on the planting density used in the experiment. The highest estimates of nitrogen fixation were observed with SU343 and WSM2323 for *D. rectum* and *D. hirsutum* respectively, indicating effective host/rhizobia interactions.

Wanjiku *et al.* (1997) found that the above ground amount of nitrogen fixed by *D. hirsutum* was 71 kg/ha/year. This finding is supported by the estimated fixation of 77 kg/ha/yr of nitrogen by the WSM2323/*D. hirsutum* combination. Bowman *et al.* (2002) stated that the semi-dormant type of lucerne (*Medicago sativa*), had the capacity to fix 74.8 kgN/ha/yr. The results (see Table 10.4) demonstrated that a number of the rhizobia/*Dorycnium* spp. combinations have the ability to fix nitrogen in quantities greater than reported for lucerne. This demonstrated that *Dorycnium* spp. are effective nitrogen fixing legume when inoculated with an appropriate root nodule bacterium. The ability of *Dorycnium* spp. to fix nitrogen effectively combined with their tolerance of moisture stress makes them a useful alternative in arid regions.

The ability of *Dorycnium* spp to fix nitrogen in substantial quantities is very important for a number of reasons. The target areas for these plants, i.e low rainfall, low soil fertility, will be limiting in terms of production due to these factors, but the fixation of nitrogen will substantially improve plant production. Nitrogen fixation will improve the nitrogen content of the plants and hence the forage quality in terms of crude protein content. In a low fertility soil system the input of nitrogen from legumes can significantly improve the nitrogen content of the soil and therefore overall soil fertility.

10.4.5 Cross Row Analysis

The analysis of the cross rows (see Tables 10.5 and 10.6) revealed that DM production was highly variable between treatments and between inner and outer sections of the cross rows. There appeared to be some major differences between the DM production/plant within the cross rows suggesting that there may have been some competition effects occurring. No definitive trend was observed between these sections, such as, inner DM production/plant < outer DM production/plant or vice versa, so these differences cannot be attributed to any one factor i.e competition, nodulation etc. Alternatively a background rhizobia population or adequate soil nitrogen levels may have interacted with the introduced strains and limited their effect on DM production.

The removal of plants from the soil was difficult due to variable soil conditions and subsequently large numbers of nodules were lost, and so exact nodule numbers could not be obtained. Dissection of exhumed nodules revealed that all were at least partially effective. The majority of the nodules were rated at approximately four (on a scale of one to five), which translates to pink colouration throughout the nodule, and hence, the presence of leghaemaglobin. An indication of the effectiveness of nodule functioning is an abundance of leghaemaglobin (Vincent, 1970) with pink internal colouring (Somasegaran and Hoben, 1994).

The highest average nodule colour rating for *D. hirsutum* was with SU343 and WSM2323, and for *D. rectum* WSM2338 and WSM1284. This provides further evidence that these strains are suitable for the inoculation of the respective *Dorycnium* spp.

All of the control plants examined were nodulated, with the nodules being considered effective. The comprehensive nodulation of control plants revealed that a background population of root nodule bacteria were present in the soil, and the nodulation of these plants was not simply a result of cross contamination from introduced inoculants.

10.4.6 Rhizobia Persistence and Spread

All of the cross rows examined, including inner and outer sections were nodulated well with effective nodules. This suggested that the introduced inoculants have persisted through the ley period and infected the sterilised seed upon sowing. The ability of the introduced rhizobia to persist and spread is important for effective widespread nodulation perennially and following ley periods. Brockwell *et al.* (1995) stated that the breakdown of nodules releases large numbers of rhizobia into the soil, which have a high potential for the subsequent nodulation of future crops. To determine whether or not the inoculants had persisted through the ley period, or whether interaction with background rhizobia was occurring, isolates from the nodules exhumed were analysed using PCR.

10.4.7 Selection of PCR Primers

Preliminary work examining the effectiveness of the primers ERIC and RPO1 revealed quite different effectiveness on the reference strains. ERIC facilitated very little or no band separation, whereas, RPO1 effectively separated all reference strains. An optical density (OD_{600}) of two and four both facilitated efficient band separations, whereas, OD_{600} of one and the pure cells separated and defined bands poorly. This poor band separation suggested that the DNA biomass was far too low. In addition to this, the suspension of pure cells without centrifugation suggested that exopolysaccharides and other cell wall material compromised the DNA samples. The examination of primers ability to separate bands prior to the undertaking of the main PCR analysis, highlights the importance of this process and selection of primers in relation to isolates being examined.

10.4.8 Nodule Isolate Analysis

With the analysis of isolates using the two software programs, an arbitrary 80 % similarity index was adopted as a threshold. There were a number of matches made for both *D. rectum* and *D. hirsutum* between isolates of the same origin, and reference strain with original inoculants for a particular cross row (see Figures 10.8 through to 10.11). Matches between isolates of the same origin, displayed that similar or identical rhizobial strains were isolated. However, this did not determine that the strain isolated was the original introduced inoculant. Matches between isolates and reference strains and isolates originally inoculated with this inoculant

demonstrated the ability of the strain to persist in the soil following a ley period, and hence inoculate subsequent *Dorycnium* spp. plants.

A large number of isolates were matched from neighbouring rows. For example isolate denoted replicate 2 *D. hirsutum* WSM2338 Outer 1 was matched with replicate 2 *D. hirsutum* WSM2323 Outer 4, and these two cross rows were neighbouring in the field (see Figure 10.2). This match suggetsed a commonality between the two, either in terms of one introduced strain persisting and spreading, or the infection of both rows by a common background strain. The spread of introduced strains may be due to natural processes, or by traffic/water movement within the site, and hence contamination. The comprehensive nodulation of all plants examined, including controls, suggests a number of possibilities, the persistence and spread of introduced strains, more likely though, the nodulation by a background population.

The low proportion of matches between reference strains and isolated strains from inoculated rows suggests a number of possibilities. Firstly the presence of a background population, with the comprehensive nodulation of all plants and resulting in a non-significant ($P>0.05$) difference in average DM production for inner and outer cross rows for both *D. rectum* and *D. hirsutum* (see Table 10.7). Whereas, there was a significant ($P<0.05$) difference in DM production between strains following the 1st harvest of plant material which suggested a background strain may have out competed introduced strains and inoculated the 2nd planting of *Dorycnium* spp. where sterilised seed was used. Secondly, the software packages may have been inaccurate or too stringent with band matching, when comparing lanes. Thirdly, exposure to unique soil micro and macroclimates may have led to changes in genetic makeup occurring with the adaptation of the inoculants. When compared to the original reference strains stored in a stable environment the changes would be apparent. Finally, the use of RAPD (random amplification of polymorphic DNA) may not produce the same results each time this system is used, and hence matching of bands will be reduced.

The nodulation of all plants and roots exhumed and the wide range of isolate matches that were made using the software packages, revealed that *D. rectum* and *D. hirsutum* are promiscuous.

There appeared to be no relationship between the introduced strains and or its persistence and spread. The strains believed to have been retrieved from cross row isolates which were originally introduced displayed an ability to persist, however, there was no decisive pattern in terms of spread from the initial inoculation row within the cross rows, or further to neighbouring plots. This was the case with both *D. rectum* and *D. hirsutum*. The results of isolate matching, suggest that there has been limited persistence or competitiveness of the introduced inoculants, and a background population has effectively infected cross row plants.

10.4.9 Comparison of Software Analysis

The analysis of the lanes using the two software packages produced quite different results in terms of matching. This displayed the inherent differences in analysis of gels by these software packages, however, as a reference, all of the Promega 1kb DNA ladders were matched on each gel. The matching of isolates of similar origin, or with reference strains was lower than expected. This may be interpreted as either ineffective analysis by the software or interactions with a background population. When all reference strain lanes were compared in Phoretics™ Database there was poor matching of reference strains of the same origin. This highlights the fallibility of such analysis with software and the inherent differences in individual gel conditions from one to another. However, it must be kept in mind that the RAPD may not be reproducible between runs. The comparison of all reference strains using Phoretics™ Database suggested in some instances that some of the different reference strains may have very similar banding patterns (see Figure 10.7), and hence genetic makeup.

10.5 Conclusions

The results of these experiments revealed that SU343 is a suitable inoculant for *D. rectum* TAS1274. Rhizobial strains WSM1284 and WS2338 were also identified as equally suitable or complementary strains to SU343 for this species. The DM production and nitrogen fixation performance of *D. hirsutum* was significantly ($P<0.05$) improved by the WSM2323 inoculant over the previously used SU343, and therefore this would be a suitable replacement strain. WSM2338, SU343, WSM1284

and WSM2323 all displayed suitable performance following the initial introduction, however, competition from background populations may seriously impede subsequent infection following ley periods.

10.6 Acknowledgements

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Chapter 11. General Discussion

This chapter examines the results of the previous experimental sections with a broader outlook. Consideration will be given to issues not examined by this research, but that are necessary to investigate for the commercial use of this genus, and may formulate a basis for future research.

11.1 Germination Characteristics

11.1.1 Extrapolation of Scarification Techniques

The work conducted on seed germination as part of this project, focused on *D. hirsutum* for a number of reasons outlined in Chapter 3. *Dorycnium rectum* and *D. pentaphyllum* have both also displayed issues relating to restricted water uptake during germination experiments. The results of this work could be extrapolated to the other two species. The application of mechanical scarification could be readily tested on these species with some experimentation with treatment time and coarseness of abrasive surfaces, both of which are known to be critical factors in effective seed treatment.

11.1.2 Commercial Application of Scarification Techniques

The commercial application of scarification treatments needs to be considered for large scale operations. The scarification techniques proved to be highly effective and repeatable, however, on a large scale a number of these treatments are not suitable. The nicking of the seed coat using a scalpel removed the seed coat barrier, however, this is a tedious method and is not viable for seed quantities required in the field. The use of severe mechanical scarification to achieve the same results led to abrasion of the embryo and total removal of the seed coat in some instances, which is unacceptable. Mechanical scarification using machinery that is easily manipulated and controlled has proven to be highly effective for the scarification of *D. hirsutum*. However, caution must be taken to ensure damage to the embryo does not occur.

Concentrated sulphuric acid whilst being highly effective in softening the seed coat, and easily manipulated in terms of treatment duration, is an extremely hazardous chemical. The large scale use of sulphuric acid would not only be costly, but would

require highly trained operators and extensive safety measures to be in place. Chemical scarification may occur naturally in the grazing environment, where animal consumption of flowering stem may facilitate chemical scarification of seed via digestion acids, and hence mimic the use of sulphuric acid.

11.1.3 Commercial Seed Production

The commercial release of these plants should be done following extensive breeding and selection for desirable genotypic and phenotypic characteristics. As a result the inherent variability within the existing accessions would be reduced. The potential commercial release of these plants presents a range of issues in relation to seed quality, quantity and production.

Allowing the seed to mature completely on the plant presents the problem of major seed losses due to dehiscence. To overcome this issue, there are a number of solutions that may be considered. Breeding and selection will be required to eliminate or reduce the impact of the dehiscence trait, and hence promote seed retention. Seed may be harvested earlier and subject to some post harvest ripening treatments or storage techniques that ensures the quality of the seed. The environmental conditions under which the seed is produced may also promote more even seed maturation and lower incidence of dehiscence. There has been anecdotal evidence of this in seed produced in milder climates with more reliable rainfall during flowering and seed set.

11.1.4 Weed Potential

Dorycnium spp. like many pasture species posses what may be considered to be 'weed' like characteristics. The production of substantial quantities of seed, ability to lay dormant in the soil, and be readily spread by animals and traffic, may facilitate uncontrolled spread and infestations. The promiscuous nature of *Dorycnium* spp. in forming symbiotic relationships with a broad range of root nodule bacteria provides greater potential for this genus to establish in non-target areas.

The responsible commercial release of these species should be preceded by investigations into plant control, and an assessment of weed potential. Like any other

crop or pasture plant, the ability to propagate must be balanced by the ability to control the plants in question.

11.2 Forage Quality

11.2.1 Forage Quality and Growth Stage

The time of grazing/harvest of the *Dorycnium* spp. will be critical in optimising the nutritional value for livestock of the forage. As demonstrated for *Dorycnium* spp. in this study, the optimum time of harvest would be when the plants are in the early stages of flowering, which occurred in September, September-October and November-December for *D. pentaphyllum, D. hirsutum* and *D. rectum* respectively. However, for *Dorycnium* spp. the proposed time of grazing is in late summer/early autumn when the plants are in an advanced stage of reproductive development and overall quality is less than optimum. The stage of plant growth is a useful means of describing forage quality.

11.2.2 Plant Management

The management of plants may also be a useful tool for optimising the quality and quantity of harvested forage, in relation to an animal's requirement when other feeds are in limited supply. There has been little investigation into the management of *Dorycnium* spp., however, observations made in relation to grazing/harvesting of these plants allows us to speculate on the management and potential response of these plants. *Dorycnium pentaphyllum* whilst providing a source of CP and ME has very little scope to provide 'useful' DM production. The DM quality and quantity characteristics of *D. hirsutum* and *D. rectum* suggested that grazing management of these plants could optimise quantity and quality. *Dorycnium hirsutum* and *D. rectum* have both displayed excellent regrowth following grazing/harvest during the spring/summer period. Therefore, during this period a number of cuts/grazing may be undertaken with the growth stage (early-late bud) the determining factor for the timing. The provision of *Dorycnium* spp. forage is dependant on the availability of regular feed in the grazing system and the timing of this. Hypothetically, when *Dorycnium* spp. is at its optimum quality, but not required at that point in time, it may be harvested and stored for distribution at a later date.

Dorycnium spp. produce significant amounts of woody stem, in particular *D. rectum*, with plants becoming progressively more woody with maturity. The production of woody stem uses valuable plant resources and nutrients, and reduces the potential for the production of higher quality fresh forage. However, the regular removal of this woody stem stimulates the regrowth of fresh digestible stem and leaf. The establishment of a substantial root and reserves system allows these plants to survive harsh pruning and vigorously produce fresh leaf and stem in response. Beever *et al.* (2000) stated that management decisions in terms of harvesting a forage crop via cutting or grazing will affect the quality of the forage, yield and nutrients obtained.

Although *Dorycnium* spp. are described as shrubs, rigorous management may facilitate them being incorporated into a pasture mix (see Figure 11.1), or as a stand alone forage crop with a growth habit similar to that of lucerne.

Figure 11.1 *Dorycnium hirsutum* growing as part of a mixed pasture.

11.2.3 Condensed Tannins

The CT work was limited to some extent due to the inherent cost involved, so detailed seasonal changes were not obtained, nor the influences of leaf and stem fractions. The CT content of leaf and stem when separated demonstrates the relative

influence of each component on CT content and how that may relate to a grazing animal, which can be selective to some degree. As shown in the forage quality work, the influence of leaf and stem as separate components is quite significant, and the relative quality of the forage can be determined by leaf:stem ratio to a certain extent.

The use of *Dorycnium* spp. as a primary forage source is questionable due to its lower quality when compared with a legume such as lucerne. However, *Dorycnium* spp. are seen as a source of forage for utilisation during times of low rainfall, and species such as lucerne have limited growth potential under such conditions. *Dorycnium* spp. may have uses outside of the summer/autumn period as a supplement. The provision of fibre or protein when incorporated into a feed may be valuable. In addition to this the CT content may act as a preventative for bloat when added to a feed. Ultimately, the optimum use of *Dorycnium* spp. as a source of forage will require careful consideration of the attributes of the plants and matching those with environmental conditions, and stock requirements.

11.3 Rhizobia Selection and Evaluation

11.3.1 Rhizobial Competition

Despite the performance of the four inoculants (WSM1284, WSM2323, WSM2338 and SU343) following the initial introduction in the field, their ability to compete with a background population was questionable. Therefore, consideration must be given to assessing background competition and soil conditions, in terms of crop history, use of inoculants, fertilizers and herbicides. Background competition may result in the introduced inoculant being completely out competed or suppressed, leading to a reduction in nitrogen fixing capacity. Failure to do so may compromise the competitive ability of the introduced inoculant, its long-term survival, activity and spread. This may translate into economic and production gains/losses with the use of a particular inoculant/s. The ability of an inoculant to perform its desired role in the soil system is critical for inoculant selection and subsequent host/rhizobia functioning and production.

11.3.2 Negative Rhizobial Interactions

A number of issues were identified through the rhizobia selection and evaluation experiments regarding the interaction between inoculant strains and other agriculturally significant pasture legumes. The formation of negative interactions, or ineffective nodules, results in little or no nitrogen fixation, and hence growth potential is compromised. Careful consideration needs to be given to the widespread release of new inoculants due to the possible negative associations that may occur. Consideration must be given to the inclusion of particular legumes and inoculants into a rotation or pasture system in terms of future uses. The competitive ability of a recently introduced inoculant against a pre-existing one must be considered, along with host/rhizobia selectivity or promiscuity.

11.4 Potential Alternative Uses of *Dorycnium* spp.

Literature pertaining to *Dorycnium* spp. has outlined a number of potential uses of this genus other than that of a source of forage. Observations of these plants during this study demonstrated their excellent ability to colonise degraded land, especially when care is taken during the establishment phase. The ability to establish *Dorycnium* spp. on poorer sites, in particular north facing slopes, provides excellent ground cover, soil stability, and nitrogen input via biological nitrogen fixation. Honey production from bees 'working' *Dorycnium* spp. flowers is known to occur. The honey produced from *Dorycnium* spp. has a very distinctive and pleasant taste (Hall pers. comm., 2001), and has the potential to form a niche market. *Dorycnium pentaphyllum* and *D. hirsutum* are considered by some to possess aesthetic qualities. In full flower these two species have decorative qualities that may see them used for landscaping purposes. Persistence and hardy nature of *Dorycnium* spp. allow for use in situations where conditions may be harsh and little or no maintenance provided.

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Appendices

Appendix 1: Rhizobial Nutrient Solutions

Compound	g/L
$MgSO_4.7H_2O$	12.3
KH_2PO_4	6.8
K_2SO_4	17.5
EDTA	2.5
H_3BO_3	0.464
Na_2MoO_4	0.018
$ZnSO_4$	0.539
$MnSO_4$	0.042
$CoSO_4$	0.141
$CuSO_4$	0.125
$CaSO_4$	2.04
KNO_3 (+N)	0.5
$KNO_3 + NH_4NO_3$ (+N)	2.5

Appendix 2: ½ Lupin Agar and Nutrient Solution

Compound	g/L
Mannitol	5.0
D-glucose	5.0
Yeast Extract	1.25
$MgSO_4$	0.8
NaCl	0.2
$CaCl_2.H_2O$	0.2
K_2HPO_4	0.87 (20 ml)
KH_2PO_4	0.68 (20 ml)
$FeSO_4$	0.5 (10 ml)
Trace Elements	(1 ml)
Agar (for ½ LA plates)	18.0
Trace Elements	
H_3BO_3	1.52
$MnSO_4.4H_2O$	2.03
$ZnSO_4.7H_2O$	0.22
$CuSO_4.4H_2O$	0.08
$Na_2MoO_4.2H_2O$	0.126