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The Biology of *Trifolium repens* L. (White Clover)

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This document provides an overview of baseline biological information relevant to risk assessment of genetically modified forms of the species that may be released into the Australian environment.

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TABLE OF CONTENTS

PREAMBLE

This document addresses the biology of *Trifolium repens* L. (referred to as white clover) with particular reference to the Australian environment, cultivation and use. Information included relates to the taxonomy and origin of white clover, general descriptions of its morphology, reproductive biology, biochemistry, and biotic and abiotic interactions. This document also addresses the potential for white clover to transfer genes via pollen and seed movement and for weediness. It should be noted that due to the large number of white clover cultivars as well as the highly heterozygous nature of white clover populations which results in many genotypes, it has been necessary to generalise much of the information provided in this document and exceptions may be common. The purpose of this document is to provide baseline information about the parent organism for use in risk assessments of genetically modified (GM) *Trifolium repens* L. that may be released into the Australian environment.

White clover is the most important pasture legume in many temperate parts of the world and in Australia, it is the main legume grown in perennial pastures in cool temperate, summer rainfall zones (Archer & Robinson 1989). White clover is mostly grown in conjunction with grasses, and is used for grazing, pasture hay and ground cover in horticultural situations, where it supplies a rich source of proteins and minerals to grazing livestock, and fixes large amounts of nitrogen in pastures, thereby improving soil fertility and reducing the need for fertilisers. While white clover is regarded as a perennial plant, it is able to behave as an annual in warm climates or under moisture stressed conditions. The plant is capable of both asexual (or vegetative) reproduction through the generation of stolons, and sexual reproduction through seed production and dispersal.

SECTION 1 TAXONOMY

White clover is also commonly known as Dutch clover, white trefoil, creeping trifolium, ladino clover or honeysuckle clover (Shaw 1906). The genus name for clover derives from the Latin tres, "three", and folium, "leaf", so called from the characteristic form of the leaf, which has three leaflets (trifoliate); hence the popular name trefoil. The specific name repens refers to the creeping and rooting stems.

White clover, *Trifolium repens* L., is one of the most agronomically important of the 250-300 species in the genus *Trifolium*. *Trifolium* belongs to the tribe Trifolieae of the subfamily Papilionoideae (also known as Faboideae) of the family Fabaceae (Australian Plant Name Index), although older taxonomic classifications placed the *Trifolium* genus in the family Leguminosae (Williams 1987b).

A recent molecular phylogeny analysis of the *Trifolium* genus confirmed the monophyly of *Trifolium* but at the same time proposed a new infrageneric classification of the genus (2006). They indicate that only 22 of the *Trifolium* species are polyploid and that 5 or 6 are of putative hybrid origin. They confirmed the ancestral chromosome number as 2n=16. *T. occidentale* and *T. pallescens* have been identified as the likely progenitors of *T. repens* within a new section *Trifoliastrum*.

White clover is a natural tetraploid with a chromosome number of 2n=4x=32 (Voisey et al. 1994). Due to the addition of divergent genomes, inheritance in allopolyploids (which includes white clover) is disomic; ie pairing behaviour during meiosis is similar to that of nonhomologous pairs of chromosomes in diploids (Voisey et al. 1994). Within the *Trifolium* genus there is variation in the chromosome number and ploidy (see Table 1 for some examples).

Table 1: A selection of *Trifolium* species with their chromosome number, ploidy, compatibility and perreniality*

Species	Chromosome number	Ploidy	Compatibility	Annual/Perennial
T. repens	32	4x	Self incompatible	perennial
T. pratense	14	2x	Self incompatible	Biannual/perennial
T. nigrescens	16	2x	Self compatible	annual
T. ambiguum	16	2x, 4x, 6x	Self incompatible	perennial
T. occidentale	16	2x	Self compatible	perennial
T. hybridum	16	2x, 4x	Self incompatible	annual
T. pallidum	16	2x	Self incompatible	annual
T. pallescens	16	2x	Self incompatible	perennial
T. alpestre	16	2x	Self incompatible	perennial

* Adapted from Abberton (2007)

Additionally, white clover has a well-developed genetic gametophytic self-incompatibility mechanism with only a small proportion of plants in a population being quite strongly self-compatible (Thomas 1987a). In essence, this means that autogamous self-pollination is infrequent in white clover. The self-incompatibility system is based on the multiple oppositional alleles of the S locus which means that incompatibility occurs when the S allele in the pollen is the same type as that present in the style (Williams 1987a). However, white clover also shows frequent pseudo-self-incompatibility (the breakdown of the S allele system). Therefore, self-incompatibility can be somewhat dependent on genotype and environmental conditions such as temperature, resulting in a limited degree of self-pollination (Thomas 1987a).

White clover cultivars are classified arbitrarily by the size of the plants, as small, intermediate and large.

Predominant outbreeding and disomic inheritance means that white clover populations are composed of a heterogeneous mixture of highly heterozygous individuals. This results in high levels of genetic variation both within and between populations (Voisey et al. 1994). By having high variability, white clover is more adaptable to competitive microenvironments. This is an important attribute as white clover does not naturally cross with other *Trifolium* species (See Section 9.2) and therefore cannot gain genetic variation by forming hybrids.

SECTION 2 ORIGIN AND CULTIVATION

2.1 Centre of diversity and domestication

The genus *Trifolium* is thought to have originated in the Mediterranean in the early Miocene period, 16-23 million years ago (Ellison et al. 2006). White clover also originated from the Mediterranean region of Europe and was spread through Europe and Western Asia with migrating animals before recorded history. Domestication of white clover occurred 400 years ago in the Netherlands and migrated along with European settlers to various continents on which it is now considered naturalised (Zeven 1991; Lane et al. 1997). White clover has tended to naturalise in temperate regions of the world with greater than 750 mm annual rainfall (Jahufer et al. 2001). The main white clover growing areas in the world are in Western Europe, North America, Australasia and New Zealand.

It is generally accepted that European settlers accidentally introduced white clover into Australia in the late 18th century and deliberate sowing of Dutch and English strains of white clover occurred soon after with the establishment of pastures (Lane et al. 1997). It is now considered a naturalised pasture legume in the coastal regions and tablelands of eastern Australia and has a significant presence in 7.8 million hectares of pasture in Australia (Hill & Donald 1998).

Many other *Trifolium* species are also widely distributed throughout temperate and subtropical parts of Australia including species commonly sown in pastures such as subterranean clover (*T. subterraneum*), arrowleaf clover (*T. vesiculosum*), red clover (*T. pratense*), persian clover (*T. resupinatum*) and strawberry clover (*T. fragiferum*) (NSW Agriculture and Grassland Society of NSW Inc 2001).

2.2 Commercial uses

White clover is used in a mixed sward with grasses. It is used for grazing, pasture hay and ground cover in horticultural situations. It is highly important in the dairy, meat and wool industries, significantly improving yields of these products (Ayres et al. 2000). For example, it has been estimated that in Victoria white clover is worth $A 267 million to the diary industry alone (Mason 1993) and in some areas in New Zealand, the value of white clover has been estimated to be worth $NZ 380-435 per hectare per year on an average diary farm (Harris 1998). The presence of white clover in pastures allows a many fold increase of stocking rates, eg greater than 10 sheep per hectare compared to 1.8 sheep per hectare on native pastures of Australia (Cotsell & Edgar 1959). A density of greater than 25% ground cover is commonly sought by pastoralists (Garrett & Chu 1997).

There are many advantages to using white clover in pastures. It has a high nutritive value because it supplies a rich source of proteins and minerals, and has high voluntary intake by grazing animals thereby making an important contribution to feed supply throughout the year. Furthermore, it is adaptable to a wide range of soil and environmental conditions and combines well with many perennial grasses. Additionally, it has dual regenerative capabilities via stolons and seed bank (discussed in Section 4.1) making it suitable for grazing (Frame 2003; Betts & Ayres 2004).

A highly desirable feature of white clover in pastures is its nitrogen fixing ability, a result of the symbiotic relationship between white clover and the bacterium *Rhizobium trifolii*, resulting in the formation of nodules in which the *Rhizobium* fix atmospheric nitrogen. Environmental factors such as soil pH, light, defoliation, temperature, soil nutrient status and water stress can influence *Rhizobium* infection, nodulation, and nitrogen fixation processes, as well as host growth and nitrogen demand (Crush 1987). Nitrogen fixation improves the nitrogen content of soil, lessening the need for fertilisers. *Rhizobium* infection is essential for white clover establishment on nitrogen-deficient soils.

More recently white clover has been investigated for its potential in phytomanagement of metal polluted soils. Metals such as cadmium, zinc and lead were shown to be taken up and accumulate in the roots of the plant. In white clover, the oxidative stress levels appeared to be controlled by the plant (Bidar et al. 2007).

White clover is a valuable plant for honey production in a number of countries including Australia, New Zealand, Britain, northern parts of the United States, Canada, and parts of Europe (Howes 2007). It has been stated that a greater quantity of honey is obtained from this plant throughout the world than from any other individual plant (Howes 2007). In Australia, white clover flowers are an important source of nectar and pollen for commercial honey production by honey bees (Somerville 1999; Malone 2002). In years with good white clover growth beekeepers situate their hives in white clover growing areas in Spring to breed bees and collect the high quality clover honey (Stace 1996). The pollen of white clover is a good source of nutrition for bees, having a crude protein content of 22.5% to 25.4%. However, there is some conflicting data on whether the pollen is lacking in the amino acids isoleucine and valine (Stace 1996; Somerville 2001).

There are some disadvantages to growing white clover. White clover has variable persistence and yield from year to year due to poor drought and heat tolerance, and therefore good summer rainfall or irrigation is required. Soils with medium to high fertility containing phosphorus and sulphur are needed for good yields and careful grazing management is required. Although white clover improves the nitrogen content of soil, the nitrogen produced is less predictable than fertiliser application (Frame 2003; Betts & Ayres 2004). Additionally, white clover is adversely affected by several pests and diseases (See Section 7.2) and contains some toxic and anti-nutritional compounds (See Section 5.2).

2.3 Cultivation in Australia

White clover is grown for seed production in regions with rainfall greater than 750 mm or under irrigation. In inland areas, perennial white clover grows only at altitudes of greater than 700 m (Cregan & McDonald 1984).

As mentioned above, white clover is predominantly grown in conjunction with grasses in pastures. The type of companion grasses commonly used include perennial ryegrass, phalaris, cocksfoot, fescue, paspalum, Italian ryegrass and kikuyu, although exact combinations are dependent on the area in Australia in which the pasture is grown (Betts & Ayres 2004)

2.3.1 Commercial propagation

White clover seed is predominantly produced in South Australia and Victoria. The Australian Seeds Authority recorded production of 3946 tonnes of white clover seed in 2005-2006 under all certification schemes, approximately 65% of which was of the cultivar Haifa (Australian Seeds Authority Ltd. 2007). Approximately 82% of this seed was produced under the OECD seed certification scheme, which is used to comply with export requirements

Two classes of seed are certified under the OECD scheme, basic and certified (OECD 2008). Basic seed is used for production of certified seed, and has the most stringent production requirements to maintain varietal purity. Basic seed may be harvested from plantings for two years, which are inspected to ensure white clover off-types and contaminating species are present at a density of no more than one plant per 30 m^2. Certified seed is used for production of further certified seed or for pasture planting. It may be harvested from plantings for four years, and presence of white clover off-types and contaminating species is allowed up to one plant per 10 m^2. Other seed certification schemes exist in Australia, for example the South Australian Seed Certification Scheme, requirements of which mirror OECD standards for white clover production (Smith & Baxter 2002). Separation distances also apply, and are discussed in Section 4.2.

Weed infestation in commercial white clover seed production areas can cause a significant drop in profit. The lack of options for weed control remains one of the main limiting factors for achieving high seed yield and cuts into profits as the costs of weed control can be up to 25% of the annual production costs (Riffkin et al. 2005). Control of weeds is discussed further in Section 7.1.

2.3.2 Scale of cultivation

White clover is part of the legume-based pastures that are used in Australia as an important crop that supports the grazing industry due to its contribution to feed quality. It may also help conserve water resources, sustain soil fertility and provide crop protection in farming systems where it is used in crop rotations (Ayres et al. 2007).

The white clover growing zone is about six million hectares and has the potential to increase to at least sixteen million hectares (Ayres et al. 2007). The white clover zone extends from south-eastern Queensland along the coast and adjacent tablelands of New South Wales and Victoria to Mt Gambier in South Australia as well as irrigated regions of south-west Western Australia and high rainfall areas of the Australian Alps and most of Tasmania (Jahufer et al. 2001). The maps at Appendix A (Figure 1 A, B) show the current distribution of white clover in Australia and Figure 2 (A, B) shows the areas of Australia that white clover could potentially inhabit (Hill & Donald 1998).

White clover seed production is based predominantly in western Victoria and south eastern South Australia. These regions produce about 90% of the white clover seed in Australia as a result of the availability of ground water for irrigation and land that has been free of white clover seed.

Data is available on exports and imports of clover seed. This data includes a number of different clover species. However Australian certified seed production statistics indicate that white clover and subterranean clover (*Trifolium subterraneum*) comprise 48% and 45% of all clover seed produced in 2002-3. In 2003, Australia produced 10% of world clover exports, with a value of $10 million. A small volume of clover seed is also imported (236 tonnes in 2003/04) (Foster et al. 2005).

2.3.3 Cultivation practices

White clover requires particular soil conditions to ensure good seedling establishment and to maintain high production. White clover performs well on soils with medium to high fertility containing good levels of phosphorus, sulphur, potassium and molybdenum. It prefers soils that have a pH above 4.5 and have good water-holding capacity but are well draining (Frame 2003; Betts & Ayres 2004). White clover pastures often require fertilisers to maintain productivity. The type of white clover cultivars planted depends on the final use (eg pasture or seed production), and environmental conditions such as pest and disease pressures, soil type and temperature.

White clover can be established a number of ways as a pasture component. White clover-grass mixtures can be seeded onto freshly prepared soil. Alternatively, white clover can be seeded into existing grass pastures by either surface sowing, direct drilling (sod seeding) or partial cultivation (Frame 2003). Such oversowing into grass swards is important if the white clover population has declined due to drought or overgrazing. The highest yields of white clover are in spring and summer, with mixed swards producing peak yields later in the season compared to clover monocultures (Frame 2003).

Various white clover sowing rates are reported, depending upon variety and the type of planting: 2-5 kg/ha for production of a dense sward; 0.5-3 kg/ha in pasture mixtures; 3-5 kg/ha for seed production (Betts & Ayres 2004; Anon. 2007d; Anon. 2007e; Anon. 2007f).White clover is sown in a fine firm seed bed to optimise seed-soil contact and covered with up to 15 mm of firm soil. Seed is often pre-treated, for example with systemic pesticides, trace elements and rhizobia as a cost effective way to optimise establishment of the emerging seedlings (Anon. 2008b).

During establishment in a pasture, white clover performs best under rotational grazing cycles of 1-2 days of grazing every 20-30 days. Established clover can be grazed continuously during the winter, spring and early summer when it is actively growing. White clover should not be grazed until the plant is 15-20 cm tall and not grazed below 5 cm tall. Grass is best kept at below 12 cm so as not to compete with white clover for light (Anon. 2007e; Anon. 2008b).

In the Murrumbidgee and Murray Valleys, it is estimated that the total cost per hectare for sowing, establishment and maintenance of a winter irrigated white clover/rye grass crop, which includes insect spray, broadleaf weed spray and fertilizer and labour is approximately $620/ha per annum (Anon. 2007b).

White clover is grown in horticultural and viticultural situations as a cover crop often in conjunction with ryegrass. It provides breeding areas for beneficial insect species, improves soil structure, improves soil fertility through the supply of nitrogen and suppresses summer weeds (Pocock & Panagiotopoulos 2003; Madge 2007).

White clover has also been investigated for use in intercropping in organic or low input farming systems, where a groundcover crop, often a legume, is used in conjunction with a grain crop. The legume is thought to provide nitrogen to the cereal crop, reduce nutrient leaching by providing permanent soil cover and to reduce pests and diseases (Thorsted et al. 2002). Increased crop yields with white clover have been seen in oats (Thorsted et al. 2002) and rice (Cho et al. 2003) whereas more variable results have been seen in wheat (White & Scott 1991; Thorsted et al. 2002; Thorsted et al. 2006; Hiltbrunner et al. 2007) and rye (White & Scott 1991).

2.4 Crop Improvement

In the USA between 1966 and 2000, the average increase in yield per area was around 2.2% per year (0.11 t/ha/year, based on data from the US Department of Agriculture, National Agricultural Statistics Service, www.usda.gov/nass/). Breeding of new cultivars in Australia has concentrated on reducing sensitivity to summer moisture stress and had led to the release of the cultivar Trophy (Ayres et al. 2007). Other cultivars such as Canterbury have been bred for increased early flowering and increased seed and forage production (Seed Genetics Australia 2008).

2.4.1 Breeding

A number of varieties are being promoted for their use in different farming regions. The creation of interspecific hybrids opens up the opportunity to generate clover varieties with advantageous traits. Table 2 outlines some of the desirable traits of related clover species that could potentially be used to create new *T. repens* hybrids.

Table 2: Advantageous traits in related species*

Desirable trait absent in *T. repens*	Putative hybrid parent species
Deeper roots	*T. ambiguum, T. uniflorum*
Presence of rhizomes	*T. ambiguum*
Faster seedling development	*T. thalli*
Profuse flowering	*T. nigrescence*
Drought tolerance	*T. ambiguum, T. uniflorum*
Salt and drought tolerance	*T. occidentale*
Heat tolerance	*T. nigrescence*
Nematode resistance	*T. nigrescence, T. ambiguum,*
Virus resistance	*T. ambiguum*

* Adapted from Williams et al. (2007) and Abberton (2007).

Quantitative trait loci (QTL) have been identified for several traits in white clover, including seed yield and components, plant height, plant spread, leaf length, leaf width, petiole length, leaf area and internode length (Barrett et al. 2005; Cogan et al. 2006). These are likely to be useful in marker-assisted breeding programs.

2.4.2 Genetic modification

White clover has been genetically modified for several traits including White clover mosaic virus resistance (Voisey et al. 1994), Alfalfa mosaic virus (AMV) resistance (Emmerling et al. 2004), insect resistance (Dudas et al. 1998) and altered nutritional quality (Christiansen et al. 2000). A range of other traits are reported to be in development including delayed leaf senescence, nutrient efficiency, aluminium tolerance and bloat safe lines (Anon. 2007a). However, there are currently no commercial approvals of GM white clover.

SECTION 3 MORPHOLOGY

3.1 Plant morphology

White clover is a prostrate legume and tends to be a short-lived perennial but can behave as an annual under moisture stressed conditions (Hutchinson et al. 1995).

The basic structural component of a mature white clover plant is the stolon (an above ground creeping stem with roots at the nodes). The stolon consists of a series of internodes separated by nodes. Each node bears a trifoliolate leaf, two root primordia and, during vegetative growth, an axillary bud which is capable of growing into a lateral stolon. If the node comes into contact with moist soil, adventitious roots may form from the root primordia closest to the ground (Thomas 1987b). The primary root is a shallow tap root with small crowns, which may grow to about one metre (Smoliak et al. 2008).

A 10-week-old seedling consists of a central primary axis of stem and root from which several secondary stolons can grow forming a branched network radiating from the initial seedling. When the primary root dies off, the lateral stolons are nutritionally supported by adventitious roots and become independent plants. The development and establishment of new stolons from the tips of the plant is continuous and older tissue gradually dies off (Thomas 1987b).

Leaf size is highly dependent on type and cultivar of white clover as well as environment, ranging from very small (leaflets less than 1 cm long) in prostrate short petioled types to large (leaflets more than 2 cm) in the more erect, longer petioled types. The cultivars Waverly, Haifa, Quest, and Will Ladino are deemed to have large leaves, while Prestige has small leaves and Tribute intermediate sized leaves (Anon. 2007d; Anon. 2007e; Anon. 2007f; Anon. 2008b). The trifoliate leaves on stolons are arranged alternately and tend to be attached to the side of the stem relative to the surface of the ground. Leaflets are generally elliptical, egg-shaped or heart-shaped with minute serrate margins and are uniformly green or patterned with whitish V-shaped marks and/or purplish colourations (Thomas 1987b).

3.2 Reproductive morphology

Flowers are produced from active apical buds. Each bud can contain up to seven developing leaves but not necessarily with an associating flower head. There may be two to three leaves between flowers (Anon. 2005c). Inflorescences are globular racemes and are supported by long peduncles. Each inflorescence consists of 20-40 florets which are white and commonly tinged with pink. Up to 3-4 seeds per pod can be produced after pollination. The seeds are smooth, heart-shaped and range in colour from bright yellow to yellowish brown, and darken with age (Frame 2003).

SECTION 4 DEVELOPMENT

4.1 Reproduction

White clover possesses two complementary mechanisms of reproduction: regeneration by seedling recruitment and vegetative perennation through the stolon system (Lane et al. 2000). The primary adoption of either one of these mechanisms depends on the influence of the environment on stolon survival. For example, in the cool temperate regions of the UK, reproduction of white clover from seed is rare, and stolon growth is the main means of reproduction. In subtropical environments, stolon survival is less common and instead white clover persists through seedling regeneration (Archer & Robinson 1989). There is an inverse relationship between stolon branching (and hence persistence) and flowering vigour. Therefore, profusely flowering white clover tends to have fewer stolons and has the potential to produce a large number of seeds (Thomas 1987a). The persistence of white clover from one growing season to the next is influenced by the strength of its flowering (Thomas 1987a).

4.1.1 Asexual reproduction

White clover is capable of vegetative reproduction through the generation of stolons. The stolon has two nodal root buds from which roots grow if they come into contact with moist soil. Further branching occurs at the lateral buds. Each growing tip has an apical bud from which leaves, flowers and lateral buds form. When the older stolons die the nodal roots support the new growing tips (Anon. 2005c).

4.1.2 Sexual reproduction

White clover is capable of sexual reproduction through flowering and seed production when environmental conditions are not favourable for vegetative reproduction. Unfavourable environmental conditions that encourage sexual reproduction include drought, cultivation or overgrazing (Archer & Robinson 1989). As a result, sexual reproduction is an important survival strategy for white clover (Archer & Robinson 1989).

Many factors influence flowering, including genotype, photoperiod, temperature, nutrition, grazing management and the amount of moisture in the soil (Lane et al. 2000). Flower development requires the appropriate amount of light and light intensity; abortion of flower formation can occur when days are short and light intensity is low. White clover is generally regarded as a long-day plant (LDP), with inflorescence development also strongly influenced by low temperatures. However, the relative importance of these two factors depends on the geographical origin of cultivars. For example, plants with high latitude origin are more sensitive to long-days, while plants of low latitude origin are more sensitive to low temperatures (Thomas 1987a).

The rate of flower emergence is dependent on the rate of leaf emergence from the apical bud (Thomas 1987a). Under normal conditions, the time of full flower emergence from the appearance of the first leaf is about nine weeks, with one leaf emerging per week (Anon. 2005c). White clover produces flowers from the axillary buds at the nodes, with each flower being made up of 20-150 florets (Lane et al.

2000). Each flower head can produce between 100 and 200 ovoid-truncate seeds (Riffkin et al. 2005).

4.2 Pollination and pollen dispersal

As white clover is predominantly self-incompatible (see Section 1 for details), cross–pollination is essential for significant seed set. Because white clover pollen is not easily dispersed by wind and any airborne pollen does not result in effective pollination, insect pollinators are normally required to transfer pollen between individual clover plants. In Australia, the honey bee (*Apis mellifera*) is the most important pollinator of white clover. A study in western Victoria recorded that 88% of insect visits to white clover flowers were made by honeybees, with the next most common visitors being native bees (*Lasioglossum* sp., 4.3%) and blowflies (Calliphoridae, 3.5%) (Goodman & Williams 1994). The same study found that excluding insect pollinators from white clover resulted in a 30-fold reduction in seed set. *Diptera* (flies) and *Lepidoptera* (butterflies and moths) may visit flowers but are not effective pollinators (reviewed by Harris 1987).

Bees are attracted to the nectar produced by the flower (Anon. 2005c). Cool temperatures, below 18ºC, reduce the nectar flow and thus bee attraction which results in a reduction in pollination activity. Nectar flow and pollen viability are also reduced in periods of water stress. However, a certain amount of stress can reduce vegetative bulk, which in turn may encourage an increase in bee activity leading to increased seed set (Anon. 2005c).

Self-pollen is always present by the time a pollinator visits the flower (possibly up to 50% saturation of the stigma) but self-incompatibility normally prevents self-fertilisation. Additionally, self-pollen that has not been deposited by bees may have inadequate contact with the stigma for pollen germination and fertilisation to occur. Seed set can be increased by artificially pollinating florets by hand pollination or rubbing the flower heads together to promote some selfing, as mechanical damage has been shown to be important in stimulating pollen germination (Harris 1987). There is evidence that bees improve the efficiency of pollen deposits and several visits by bees to a floret maximises pollen tube access to ovules (over 90%) (Rodet et al. 1998). In a report where inflorescences had an average of 54.9 florets, an average of 22.4 florets contained seeds, which represented 86% of florets that had been probed by a bee (Michaelson-Yeates et al. 1997).

Although honey bees can travel up to 10 km and distances of 2.5 km are regularly recorded (Beekman & Ratnieks 2000), when there is an abundant nectar source, the forage area is a lot smaller (Williams 2001). In a study of honey bee foraging on white clover, 60.9% of flower heads visited were within 10 cm of the previous head visited and only 13.6% were beyond 25 m (Weaver 1965). It has been reported that when a bee visits a pollen donor, that particular pollen is deposited on the next 15 to 20 inflorescences visited and then sporadically up to the 50th inflorescence (Marshall et al. 1999). Bees have been reported to visit an average of 2.5-3.5 white clover florets per inflorescence (Weaver 1965; Michaelson-Yeates et al. 1997) and generally the pollen most effective in fertilisation comes from the last pollen donor visited by the bee (Michaelson-Yeates et al. 1997).

Glover (2002) reviewed factors contributing to gene flow in Australian crops, and listed several factors contributing to the likelihood of gene flow. After considering that white clover is out-crossing and bee-pollinated, important factors contributing to cross-pollination between two flowering white clover plants include: the separation distance and relative plot sizes of donor and receptor plants; environmental factors affecting bee activity; availability and attractiveness to bees of other flowers; factors contributing to successful pollination such as presence of competing pollen and pollen viability. Pollen biology is an important component in the assessment of potential for gene flow, and in the evaluation of a need for and the type(s) of pollen confinement strategies such as buffer rows or isolation distances.

There are a number of reports of outcrossing rates for white clover in the scientific literature. Any differences are likely to be due to the cultivars used, experimental design, differences in the size of the pollen source and pollen sink and their spatial arrangement, local topology and environmental conditions (Eastham & Sweet 2002). Pollination rates of 50% were measured between two adjacent blocks of white clover plants at 0.75 m, dropping to approximately 10% at 4.5 m apart (Marshall et al. 1999). Clifford et al. (Clifford et al. 1996) studied gene flow in white clover over two seasons, reporting 0.7% and 1.3% of seed was produced by cross-pollination at 2 m from a pollen source. At 10 m, these figures had dropped to 0.02% and 0.04%. Another study on white clover similarly reported outcrossing rates of 0.68% and 0.02% at 2 and 10 m, respectively (Woodfield et al. 1995). These authors concluded that an isolation distance of 100 m is adequate to minimise gene flow.

Pollen viability is thought to be high. Long term viability of white clover pollen when carried by bees is unknown, although the pollen remains viable for at least 60 mins in a sucrose solution (Erith 1924 as cited in Thomas 1987a).

Separation distances are used for commercial seed production to maintain varietal purity. In the USA, general clover standards for fields of less than 2 ha require an isolation distance of 274 m for foundation seed (maximum 0.1% contamination with other varieties permitted), 137 m for registered seed (maximum 0.25% contamination with other varieties permitted) and 50 m for certified seed (maximum 1.0% contamination with other varieties permitted) (Association of Official Seed Certifying Agencies 2003). In Europe, an isolation distance of 200 m is required (for fields of 2 ha or less) for basic legume seed, or 100 m for certified seed, with half these distances applicable to fields greater than 2 ha (OECD 2008). OECD standards for legumes are consistent with South Australian white clover standards (Smith & Baxter 2002) and the New Zealand seed standards for legumes (MAF Biosecurity 2007).

4.3 Fruit/seed development and seed dispersal

Seed development from pollination to full ripening takes 26 ± 5 days. Seed first starts becoming viable at about day 10 with approximately 90% of seed viable by day 15. The seeds are contained within a 4-5 mm seed pod (Anon. 2008a). The seed is shed once maturity is reached (Harris 1987). Under permanent pasture, seeds are confined largely to the top five centimetres of soil (Hyde & Suckling 1953).

Components that affect seed yield include the number of flower heads per unit area, the number of florets per flower head, number of seeds per floret, and the weight of ripe seeds. Each of these components are dependent on environmental and developmental factors and genotype (Thomas 1987a; Marshall 1994). For example, cold temperatures at flowering increase pollen sterility, high temperatures increase bee foraging and hence increase pollination, increased ovule age at pollination decreases seed set, long day length decreases flower initiation and close grazing during spring minimises stolon shading and can lead to high seed production (Thomas 1987a; Jakobsen & Martens 1994). In general, seed set decreases with decreasing temperatures. Interestingly, seed set is not influenced by pollen age (up to 5 days after anthesis) (Jakobsen & Martens 1994) and it appears that failure of high seed yield is not a result of inadequate pollination or the self-incompatibility system (Cowan et al. 2000).

White clover seeds can be dispersed long distances by human activities and through the digestive tract of birds and grazing animals. Alternatively, short distance dispersal may occur by dehiscence, stock trampling, worms, ants, and to a small extent by wind. Seeds can remain viable after passing through the digestive tracts of sheep, cattle and goats several days after consumption (Suckling 1952; Yamada & Kawaguchi 1971; Yamada & Kawaguchi 1972). Viable white clover seed can also be recovered from birds such as sparrows, pigeons, pheasants and rooks (Krach 1959), and it is eaten by species including crimson and Adelaide rosellas (*Platycercus elegans*) and galahs (*Elophus roseicapilla* syn. *Cacatua roseicapilla*) (Tracey et al. 2007) . However, white clover seed is thought to be relatively unattractive to birds due to its very small size. Ingestion of white clover seeds by earthworms does occur and viable seed is found in worm casts (McRill & Sagar 1973). Distribution by common Australian animals and birds has not been studied. However, ants have been shown to carry white clover seeds in Australian pastures (Campbell 1966).

4.4 Seed dormancy and germination

Mature white clover seed can be either ‘soft’ (ie permeable to water and able to germinate readily) or ‘hard’ (impermeable to water leading to delayed germination); the ratio is dependent on the conditions under which they ripen. If the seed ripens under dry conditions then nearly all seed is in the dormant hard condition, whereas under moist conditions the seed will immediately be able to germinate (Harris 1987). One study which ripened seed under five different relative humidities found that seeds ripened at 70% relative humidity were all soft whereas at 30% relative humidity most were hard indicating a high negative correlation between moisture content of the seed and duration of impermeability (Hyde 1954). In general, most naturally reseeded white clover seed is hard (Harris 1987). Under very humid conditions vivipary has been observed in white clover, where seed germinates on the dried flower heads with little or no dormancy period (Majumdar et al. 2004).

Longevity of white clover seed is highly dependent on the level of hard seed and is influenced by levels of aeration and temperature. Under New Zealand conditions, a five year break from white clover cultivation coupled with either annual cultivation or herbicide treatment is successfully employed in 90% of cases when white clover cultivars are changed for seed production, reducing the volunteer plants to less than 1 per 10 m^2 (Clifford et al. 1990; Clifford et al. 1996). In general, viability is retained for longer at low temperatures. Without active control, viability of hard seed in various undisturbed soil conditions has been shown to be reduced to 1% after 20 years (Lewis 1973) and there are rare reports of white clover seed being viable for longer than 20 years (Harris 1987).

The breaking of seed dormancy is again dependent on the cultivar (adaptability) and environment, and scarification may be required for some white clovers (Harris 1987). Germination of seed occurs in winter and spring and timing is dependent on environmental conditions (Archer & Robinson 1989). Ideally, scarification, pre-exposure to low temperatures (less than 15°C) and adequate levels of moisture will ensure maximum germination of white clover seeds. The maximum growth rate of white clover plants occurs at about 24°C but is very similar between 18°C and 30°C (Harris 1987).

4.5 Vegetative growth

As discussed in Sections 3.1 and 4.1.1, white clover is capable of vegetative reproduction through the generation of stolons and stolon growth and survival has been shown to be vital as a regenerative strategy for white clover in determining the persistence of white clover in pastures (Hutchinson et al. 1995). Stolon growth ensures that the white clover plant persists as a perennial. As discussed earlier, a branched network of stolons radiates out from the primary plant and each stolon establishes itself as an independent plant (Thomas 1987b). Vegetative spread by stolon formation has led to patches of clonal white clover up to 2 m wide in sand dunes in Scotland (Harberd 1963), although this spread does depend on the availability of areas of bare ground (Clatworthy 1960 as cited in Harris 1987). Drops in soil moisture lead to a decline in white clover mass but once soil moisture conditions improve in cooler months, surviving stolons resume growth. Maximum growth potential of white clover is only achieved by stolon survival as seedling growth is slow and variable (Archer & Robinson 1989). White clover differs from red clover and alfalfa in that it is capable of continuous leaf generation (Anon. 2005a).

Branching of stolons occurs at the lateral buds. The apical bud at the growing tip of the stolon is where the leaves, flowers and lateral buds form. More branches produce more growing tips thus more laterals, leaves and subsequently flowers. Branching from the lateral buds is stimulated by the formation of nodal roots and light. Conversely, shading and a lack of nodal development reduce branching (Anon. 2005c).

The cultivar characteristics determine the height, leaf and flower size of the plant. Large apical buds also produce larger leaves, usually earlier in the growing season. Towards the end of the growing season, leaf and flower size decreases (Anon. 2005c).

SECTION 5 BIOCHEMISTRY

5.1 Toxins

White clover is not a pathogen and is not capable of causing disease in humans, animals or plants. However, white clover can potentially be toxic to grazing animals if ingested in large quantities or under particular situations because of the presence of toxic and anti-nutritional compounds.

5.1.1 Bloat

Some legumes including white clover can cause bloat in ruminants. Bloat occurs if foams containing gases form and stabilise in the rumen during microbial fermentation leading to respiratory and circulatory malfunction. Ingestion of foliage containing high levels of starch and carbohydrates may promote bloat, and saponins, colloidal particles, and soluble proteins present in white clover may all play a role in bloat (Hart 1987; Lane et al. 2000).

5.1.2 Cyanogenesis

Cyanogenesis is the release of hydrogen cyanide from damaged plant tissue. White clover is polymorphic for cyanogenesis and therefore populations can be composed of both acyanogenic and cyanogenic individuals. White clover plants that are cyanogenic contain the cyanogenic glucosides laminarin and lotaustralin and the enzyme linamarase which is responsible for hydrolysing cyanogenic glucosides and releasing hydrogen cyanide (Hart 1987). The cyanogenic phenotype is dependent on two complimentary dominant genes; one which determines the production of cyanogenic glucosides and the other which determines the production of linamarase (Collinge & Hughes 1984; Hart 1987). Levels of cyanogenic activity differ among cyanogenic plants and, although this variation does have a heritable basis, it also can be influenced by other factors such as light intensity, plant maturity, temperature, moisture stress and phosphorous application (Vickery et al. 1987).

The level of the glucosides in the foliage may be sufficiently high for grazing animals to suffer from direct cyanide poisoning but the risk of this is low. However, cyanide metabolites interfere with selenium metabolism which in turn causes problems in ruminants. Highly cyanogenic white clover has been implicated in nutritional myopathy, a muscle wasting condition of young rapidly growing animals, from exposure to highly cyanogenic forage during pregnancy (Lehmann et al. 1991; Gutzwiller 1993). High levels of cyanogenesis may also increase the risk of goitre formation in ruminants such as sheep depending on body iodine reserves (Gutzwiller 1993). It is recommended that only white clover with levels of less than 700 μg hydrogen cyanide/g dry matter be used to ensure limited disruption to selenium metabolism (Lehmann et al. 1991). The white clover plants benefit from limited levels of cyanogenesis by having increased resistance to insect herbivory and increased seedling establishment (Pederson & Brink 1998).

5.2 Allergens

The pollen and foliage of white clover are described as suspected allergens in unsubstantiated reports found during web searches, and specific white clover pollen allergy tests are reportedly available internationally. However, a search of medical literature for references to white clover allergenicity (PubMed, September 2008) found only one report of a single subject showing an eczematous skin reaction to patch testing with white clover leaves (Jovanovic et al. 2003).

5.3 Other undesirable phytochemicals

Levels of phytoestrogens, including isoflavones (Genistein, daidzein, formonontein, biochanin-A) and coumesterol produced by other clovers are associated with reproductive problems in female cattle and sheep (Adams 1998), but there have been only sporadic reports of these conditions being linked to white clover (Wright 1960). In white clover, levels of phytoestrogens are low (0.01%-0.06% of dry matter). Formononetin comprised 90-95% of the total estrogenic isoflavones, with small amounts of genistein detected (Saloniemi et al. 1995). Coumestrol, an oestrogenic flavonoid, is present in white clover and can interfere with reproduction or have a growth enhancing effect on foetuses (Saloniemi et al. 1995). Most white clover cultivars have little oestrogenic activity, although fungal infection has been shown to increase levels of coumestans and produce detectable effects in mice (Wong et al. 1971).

5.4 Beneficial phytochemicals

White clover is high in protein and minerals (Table 3). It contains 22-28% crude protein, 2.7-3.3% crude fat, 9.4-11.9% ash, 6.6-7% lignin and a crude fibre content of 15.7-21.1% (Anon. 2005a). It is deemed more digestible than other temperate forage legumes and therefore the ingested nutrients can be used more efficiently. Tannins accumulate in the flowers of white clover but not in the leaves or stolons (Anon. 2005a). As animal forage, white clover functions optimally as a 10-20% component when grown in conjunction with other grasses (Anon. 2005a).

Table 3. White clover mineral composition *

Chemical constituent	Content range (g/kg DM)
Nitrogen	26.6-5.3
Phosphorus	1.9-4.7
Potassium	15.4-38.0
Magnesium	1.4-4.8
Sulphur	2.1-4.3
Calcium	12.0-23.1
Sodium	0.5-4.6
	Content range (mg/kg DM)
Iron	102-448
Molybdenum	1.3-14.2
Manganese	40-87
Copper	5.4-9.7
Zinc	22-32
Boron	26-50

* data compiled from OECD (Anon. 2005a) and Frame and Newbould, 1986, Frame 2003

SECTION 6 ABIOTIC INTERACTIONS

6.1 Nutrient requirements

White clover requires good levels of nitrogen, sulphur, potassium and molybdenum especially when grown with other grasses. High levels of nitrogen can inhibit white clover growth (Anon. 2008b).

6.2 Temperature requirements and tolerances

The soil temperature should ideally be above 10ºC, with the maximum growth of white clover occurring between 18 and 30ºC. High temperatures in the first months of growth can also limit establishment. Sensitivity to temperature can be somewhat cultivar dependent (Anon. 2008b). In cooler climates, white clover tends to exist as a perennial/biannual while in the warmer climates it exists as an annual.

6.3 Water use efficiency

White clover is intolerant to long periods of waterlogging and is sensitive to drought (Anon. 2008b). Unlimited water supply can lead to excess leaf development resulting in a shading canopy. This, in turn, can lead to ovule abortion, a late maturing crop and infection by fungal diseases. Tolerance to drought depends somewhat upon cultivar and on whether the crop is grown as fodder or for seed production. Severe water deficit affects vegetative and reproductive growth/processes, but moderate water deficit enhances some (Bissuel-Belaygue et al. 2002a; Bissuel-Belaygue et al. 2002b). However, inadequate water will result in a reduction in the number of stolons produced per plant as well as phytomers (structural subunit made up of a leaf, an internode, a node and an axillary bud) per stolon (Bissuel-Belaygue et al. 2002a).

6.4 Other tolerances

Grazing/cutting can be detrimental to white clover although some cultivars (eg Tribute) can be more persistent under hard grazing. White clover does not grow well in shady conditions and, although it can adapt to shade, the adaptation is more successful if it occurs gradually. Moderate to hot fires will kill or severely thin white clover, and as white clover does not bury its seed, the recovery after fire can be patchy and depends on the growth stage of the plant (McGowen 1997).

White clover seeds are small and have low reserves for sustaining seed development, and sowing too deeply or in compacted soil can have adverse effects on germination and establishment (Murray & Clements 1993). White clover does not tolerate soil that is too acid or too alkaline, it performs optimally in soils with a pH range of 5.6-6.5 (in water) (Anon. 2008b).

SECTION 7 BIOTIC INTERACTIONS

7.1 Weeds

Six main problems as a result of weed infestations in commercial white clover production have been identified: a reduction in seed yield through competition with weeds for water, nutrient and space availability; contamination of seed resulting in rejection from certification; rejection from overseas markets; cost associated with herbicide application; possible development of resistance to the herbicides; chemical and herbicide use can potentially lead to environmental and social problems (Riffkin et al. 2005).

Grower groups have identified four major weed species that are problematic in the clover seed production industry (Riffkin et al. 2005) and these are discussed below.

Annual ryegrass (*Lolium rigidum*) has been found to be particularly difficult to control in 4-5 year old stands of clover. Effective control methods include grazing regimes and application of herbicides such as trifluralin, metolachlor, glyphosate, imazethapyr, carbetamide and clethodim.

Sowthistle (*Sonchus oleraceus*) is a late season germinating weed and is a problem in 2-3 year old stands, especially since it overlaps with white clover flowering, making herbicide or control through grazing difficult. The infestations are worst in bare patches, header rows and on heavy soils. Some effective herbicides include simazine, paraquat, diquat, 2,4-D ester and diflufenican (Riffkin et al. 2005).

Maltese cockspur (*Centaurea melitensis*) is a problem mainly in the first year of clover crops. It favours cultivation and heavier soil types. Late germination results in an overlap in flowering with clover, resulting in contamination of clover seed and subsequent reduction of marketability. MCPA, glyphosate and clopyralid are some of the herbicides used as a control method.

Jersey cudweed (*Pseudognaphalium luteoalbum*), once established, can be very aggressive and has the ability to destroy a white clover stand by the third year. It initially establishes in bare patches on loam and heavier soils. It can also block machinery at the time of harvest. Paraquat, metribuzin, glyphosate and imazethapyr are some of the herbicides used to control the weed (Riffkin et al. 2005).

In general, white clover is not affected by herbicide treatments as they are usually pre-emergence treatments, however high rates of simazine and glyphosate can lead to a reduction in yield (Riffkin et al. 2005). Grower groups have been advised not to use glufosinate in white clover seed crops as it significantly reduces seed yield. The effectiveness of herbicide treatment varies with the farming practices, climatic factors and the presence of resistant weed populations (Riffkin et al. 2005).

7.2 Pests and pathogens

Insects and other invertebrates, fungi and viruses can affect white clover. However, there is only limited knowledge about fungal, viral and nematode influences and their relative effects on productivity in pastures containing white clover (Jahufer 1991). Limitations of growth of white clover probably result from a complex interaction of one or more diseases with other constraints such as moisture stress, soil fertility, grazing pressure and competition (Latch & Skipp 1987). Pest and disease susceptibility is also dependent on the white clover cultivar. For example, the cultivar Haifa is particularly susceptible to infections by Cucumber mosaic virus, Alfalfa mosaic virus, *Sclerotinia* moulds and *Botrytis* moulds (Jahufer 1991). Furthermore, pest and disease problems depend on the region of Australia and the season. For example in West Gippsland, Victoria, lucerne flea is the major pest that attacks white clover in spring (Jahufer 1991).

7.2.1 Insects and other invertebrate pests

The range of invertebrate pests in Australia varies according to location and whether the white clover is used for seed production or grazing. Some important pests which affect the establishment of white clover in Australia include red-legged mite (*Halotydeus destructor*), blue oat mite (*Penthaleus major*), lucerne flea (*Sminthurus viridis*), corbies, pasture web worms and related caterpillars (*Oncopera* spp.), blackheaded pasture cockchafer (*Aphodius tasmaniae*), pink cutworm (*Agrotis munda*) and reticulated slug (*Deroceras reticulatum*). The main pests affecting white clover seed-crops include native budworm (*Helicoverpa punctigera*), clover casebearer (*Coleophora frischella*) and bluegreen aphid (*Acyrthosiphon kondoi*) (Berg 1993). Another pest that requires control and close monitoring is the pea aphid (*Acyrthosiphon pisum*) (Anon. 2007d; Anon. 2007e; Anon. 2007f).

There is little information published on the economic impact of invertebrate pests on white clover productivity. The red-legged mite and lucerne flea may have the greatest impact on white clover productivity especially in the establishment of pastures in Victoria (Berg 1993). Short-lived insecticides such as organophosphate are the main chemicals used to control invertebrate pests in white clover (Berg 1993), and pre-planting insecticide treatment of seeds can assist establishment and reduce plant losses due to attack of insects such as the red legged earth mite (Anon. 2008b).

Nematodes may significantly reduce white clover performance by reducing root growth and nitrogen fixation, and by stunting both leaf and stolon growth (Lane et al. 2000). Examples of nematodes found in white clover swards in the subtropics of Australia include the root-knot nematode (*Meloidogyne* spp.) the clover cyst nematode (*Heterodera trifolii*) and a free-living nematode (*Helicotylenchus dihystera*) (Zahid et al. 2001). Other nematodes that attack white clover include the stem nematode (*Ditylenchus dipsaci*), and root lesion nematodes. The cultivar Will Ladino has been developed to resist stem nematodes (Anon. 2008b).

7.2.2 Vertebrate pests

As discussed in Section 4.3, white clover seed is eaten by a range of birds. Kangaroos, rabbits and possums are pests known to decrease yield of improved pastures and can be assumed to feed on white clover, although this is not specifically documented in the scientific literature.

7.2.3 Pathogens

Fungal disease is associated with damage to taproots and stolons and can lead to the subsequent death of these structures. The fungus *Sclerotinia trifoliorium* causes clover rot and can have a great impact on white clover productivity. Outbreaks occur most years although the incidence and severity of the disease varies from year to year. Other minor fungal diseases include grey mould, which is caused by the fungus *Botrytis cinerea* and wart disease, which is caused by the fungus *Physoderma trifolii* (Clarke 1999b). Fungi that cause leaf spot diseases in white clover include *Leptosphaerulina trifolii* (Pepper spot), *Pseudopeziza trifolii* (Common leaf spot), *Cymadothea trifolii* (Black/Sooty spot), *Stemphylium spp.* (Stemphylium leaf spot), *Stagonospora spp.* (Stagonospora leaf spot), *Peronospora trifoliorium* (Downy mildew) and *Erysiphe trifolii* (Powdery mildew) but all rarely cause significant losses (Clarke 1999c).

7.2.4 Viral diseases

The presence of White clover mosaic virus (WCMV), Alfalfa mosaic virus (AMV) and Clover yellow vein virus (CYVV) in clover is widespread throughout Australia and all have an impact on white clover productivity. AMV can cause severe losses, with reports of up to 60% damage to pasture legumes (Garrett 1991). AMV and CYVV are transmitted by aphids only whereas WCMV is not aphid- or seed-borne but is readily spread by machinery (Garrett 1991). Incidences of viral infection in stands older than two years are commonly 20% or more (Clarke 1999a), although in one study, infection of white clover by AMV and WCMV exceeded 90% (Norton & Johnstone 1998) and in another greater than 86% infection by AMV was found (Mckirdy & Jones 1995). Glasshouse studies have shown dry mass losses of up to

24% due to the presence of WCMV (Clarke 1999a) and up to 60% due to AMV (Kalla et al. 2001). In the field, AMV, CYVV and WCMV may reduce white clover pasture production by up to 30% through reduced foliage yield and quality, reduced nitrogen fixing capacity and reduced vegetative persistence (Kalla et al. 2001).

Other viruses known to infect white clover in Australia include Bean common mosaic virus, Clover yellow mosaic virus, Peanut stunt mosaic virus, Subterranean clover red leaf virus and Rugose leaf curl virus, with many other viruses also being detected in white clover (Garrett 1991). Most viruses that affect white clover, are predominantly present in pastures, whereas CYVV is also present in natural environments (Godfree et al. 2004). It is estimated that the agronomic impact of viruses on white clover equates to a loss of $30 million annually to the Australian diary industry (Garrett 1991).

SECTION 8 WEEDINESS

There are a number of attributes that may contribute to making a plant weedy. White clover has some of these weedy characteristics such as a proportion of seeds being hard and able to persist for many years in the soil, high seed output and long distance seed dispersal by animals. Other characteristics of white clover that increase its potential to persist in the environment include its ability to regenerate by either seedling recruitment or by vegetative perennation through the stolon system, profuse flowering throughout spring and summer providing prolonged opportunity for pollen and seed dispersal, and its highly heterozygous nature due to outcrossing, which allows rapid adaptation (See Section 1.5). However, the degree to which these attributes may lead to successful establishment and persistence is highly dependent on environmental conditions as well as genotype.

As discussed in Section 2.3, optimum growth conditions for white clover include an annual rainfall of above 750 mm, a soil pH of >4.5, sufficient levels of phosphorous, potassium, and sulphur in the soil, medium to well drained soils, and temperatures between 18 and 30°C (Harris 1987; Lane et al. 2000; Betts & Ayres 2004).

8.1 Weediness status on a global scale

Weeds are plants that spread and persist outside their natural geographic range or intended growing areas such as farms or gardens. Weediness in Australia is often correlated with weediness of the plant, or a close relative, elsewhere in the world (Panetta 1993; Pheloung et al. 1999). The likelihood of weediness is increased by repeated intentional introductions of plants outside their natural geographic range that increase the opportunity for plants to establish and spread into new environments (eg escapes of commonly used garden plants) (Groves et al. 2005).

The most comprehensive compilation of the world's weed flora is produced by Randall (Randall 2002). Most of the information contained in this book has been sourced from Australia and North American countries but also includes numerous naturalised floras from many other countries. Seventy *Trifolium* species are listed in this book and are categorised as naturalised, weeds and/or environmental weeds and many are found in a number of different countries highlighting the successful spread and establishment of the genus *Trifolium* in many countries. Randall gives a number of descriptors for the weed status of white clover in various countries. These include weed (normally an economic weed), naturalised (self-sustaining and spreading populations with no human assistance), introduced (purposely planted and therefore desirable in certain situations), garden escape (species originating from gardens), and environmental weed (invades native ecosystems).

8.2 Weediness status in Australia

In Australia, white clover is generally classified as a weed in most States (Groves et al. 2003). Persistence, as well as productivity and seed production, of white clover is limited by a number of factors in Australia as outlined by Ayres and Reed (1993). These factors include: limited growth in cold winters; low tolerance to summer moisture stress, local ecotypes more persistent than introduced ecotypes; poor performance in soils with low fertility, high acidity or poor drainage; performance affected by competition with companion grasses and close grazing; and yield and persistence affected by pests and diseases. Summer moisture stress is the primary environmental constraint limiting the persistence and agronomic performance of white clover in Australia (Jahufer et al. 2002). In a study carried out over 30 years, moisture stress caused by low rainfall and/or high temperature, and high stocking rate were determined to have significant detrimental effects on white clover persistence in pastures located in the Northern Tablelands of New South Wales, Australia (Hutchinson et al. 1995).

In summary, a complex interaction between the genotype/phenotype of the white clover, climatic conditions and the physical environment will determine if the white clover can be successful in establishing itself and persisting in a given area.

8.3 Weediness in agricultural ecosystems

Ratings have been given on a State/Territory level for white clover as a weed in agricultural ecosystems and there is a large variation in the ratings (Groves et al. 2003). The State with the highest rating is Queensland which rates white clover as 5, meaning it is known to be a major problem at 4 or more locations within the State. This is due to its presence as a weed in turf. A rating of 3 is given by New South Wales, Victoria and Western Australia, which means white clover is naturalised and known to be a minor problem warranting control at 4 or more locations within a State or Territory, with Western Australia actively controlling populations within some parts of the State. White clover has a rating of 1 in South Australia, which means it is naturalised and may be a minor problem but is not considered important enough to warrant control at any location. White clover is noted to be present in Tasmania, where it is not described as an agricultural weed.

In the US, white clover has been identified as a weed in apple orchards both because it competes with the trees for nutrients and water and also because its flowers are an unwanted attractant of bees at times when orchards are sprayed with insecticides that could harm bees (MacRae et al. 2005).

8.4 Weediness in natural ecosystems

A rating for white clover has been given for its status as a naturalised non-native species in natural ecosystems in each State and Territory of Australia, on the same rating scale as weeds of agricultural ecosystems described above. Under the categories for assessing the status of naturalised non-native species in natural ecosystems, the highest rating given to white clover is 4, which means it is naturalised and known to be a major problem at 3 or fewer locations within a State or Territory (Groves et al. 2003).

As shown in Appendix A, Figure 1, white clover is distributed in many temperate areas of Australia. These areas include potentially sensitive ecosystems such as woodlands and grasslands of montane and subalpine regions, of which some may be areas of national environmental significance and therefore included in the Environment Protection and Biodiversity Conservation Act (1999). However, to date there is limited information on the impact of white clover on native flora and fauna. Potentially, white clover may spread even further into new areas (See Appendix A, Figure 2). White clover tends to establish in areas that have been disturbed and where there is minimal competition from other plant species, such as roadsides and freshly excavated areas (Godfree et al. 2004).

In lowland grasslands of Victoria, white clover is common along roadsides, but tends to be restricted to the shoulder of the road and does not enter the adjacent native grassland verge (Garrett & Chu 1997). In the Bogong high plains, white clover is found in clearings but only at low densities of less than 1% ground cover in moist flushes on ridges, slopes and streamside flats. It has been found around recently disturbed areas around ski lodges. It does not establish under closed shrub canopies (Garrett & Chu 1997).

White clover is common in south eastern Australian subalpine grassland communities and present to a lesser degree in woodland communities (Godfree et al. 2004). In mesic grassland communities, white clover constitutes over 40% of total herb cover. Although white clover does not dominate the invaded communities (Godfree et al. 2006; Godfree et al. 2007), it is considered a significant environmental weed in south eastern Australia, mostly due to high adaptability. Where it is present in semi-natural native plant communities at a relatively high abundance it competes for growing space with native analogues and may even exclude native species altogether by forming a mat. This is especially the case along creek lines or in mesic grasslands (Godfree et al. 2006).

8.5 Control measures

Where white clover is a weed of apple orchards (MacRae et al. 2005) and lawns (Anon. 2005b; Anon. 2007c) it is described as hard to control because of its persistence by stolon proliferation and presence in the soil seed bank. In these situations, control measures focus on reducing the competitiveness of white clover relative to grasses, for example by heavy grazing or close mowing. In high nitrogen soils white clover is not as competitive as in soils where its nitrogen-fixing ability gives it an advantage, hence application of nitrogen fertiliser has also been suggested as a control measure. Where white clover is a weed in native ecosystems, spot application of herbicides and hand pulling are standard control measures. 2,4-D and MCPA known to be effective on white clover (Rolston 1987; Riffkin et al. 2005).

White clover must also be controlled in seed production areas where the cultivar in production is being changed. In this situation, legume seed certification rules require a break in cultivation of at least 3 years combined with monitoring subsequent white clover stands for off-type volunteer plants (OECD 2008). Clifford et al. (1990) found that in New Zealand conditions a 5 year cropping break combined with either annual cultivation to encourage germination or herbicide treatment to kill volunteer plants was necessary to pass contamination requirements in the following white clover seed crop. An increased rate of successful certification of seed crops has been observed after adoption of these practices as industry standards in New Zealand (Clifford et al. 1996).

SECTION 9 POTENTIAL FOR VERTICAL GENE TRANSFER

The possibility of genes transferring from *T. repens* L. to other organisms is addressed below. Potentially, genes could be transferred to: (1) domestically cultivated white clover and naturalised white clover populations; (2) other cultivated and naturalised *Trifolium* species; (3) other plant genera; and (4) other organisms. With particular regard to the possibility of gene transfer to other plants, potential barriers must be overcome before gene flow can occur successfully. *Pre-zygotic* barriers include differences in floral phenology, different pollen vectors and different mating systems such as stigmatic or stylar incompatibility systems. *Post-zygotic* barriers include genetic incompatibility at meiosis, selective abortion, lack of hybrid fitness and sterile or unfit backcross progeny. Even where *pre-zygotic* and *post-zygotic* barriers do not exist, physical barriers created by geographic separation can still limit gene transfer to other plants.

Successful gene transfer requires that three criteria are satisfied. The plant populations must: 1) overlap spatially; 2) overlap temporally (including flowering duration within a year and flowering time within a day); and 3) be sufficiently close biologically that the resulting hybrids are fertile, facilitating introgression into a new population (den Nijs et al. 2004).

9.1 Intraspecific crossing

Cross-pollination of one *T. repens* plant with another mediated via an insect vector is the most likely means by which white clover genes could be dispersed in the environment. In Australia, the honey bee (*Apis mellifera*) is the predominant pollinator of white clover.

As discussed above in Section 1, white clover has a well-developed genetic gametophytic self-incompatibility mechanism with only a small proportion of plants in a population being quite strongly self-compatible and therefore white clover is considered an obligate outbreeder. As a result of the high degree of crossing that occurs between individual plants, white clover populations are composed of a heterogeneous mixture of highly heterozygous individuals (Voisey et al. 1994).

Viable seed and fertile progeny would be produced from cross-pollination of white clover plants, irrespective of whether the white clover plants are in the semi-cultivated environment of a pasture and whether they are present in other areas such as roadsides or native grasslands.

Gene flow between white clover populations is limited by geographic distance as determined by bee foraging ranges. As discussed in Section 4.2, when abundant nectar is available, the foraging range of bees is quite small and successful cross-pollination occurs only over short distances. However, if food is scarce then bees may travel many kilometres increasing the likelihood of long distance cross-pollination.

9.2 Natural interspecific and intergeneric crossing

The species with which *T. repens* is most likely to hybridise and exchange genes are those belonging to the genus *Trifolium*. *Trifolium* species are widely distributed throughout more temperate and subtropical parts of Australia and many clover species are commonly sown in pastures, such as subterranean clover (*T. subterraneum*), arrowleaf clover (*T. vesiculosum*), red clover (*T. pratense*), persian clover (*T. resupinatum*) and strawberry clover (*T. fragiferum*) (NSW Agriculture and Grassland Society of NSW Inc 2001).

Trifolium species with similar karyotypes to *T. repens* have the greatest chance of forming hybrids (Chen & Gibson 1972). Karyotypic differences such as different chromosome numbers or dissimilar linear arrangement of genes in homologous chromosomes, limit gene transfer to other species of *Trifolium*, and therefore crosses generally only occur in nature between different *T. repens* cultivars/genotypes (Chen & Gibson 1971; Williams 1987b). The cross-incompatibility of *T. repens* with related species is due to both pre- and post-fertilisation barriers. Failure to form viable seeds from interspecific crosses may be attributed to the inability of the pollen to germinate on a foreign stigma, the inability of the pollen tubes to grow normally in a foreign style, failure of fertilisation, or seed abortion. It has been shown that pollen tube germination normally occurs in crosses between *T. repens* and many other *Trifolium* species. However pollen tube growth is slow, and swelling, bursting, coiling and/or misdirected growth is a common occurrence (Chen & Gibson 1972). Success in producing hybrids can also be dependent on which species acts as the pollen donor as well as which cultivars are used (see below for examples).

T. repens has successfully been crossed with *T. nigrescens*, *T. uniflorum*, *T. occidentale*, *T. isthmocarpum*, *T. argutum*, and *T. ambiguum* in experimental situations (reviewed by Williams 1987b). However most hybrids were recovered through tissue culture methods and many were sterile or showed abnormal development as discussed below. Of these species, only *T. nigrescens* (ball clover) is able to produce hybrids with white clover following cross-pollination and without further intervention, albeit with some difficulties; many hybrids formed have sterile pollen, have chlorophyll deficiencies or produce non-viable seeds (Chen & Gibson 1972; Williams 1987b; Marshall et al. 1995). The rate of successful hybridisation is greatest when *T. repens* is used as the female parent plant (Hovin 1962). The formation of hybrids is also highly dependent on the cultivar of *T. nigrescens* used in the cross (Hovin 1962).

T. nigrescens is present in Australia (Australia's Virtual Herbarium 2007). However, due to the difficulties of white clover forming viable, competitive hybrids with this species it is extremely unlikely that they would occur in nature.

9.3 Crossing under experimental conditions

There have been numerous unsuccessful attempts to hybridise *T. repens* with many other *Trifolium* species, even when embryo rescue methods were used. These include species commonly found in Australia such as *T. arvense* (hare’s foot clover), *T. fragiferum* (strawberry clover), *T. hirtum* (rose clover), *T. scabrum* (rough clover) and *T. subterraneum* (subterranean clover) (reviewed in Williams 1987b). In more recently published work, embryo culture and colchicine induced chromosome doubling was required to produce fertile hybrids (Hussain et al. 1997).

Hybrids of white clover and *T. ambiguum* (Caucasian or kura clover) have been produced by embryo culture (Williams & Verry 1981; Meredith et al. 1995) and subsequent backcrosses of the hybrids to the parent species have been successful (Anderson et al. 1991; Abberton et al. 2002).

White clover has been artificially crossed with *T. occidentale* (Western clover), most successfully when an induced tetraploid of *T. occidentale* was used (Gibson & Reinhart 1969). In another study, initial *T. repens* x *T. occidentale* hybrids produced using embryo culture gave rise to fertile F2 progeny (Pederson & McLaughlin 1989).

Pollination of *T. repens* by *T. uniflorum* (one flower clover) has been found to result in poor fertilisation rates and high rates of seed failure (Chen & Gibson 1971). However, abnormal and fertile hybrids can be rescued using embryo culture techniques (Pandey et al. 1987).

Of these species, *T. uniflorum* and *T. ambiguum* have both been recorded in Australia (Australia's Virtual Herbarium 2007) with *T. ambiguum* being a relatively common clover in pastures of NSW (Hackney & Dear 2007). However, due to the difficulties of forming hybrids with these species, it is extremely unlikely that *T. repens* would form viable competitive hybrids with other *Trifolium* species in nature.

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APPENDIX A

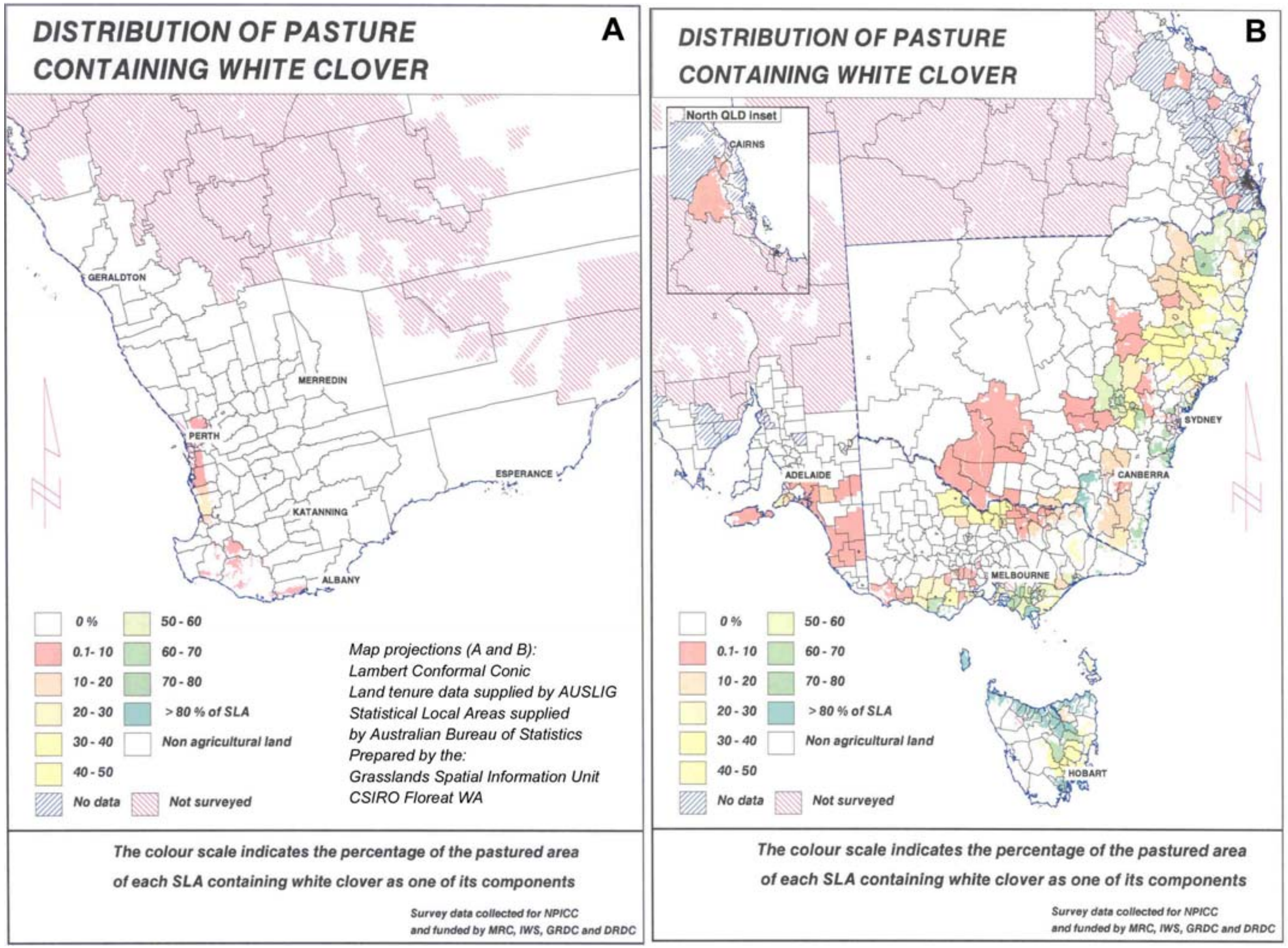

Figure 1. Distribution of white clover in Australian pastures. Maps from the Australian Temperate Pastures Database.

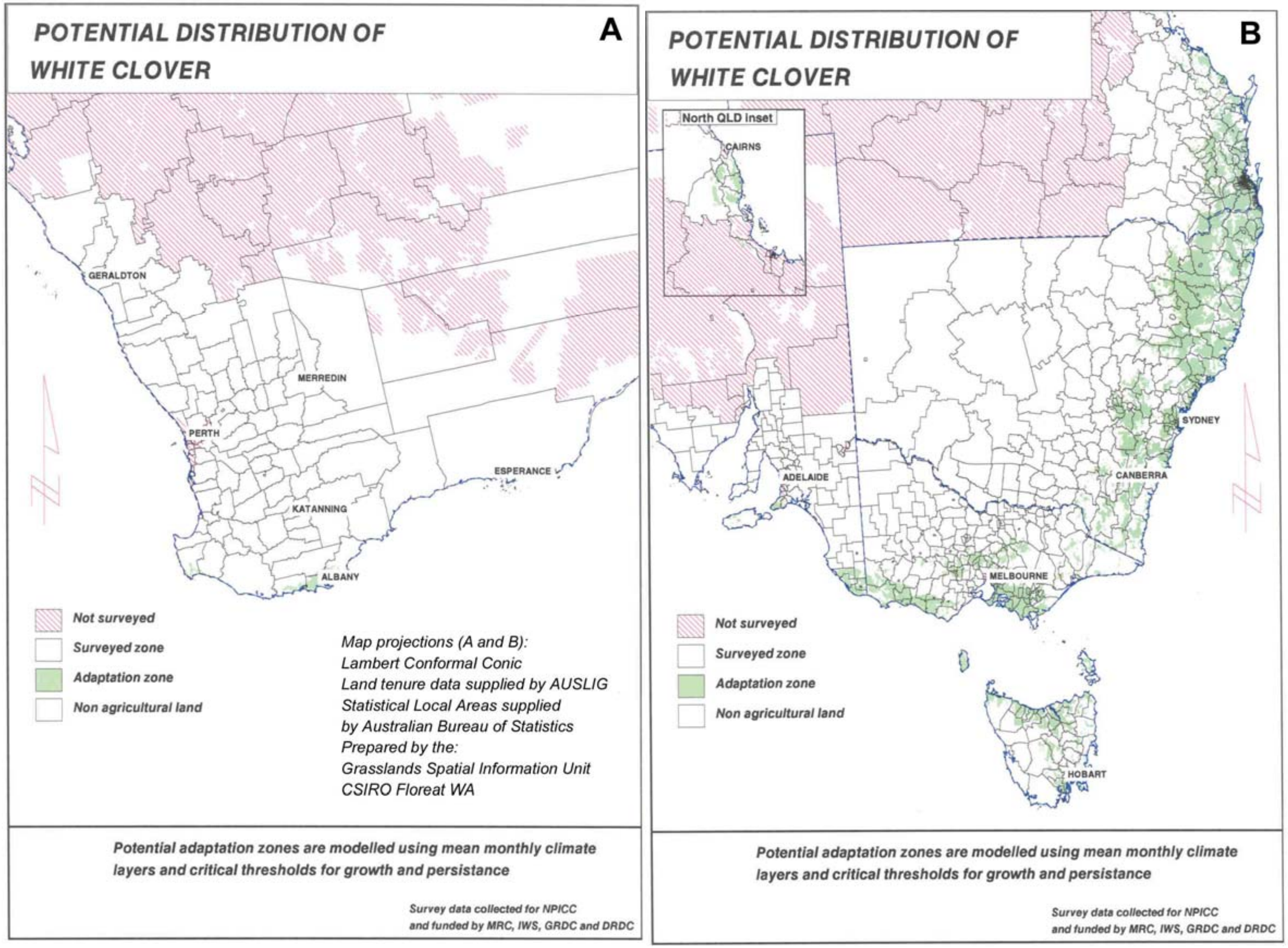

Figure 2. Potential distribution of white clover in Australian pastures. Maps from the Australian Temperate Pastures Database.