Australian Government
Department of Health and Ageing
Office of the Gene Technology Regulator

The Biology and Ecology of White Clover (*Trifolium repens* L.) in Australia

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PREAMBLE

This document addresses the biology and ecology of *Trifolium repens* L. Included is the origin of *T. repens* (referred to as white clover), general descriptions of its growth and agronomy, its reproductive biology, toxicity and allergenicity and its general ecology. 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.

1. BIOLOGY OF WHITE CLOVER

SECTION 1.1 TAXONOMY AND GENETICS

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). White clover cultivars are classified arbitrarily by the size of the plants, as small, intermediate and large. There are a great number of cultivars/varieties with many different adaptations and even individual cultivars are composed of a heterogeneous mixture of highly heterozygous individuals (Voisey et al. 1994).

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).

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).

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 4.2) and therefore cannot gain genetic variation by forming hybrids.

SECTION 1.2 ORIGIN AND DISTRIBUTION

White clover 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 they are now considered naturalised (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).

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). 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 Figure 1 (A, B) shows 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).

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).

SECTION 1.3 USES OF WHITE CLOVER

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 1.5 below) making it suitable for grazing (Frame 2003; NSW Agriculture 2002b).

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.

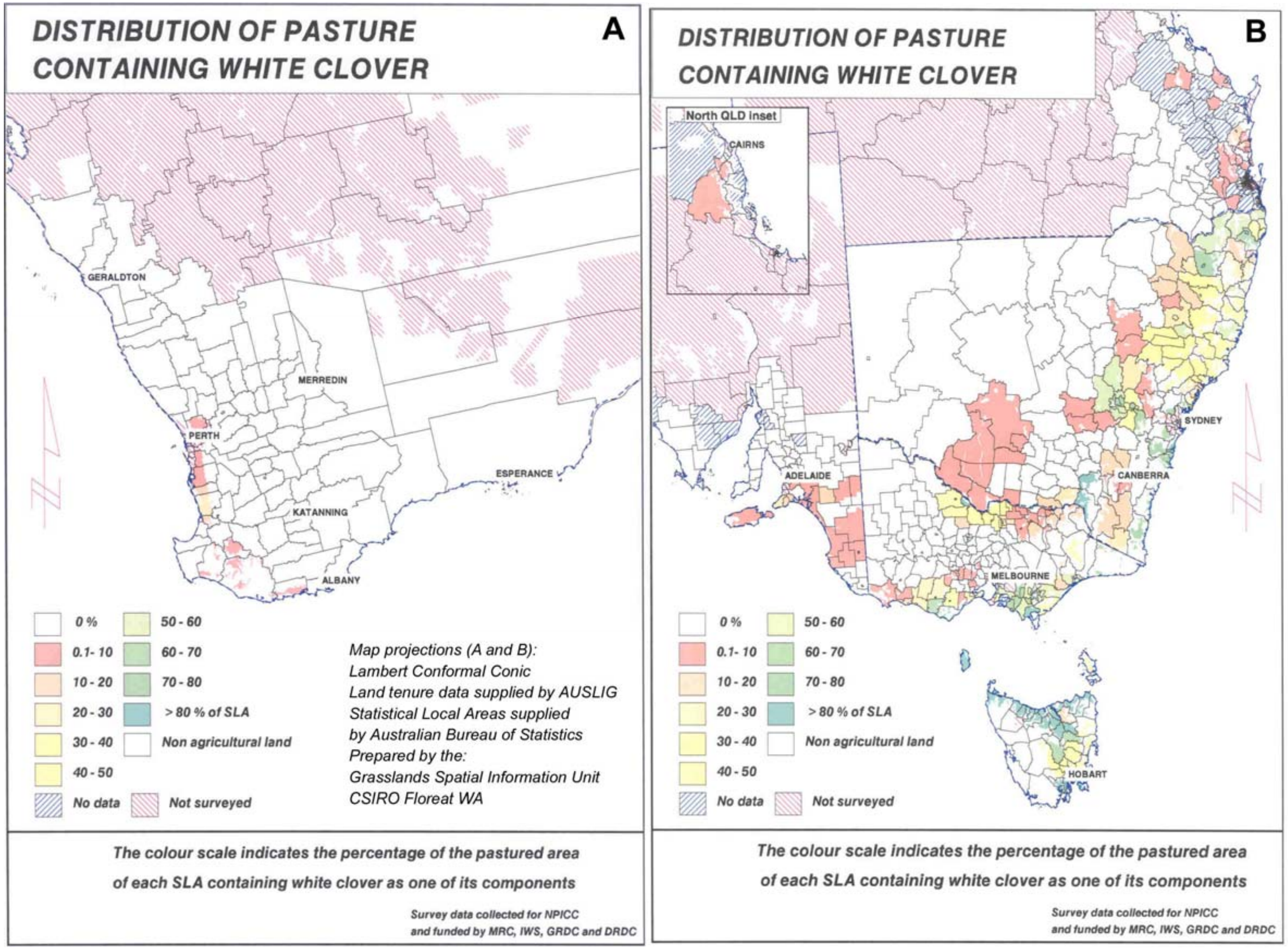

Figure 1. Distribution of white clover in Australian pastures. Maps from the Australian Temperate Pastures Database.

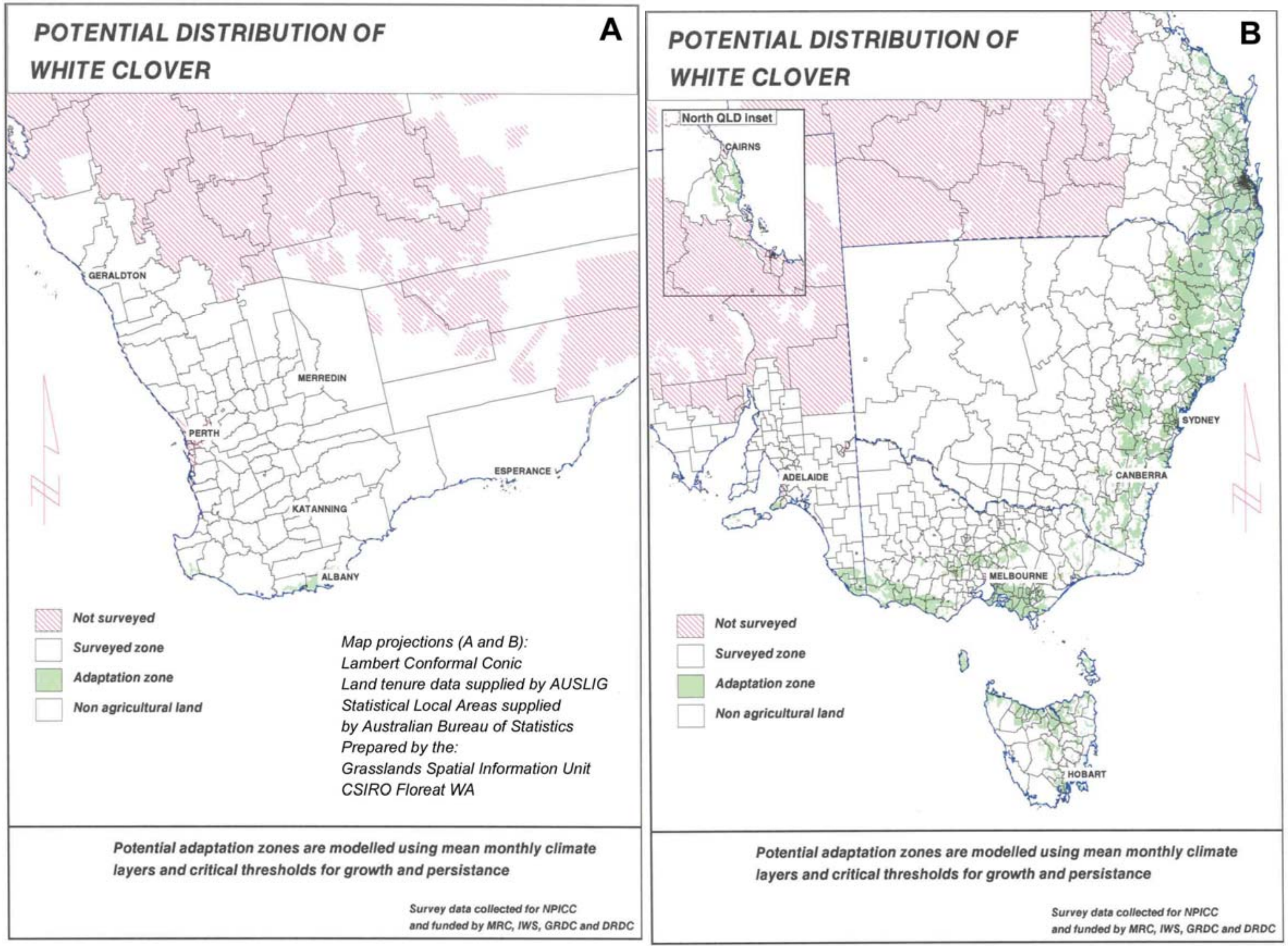

Figure 2. Potential distribution of white clover in Australian pastures. Maps from the Australian Temperate Pastures Database.

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.

However, 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; NSW Agriculture 2002b). Additionally, white clover is adversely affected by several pests and diseases (See Section 1.6) and contains some toxic and anti-nutritional compounds (See Section 2.1).

SECTION 1.4 CULTIVATION

As mentioned above, white clover is predominantly grown in conjunction with grasses. 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 (NSW Agriculture 2002b).

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 and potassium. It prefers soils that have a pH above 4.5 and have good water-holding capacity but are well draining (Frame 2003; NSW Agriculture 2002b). White clover pastures often require fertilisers to maintain productivity. The type of white clover cultivars planted depends on the final use such as 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 to an optimal depth of 10-12 mm. 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. Seed germination occurs in winter and spring.

SECTION 1.5 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. 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). 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 the 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 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).

White clover flowers through the spring and summer. 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 1.6 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. There is an inverse relationship between stolon branching (and hence persistence) and flowering vigour. Therefore, profusely flowering white clover tend to have less stolons and have the potential to produce a large number of seeds (Thomas 1987a).

1.6.1 Pollination

As white clover is predominantly self-incompatible (see Section 1.1 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. The most important natural pollinating agents of white clover are bees. *Diptera* (flies) and *Lepidoptera* (butterflies and moths) may visit flowers but are not effective pollinators (Harris 1987). In Australia, the honey bee (*Apis mellifera*) is the predominant pollinator of white clover. In the absence of pollinators, seed set is very low with reports of 0 to 2.7 seeds produced per flower head.

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 kilometres and distances of 2.5 kilometres are regularly recorded (Beekman & Ratnieks 2000), when there is abundant nectar source, the forage area is a lot smaller (Williams 2001). In a study on 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 and then sporadically up to the 50^{th} inflorescence (Marshall et al. 1999). Bees have been reported to visit an average of 2.5-3.5 white clover florets per inflorescence (Michaelson-Yeates et al. 1997; Weaver 1965) and generally the pollen most effective in fertilisation comes from the last pollen donor visited by the bee (Michaelson-Yeates et al. 1997).

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). 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 metres from a pollen source. At 10 metres, 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 two and ten metres, respectively (Woodfield et al. 1995). These authors concluded that an isolation distance of 100 metres are adequate to minimise gene flow. When separate plots of white clover were grown various distances apart, pollen transfer between the plots were reported to be high over distances less than one metre but low when distances between pollen sources were greater than two metres (Marshall et al. 1999).

Pollen viability is very high with a pollen germination test indicating 98.4% viability of freshly collected pollen (information supplied by applicant). Long term viability of white clover pollen when carried by bees is unknown.

1.6.2 Seed production

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 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 (Marshall 1994; Thomas 1987a). 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 (Jakobsen & Martens 1994; Thomas 1987a). 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).

1.6.3 Seed dormancy

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 germinable (Harris 1987). One study which ripened seed under five different relative humidities found that seed 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).

Longevity of white clover seed 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 to 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. Maximum growth rate of white clover plants occurs at about 24°C but is very similar between 18°C and 30°C (Harris 1987).

1.6.4 Seed dispersal

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 seed can also be recovered from birds such as sparrows, pigeons, pheasants and rooks (Krach 1959) but the seed is relatively unattractive to birds due to its very small size. Ingestion of white clover seeds by earthworms does occur and viable seed are 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).

1.6.5 Stoloniferous regeneration

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). 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).

SECTION 1.7 PESTS AND DISEASES

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).

1.7.1 Fungal diseases

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).

1.7.2 Insects and other invertebrates

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). 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).

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 freeliving nematode (*Helicotylenchus dihystera*) (Zahid et al. 2001). Other nematodes that attack white clover include the stem nematode (*Ditylenchus dipsaci*), and root lesion nematodes.

1.7.3 Viral diseases

The presence of white clover mosaic virus (WCMV), alfalfa mosaic virus (AMV) and 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 2 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). 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).

2. TOXICITY AND ALLERGENICITY OF WHITE CLOVER

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.

SECTION 2.1 TOXICITY

2.1.1 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).

2.1.2 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 the cyanogenic glucosides and releasing hydrogen cyanide (Hart 1987). The cyanogenic phenotype is dependent on two complimentary dominant genes; one which determines the inheritance of the cyanogenic glucosides and the other which determines the inheritance of the 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 determined 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 is low. Instead, cyanide metabolites interfere with selenium metabolism which in turn causes problems in ruminants. High cyanogenic white clover has been implicated in nutritional myopathy, a muscle wasting condition of young rapidly growing animals, by expose to high cyanogenic forage during pregnancy (Gutzwiller 1993; Lehmann et al. 1991). 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).

2.1.3 Phytoestrogens

Coumestrol, an oestrogenic flavonoid, is present in white clover and can interfere with reproduction or have a growth enhancing effect (Saloniemi et al. 1995). However, most white clover cultivars have little oestrogenic activity.

SECTION 2.2 ALLERGENICITY

In terms of allergenicity, white clover pollen is not considered to be an airborne allergen for humans due to the physical characteristics of the pollen. However, physical contact with white clover could potentially trigger an allergic response in some people although there are no reports of any major allergic responses.

3. WEEDINESS OF WHITE CLOVER

There have been numerous attempts to characterise the ‘typical’ traits of weeds (Baker 1965; Bazzaz 1986; Noble 1989; Roy 1990; Williamson & Fitter 1996). These attributes include ability to germinate, survive, and reproduce under a wide range of environmental conditions; long-lived seed with extended dormancy periods; rapid seedling growth; rapid growth to reproductive stage; long continuous seed production; ability to self pollinate but not exclusively autogamous; use of unspecialised pollinators or wind when outcrossing; high seed output under favourable conditions; special adaptations for long distance and short distance dispersal; and being good competitors. However, because environmental conditions have a big influence on these attributes, and other factors such as plant community composition also play a role in invasivness of a plant species, weedy characters alone are not enough to determine if a plant will become a weed. Therefore, the most successful predictors of weediness remain taxonomic affinity to other weedy species and the history of the species weediness elsewhere in the world (Panetta 1993; Pheloung et al. 1999).

SECTION 3.1 WEEDINESS ON A GLOBAL SCALE

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).

SECTION 3.2 WEEDINESS IN AUSTRALIA

In Australia, white clover is generally classified as a weed in most States (Groves et al. 2003). A rating for white clover has been given for its status as a naturalised non-native species in natural ecosystems and agricultural ecosystems in Australia. Under the categories for assessing the status of naturalised non-native species in natural ecosystems, white clover is given a rating of 4 in Australia which means it is naturalised and known to be a major problem at 3 or fewer locations within a State or Territory. The rating for white clover in agricultural ecosystems is 5 which means it is naturalised and known to be a major problem at 4 or more locations within a State or Territory. Individual 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. The State with the highest rating is Queensland, which rates white clover as 5 due to its presence 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. Tasmania does not give a rating for white clover as an agricultural weed probably because it is not considered a problem.

As shown in 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 Figure 2). White clover tends to establish in areas that have been disturbed and where there is minimal competition from other plant specie, 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).

SECTION 3.3 FACTORS INFLUENCING THE WEEDINESS OF WHITE CLOVER

As mentioned above, 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. The impact of the environment is discussed in greater detail below.

Optimum growth conditions for white clover include an annual rainfall of above 750mm, a soil pH of 5.3, 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; NSW Agriculture 2002b).

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 (Ayres & Reed K.F.M. 1991). 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 by 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.

4. POTENTIAL FOR GENE TRANSFER FROM WHITE CLOVER TO OTHER ORGANISMS

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.

SECTION 4.1 GENE TRANSFER TO CULTIVATED AND NATURALISED *T.REPENS*

Cross-pollination of one *T. repens* plant to another mediated via an insect pollinator 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 1.5, 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. This means that white clover populations are composed of a heterogeneous mixture of highly heterozygous individuals (Voisey et al. 1994) highlighting the high degree of crossing that occurs between individual white clover plants.

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 1.5.2, 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.

SECTION 4.2 GENE TRANSFER TO OTHER *TRIFOLIUM* SPECIES

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).

However, well-demonstrated genetic differences, such as dissimilar karyotypes (ie. different chromosome number and 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).

Trifolium species with similar karyotypes to *T. repens* have the greatest chance of forming hybrids (Chen & Gibson 1972). *T. repens* has been crossed with *T. nigrescens*, *T. uniflorum*, *T.occidentale*, *T. isthmocarpum*, *T. argutum*, and *T. ambiguum* but most hybrids were generated through tissue culture methods and many were sterile or showed abnormal development as discussed below.

White clover can cross naturally with *T. nigrescens* (ball clover) albeit with some difficulties and many hybrids formed have sterile pollen, have chlorophyll deficiencies or produce non-viable seeds (Chen & Gibson 1972; Marshall et al. 1995; Williams 1987b). Successful hybridisatio has been 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). 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 (Meredith et al. 1995; Williams & Verry 1981) and subsequent backcrosses of the hybrids to the parent species have been successful (Abberton et al. 2002; Anderson et al. 1991). However, no hybrids of these two species have been found in nature.

White clover has been artificially crossed with *T. occidentale* (Western clover) but it was most successful 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 have produced fertile F2 progeny (Pederson & McLaughlin 1989).

Cross pollination between *T. repens* with *T. uniflorum* (one flower clover) have not produced viable seed and/or viable hybrids (Chen & Gibson 1971; Pandey et al. 1987). Partially fertile hybrids have been produced by embryo culture from crosses of *T. repens* with *T. uniflorum,* again using *T. repens* as the female parent plant (Pandey et al. 1987).

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) (Williams 1987b).

Of the species that can form viable hybrids, T. *nigrescens*, *T. uniflorum* and *T. ambiguum* have been found at one or more sites in Australia (Australia's Virtual Herbarium) with *T. ambiguum* being a relative common clover in pastures of NSW (NSW Agriculture 2002a). 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.

SECTION 4.3 GENE TRANSFER TO OTHER PLANTS

Gene transfer to unrelated plant species is highly improbable because of well documented pre- and post-zygotic genetic incompatibility barriers, as discussed above. The difficulties with which white clover can hybridise with its closest relatives, and the infertility of such hybrids when they are formed, illustrates the reproductive isolation of *T. repens* from other plant groups. To date, there have been no reports of *T. repens* forming hybrids with plants not of the *Trifolium* genus. Thus, the likelihood of gene transfer between *T. repens* and other plant species is negligible.

SECTION 4.4 GENE TRANSFER TO OTHER ORGANISMS

The only by which genes could be transferred from white clover organisms other than plants is by horizontal gene transfer. Such transfers have not been demonstrated under natural conditions (Nielsen et al. 1997; Nielsen et al. 1998; Syvanen 1999). and deliberate attempts to induce them have so far failed (Coghlan 2000; Schlüter et al. 1995).

Transfer of plant DNA to bacteria has been demonstrated under highly artificial laboratory conditions (Gebhard & Smalla 1998; Mercer et al. 1999; Nielsen et al. 1998), but even then only at a very low frequency. Phylogenetic comparison of the sequences of plant and bacterial genes suggests that horizontal gene transfer from plants to bacteria during evolutionary history has been extremely rare, if occurring at all (Doolittle 1999; Nielsen et al. 1998).

Recombination between viral genomes and plant DNA has only been observed at very low levels, and only between homologous sequences under conditions of selective pressure, eg. regeneration of infectious virus by complementation of a defective virus by viral sequences introduced into a genetically modified plant genome (Greene & Allison 1994; Teycheney & Tepfer 1999).

Thus, gene transfer from white clover to organisms other than plants is extremely unlikely. A more detailed review of horizontal gene transfer from plants to other organisms is provided in the risk assessment and risk management plan that was prepared in relation to application DIR 047/2003 for the release of GM white clover into the Australian environment.

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