EFFECT OF PLOIDY ON INTERSPECIFIC HYBRIDISATION BETWEEN *Trifolium repens* L. AND RELATED SPECIES

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ABSTRACT

Two interspecific *Trifolium* hybrids were available at the time this project was initiated. The first hybrid (3*x* H-6909-5) was a sterile triploid obtained from a *T. repens* x *T. nigrescens* cross to transfer clover cyst nematode (*Heterodera trifolii* Goffart) resistance from *T. nigrescens* to *T. repens*. The second was a tetraploid hybrid (4*x* H-435) and its octoploid derivative (8*x* H-435), developed between 4*x* *T. ambiguum* and *T. repens* to transfer genes conferring longevity and virus resistance from *T. ambiguum* to *T. repens*. This was the first partially fertile hybrid reported between the two species. Chromosome doubling had increased pollen stainability in the octoploid clones (8*x* H-435).

The objectives of this project were to generate backcross progenies from these two hybrids at various ploidy levels, and to endeavour to achieve successful flow of genes between the parental species.

The triploid H-6909-5 ($2n=3x=24$) was highly sterile and produced no seed from approximately 3,000 reciprocal crosses to both parental species. It was chromosome doubled by an *in vitro* colchicine method using 0.1% and 0.05% colchicine for 48-72 h, depending on treatment. Three chromosome doubled plants (approximately 10% of the surviving meristems) from treatments with 0.05% colchicine and 48 or 60 h duration of application were obtained. Chromosome doubling resulted in a marked increase in fertility, as pollen stainability was increased from 9.9% in 3*x* H-6909-5 to an average of 89.2% (range 87.7-90.9%) in 6*x* H-6909-5. Subsequent backcrosses of 6*x* H-6909-5 and interbreeding of backcross derivatives resulted in an array of fertile hybrids at 4*x*, 5*x* and 7*x* levels, and some aneuploids. The occurrence of 7*x* BC_1F_1 progeny from the *T. repens* x 6*x* H-6909-5 (4*x* x 6*x*) cross is the first evidence of functional female 2*n* gametes in *T. repens*. The failure or success of different backcrosses, BC_1F_1 x BC_1F_1 and BC_1F_1 x 6*x* F_1 crosses supported a 2 maternal:1 paternal endosperm balance number (EBN) hypothesis to explain seed set in wide crosses. The EBN system will be helpful for predicting the success of future crosses if endosperm failure is the cause of hybrid embryo abortion. Meiotic chromosome pairing in F_1 and BC_1F_1 progeny

indicated the presence of allosyndetic pairing, suggesting that genetic exchange between the two species is possible.

T. nigrescens appeared to be a useful source of clover cyst nematode resistance. In the initial screening a mean number of 23.4 (range 0-150) cysts per plant was recorded for *T. nigrescens* in comparison to a mean number of 149.7 cysts per plant for *T. repens*. Rescreening of sixteen surviving *T. nigrescens* and five *T. repens* genotypes confirmed the initial screening results. H-6909-5 (3*x* and 6*x*) was also screened for clover cyst nematode resistance, and their resistance equalled that of the resistant *T. nigrescens* genotype. H-6909-5 (3*x* and 6*x*) had significantly fewer cysts/g root dry weight than the susceptible *T. nigrescens* and *T. repens* genotypes.

The second array of backcross progenies was generated from crosses involving 8*x* H-435 and *T. repens* and *T. ambiguum*. 8*x* H-435 was only cross fertile with *T. repens* and resulted in 145 seeds from 1,578 reciprocal crosses. Eleven out of 19 initially grown BC_1F_1 plants were all hexaploid ($2n=6x=48$). Mean pollen stainability for nine out of the 19 BC_1F_1's was 32.9% (range 19.1-64.9%). Meiotic chromosome pairing in the 6*x* BC_1F_1 plants averaged 2.1 univalents, 18.1 bivalents, 1.1 trivalents and 1.6 quadrivalents. From meiotic configurations it was not possible to conclude whether chromosomes of the two species had paired autosyndetically or allosyndetically but the occurrence of a high frequency of multivalents (up to three trivalents and four quadrivalents) indicated both types of pairing.

Backcrosses of 6*x* BC_1F_1 plants to *T. repens* resulted in 134 BC_2F_1 seeds from 760 crosses. Three out of seven initially grown BC_2F_1 plants were pentaploids ($2n=5x=40$). Pollen stainability averaged 59.3% (range 44.4-70.1%) for four 5*x* BC_2F_1 plants. On the other hand 6*x* BC_1F_1 x 6*x* *T. ambiguum* crosses did not produce any seed and only two pentaploid plants were obtained from 6*x* BC_1F_1 x 4*x* *T. ambiguum* crosses. One of these had 17.6% pollen stainability while the other did not produce normal inflorescences.

The difficulty encountered in generating 6*x* backcross progeny with 6*x* *T. ambiguum* was overcome by the creation of a fertile "bridging population". However the "fertile bridge" did not eventuate until after two generations of crossing. The 6*x*

BC_1F_1 plants were intercrossed and produced 114 BC_1F_2 seeds from 663 crosses. Two of the six initially grown BC_1F_2 plants were studied for somatic chromosome counts and were found to be hexaploid ($2n$=6x=48). The average pollen stainability was 40.8% for all six BC_1F_2 plants. One of these 6x BC_1F_2 plants was cross compatible as a female with 6x *T. ambiguum* and resulted 17 seeds from 318 reciprocal crosses.

Most of the 6x BC_1F_1 plants combined the rhizomatous and stoloniferous growth habit of the parental species and two of the ten 6x BC_1F_1 showed significant improvement in stolon number, stolon length, shoot dry weight and nodulation over 8x H-435. However, 6x BC_1F_2 are likely to be superior to 6x BC_1F_1 progeny, as they have exhibited better expression of the combined stoloniferous and rhizomatous growth habit, improved fertility, frequent nodal rooting and heavier nodulation than the BC_1F_1 progeny. Consequently the 6x BC_1F_1 plants can either be used directly in the selection programme or as a "fertile bridge" between the two parental species.

This work has resulted in the development of two arrays of fertile backcross progenies by manipulation of chromosome numbers and the production of a range of hybrid plants combining agronomic characteristics of the parent species in varying genome balances and at a range of ploidy levels. It is therefore concluded that initial sterility of the primary interspecific hybrids need not be a barrier to successful inter-breeding.

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CHAPTER 1
INTRODUCTION

White clover (*Trifolium repens* L. $2n=4x=32$) is one of the most important and widely planted forage legumes in temperate regions of the world. It occupies the prime position among the 250-300 species in the genus *Trifolium*. This genus is classified taxonomically in the tribe Trifolieae of the sub-family Papilionoideae, of the family Leguminosae (Gillett, 1985).

Although a temperate species, found in areas characterised by fertile soil and adequate soil moisture for growth, white clover is widely distributed in the world, from the Arctic circle to the subtropics, and has a wide altitudinal range, being reported from up to 6,000 m in the Himalaya range of India (Williams, W. M. 1987a). The centre of origin of white clover is believed to be the Mediterranean region (Vavilov, 1951) from where it spread naturally throughout Europe, Western Asia and Northern Africa. It has been introduced to other temperate regions including China, Mongolia, Korea, Japan, North and South America, Southern Africa, Australia and New Zealand (Williams, W. M. 1987a).

There are two reasons for the valuable contribution of white clover to pastoral farming systems. The first reason is that, as a forage legume, it provides a high quality animal feed throughout the growing season. In general it has less fibre than grasses, a higher ratio of soluble and insoluble carbohydrates, higher protein contents and greater palatability (Minson *et al.*, 1964; Rattray and Joyce, 1969; Ulyatt, 1971; Ulyatt *et al.*, 1977). These quality factors of white clover result in more milk production and increased animal growth rates (Johns, 1966; Brown, 1990).

The second reason for the importance of white clover in pastoral agriculture is its ability to fix biological nitrogen (N_2) in symbiosis with the bacterium *Rhizobium trifolii* Dangeared (Crush, 1987). The fixed nitrogen becomes immediately available for white clover growth, and in a mixed sward also promotes the growth of associated grasses.

Although a perennial, stands of white clover often decline significantly in the second or third year of growth due to the death of its tap root (Westbrook and Tesar, 1955), susceptibility to a number of stress factors including drought (Bryant, 1974; Spencer *et al.*, 1975), various virus diseases (Barnett and Gibson, 1975; McLaughlin and Pederson, 1985; Alconero *et al.*, 1986; Ragland *et al.*, 1986), nematodes (Yeates *et al.*, 1973; Skipp and Gaynor, 1987; Mercer, 1988; Pederson and Windham, 1989), and other root chewing insects (Gaynor and Skipp, 1987).

Some of the *Trifolium* species (Zohary, 1972) possess characteristics such as a rhizomatous root system, perennial tap root and resistance to viruses and nematodes. Of particular importance are *T. ambiguum* and *T. nigrescens* Viv. *T. ambiguum* M. Bieb. (caucasian clover) is tolerant to several viral diseases (Barnett and Gibson, 1975; Jones *et al.*, 1981; Pederson and McLaughlin, 1989), spreads by means of underground rhizomes rather than above ground stolons and has the ability to persist under dry conditions due to its deep well-developed root and rhizome system (Spencer *et al.*, 1975). It is also one of a few species in the genus that exists naturally in diploid, tetraploid and hexaploid forms with a basic chromosome number of $x=8$.

T. nigrescens on the other hand is an annual diploid species ($2n=2x=16$), which is reported to be closely related to *T. repens* (Hovin, 1962a; Chen and Gibson, 1970b). Although used frequently in interspecific crosses with *T. repens* (Brewbaker and Keim, 1953; Trimble and Hovin, 1960; Hovin, 1962a; Chen and Gibson, 1970b; Kazimierski and Kazimierska, 1970; Williams *et al.*, 1978), its potential as germplasm for the improvement of white clover has rarely been exploited. The species was reported to be unpromising for interspecific hybridisation with white clover as it appeared to be highly susceptible to viruses (Gibson *et al.*, 1971) and the hybrids were weakly perennial. However the species was later evaluated for resistance to nematodes and was found to be highly resistant to clover cyst nematode (*Heterodera trifolii* Goffart) (Mercer, 1988) and southern root knot nematode [*Meloidogyne incognita* Kofoid & White (Chitwood)] (Pederson and Windham, 1989).

Interspecific hybridisation of *Trifolium* species has long been suggested as a means of improving commercial white clover cultivars. Numerous attempts have been

made to incorporate economically important characteristics into white clover cultivars through interspecific hybridisation, but with limited success. Barriers to successful hybridisation among *Trifolium* species occur both before and after fertilisation, although post fertilisation barriers are reported to be the main causes of failure (Chen and Gibson, 1972). Even when successful interspecific hybrids between *T. repens* and other *Trifolium* species have been obtained, their potential as useful genetic material for the improvement of standard white clover cultivars has not been exploited. The main obstacles to the use of existing interspecific hybrids include:

a) Small number of primary hybrids or hybrid derivatives;

b) Sterility or very low fertility of the primary or secondary hybrids;

c) Inability to derive segregating F_2 and fertile backcross progeny due to self-incompatibility or crossability problems arising as a result of interspecific genomic incompatibility or differences in ploidy levels; and

d) Limited recombination between interspecific genomes.

At the time this project was initiated, two interspecific *Trifolium* F_1 hybrids were available. The first hybrid (3*x* H-6909-5) was developed from a *T. repens* x *T. nigrescens* cross. The objective of this cross was to transfer clover cyst nematode resistance from *T. nigrescens* to *T. repens* (White and Mercer, unpublished work). This triploid F_1 hybrid (3*x* H-6909-5) was known to be resistant to clover cyst nematode, but was highly sterile and did not produce any seed after backcrossing to the parental species. The first objective of the present research was to develop hexaploid forms of H-6909-5 by an *in vitro* method of colchicine doubling (Anderson *et al.*, 1991c) and to study the effects of chromosome doubling on plant fertility. If fertility was restored to H-6909-5 at the hexaploid level, the second objective was to evaluate the hybrid for clover cyst nematode resistance and generate a wide range of backcross progenies at various ploidy levels for future evaluation of economic parental characteristics.

The second hybrid (4*x* H-435) was developed by Williams and Verry (1981) between 4*x* *T. ambiguum* and *T. repens* and was the first partially fertile hybrid developed between *T. ambiguum* and *T. repens*. This hybrid (4*x* H-435) had been

created to transfer genes conferring longevity, cold and high altitude tolerance and possibly virus resistance from *T. ambiguum* to cultivars of white clover. The hybrid was partially fertile but was only cross fertile with the *T. repens* parent in backcrosses. H-435 had been chromosome doubled (Anderson *et al.*, 1991c) and 8*x* H-435 was a little more fertile than tetraploid clones but, again was only cross fertile with *T. repens* and no confirmed backcross progeny had been obtained with *T. ambiguum*. Therefore it remained uncertain whether the octoploid might be used as a "fertile bridge" between the two parental species.

The third objective of the current study was to create a fertile bridging population between *T. repens* and *T. ambiguum*, preferably at hexaploid level, by backcrossing octoploid H-435 to both of its parental species. As the two species are very difficult to cross, the creation of a "fertile bridge" would overcome the difficulty of crossing these two species and would also remove the need to derive further primary hybrids by using the sophisticated techniques of embryo rescue with nurse endosperm (Williams and Verry, 1981) or ovule culture (Yamada *et al.*, 1989). Once created, the fertile bridging plants at hexaploid level could be efficiently used for transferring characters of interest from one species to another.

The fourth objective of the current research was to estimate the extent of chromosome homology between *T. repens* and *T. nigrescens*, and *T. repens* and *T. ambiguum* by studying the pollen stainability and cytogenetics of F_1 and different backcross populations, and to find possible explanations for the crossability barriers between the species and hybrid derivatives in advanced generations of backcrossing.

CHAPTER 2
LITERATURE REVIEW

2.1 INTERSPECIFIC HYBRIDISATION OF WHITE CLOVER

Species hybridisation is a mean of extending the range of heritable variation which can be exploited by the plant breeders to improve commercial cultivars. Interspecific hybridisation of white clover has long been suggested as a method to improve the species (Brewbaker and Keim, 1953). Potential benefits of hybridisation of white clover with other *Trifolium* species include disease and insect resistance, increased persistence, an improved root system, cold and drought tolerance, the ability to produce reasonable quantities of seed, and seedling vigour.

White clover has been successfully hybridised with three annual and four perennial *Trifolium* species i.e. *T. nigrescens* Viv. (Brewbaker and Keim, 1953; Hovin, 1962a; Kazimierski and Kazimierska, 1970; Williams *et al.*, 1978; Marshall *et al.*, 1995), *T. argutum* Sol. (syn *T. xerocephalum* Frenzl.) (Kazimierski and Kazimierska, 1968), *T. isthmocarpum* Brot. (Kazimierski and Kazimierska, 1972), *T. occidentale* Coomb. (Gibson and Beinhart, 1969), *T. hybridum* (Kruse, 1971; Przywara *et al.*, 1989), *T. uniflorum* L. (Gibson *et al.*, 1971; Pandey, 1957; Pandey *et al.*, 1987), and *T. ambiguum* Bieb. (Williams, 1978; Williams and Verry, 1981; Yamada and Fukuoka, 1986; Yamada *et al.*, 1989). Most of these crosses required embryo rescue, they were obtained with difficulty and the success rates were also very low.

Zohary and Heller (1984) classified *T. repens* in section *Lotoidea* of the genus *Trifolium*. With the exception of *T. argutum*, interspecific hybridisation has so far been achieved only between species of the same section (*Lotoidea*). *T. argutum* has been taxonomically classified in section *Mistyllus* by Zohary and Heller (1984). Several other species in the genus *Trifolium* have also been tried, but with no success (Williams, W. M. 1987c).

Hybridisation between related species is normally prevented by pre-fertilisation and/or post-fertilisation barriers which can be classified into the following groups (Hovin, 1962b;

Evans, 1962b; Chen and Gibson, 1972):

(1) Inability of pollen to germinate on the foreign stigma e.g. by osmotic mismatch of pollen and stigmatic fluid;

(2) Inability of pollen tubes to grow normally in a foreign style;

(3) Inability of pollen tubes to penetrate the embryo sac i.e. failure of fertilisation; and

(4) Seed abortion.

2.1.1 Pre-fertilisation Barriers

Pre-fertilisation incompatibility in interspecific *Trifolium* crosses was observed as a pollen-pistil rejection response involving reduced pollen germination, slow pollen tube growth or distortion (swelling or coiling) and even bursting of pollen tubes (Evans, 1962b; Hovin, 1962b; Chen and Gibson, 1972). Evans (1962b) found that incompatibility of the pollen tube and stylar tissues was the most important pre-fertilisation barrier to successful interspecific *Trifolium* crosses. Hovin (1962b) also found differences in pollen tube growth in the style after interspecific *Trifolium* crosses. In certain crosses pollen tubes were observed near the ovule while in others the pollen tubes did not grow in the style. Chen and Gibson (1972) studied pre-fertilisation barriers to hybridisation of *T. repens* with six other *Trifolium* species by comparing pollen germination, pollen tube growth, and fertilisation following intra and interspecific pollination using *T. repens* as the pistillate parent. As fertilisation occurred in all interspecific crosses, Chen and Gibson (1972) therefore concluded that failure or ineffectiveness of pollen germination and of pollen tube growth are not the primary causes of cross-incompatibility of *T. repens* with other related species. Post-fertilisation barriers which prevent the development of viable progeny must therefore be more significant.

Pre-fertilisation incompatibility varies in intensity depending on the genotypes of the individual plants that are crossed (Evans, 1962b). In a wheat-rye cross, Lange and Wojciechowska (1976) found that dominant alleles of the wheat crossability gene Kr_1 and Kr_2 inhibited the growth of rye pollen tubes in the base of the style and ovary wall preventing fertilisation. Cultivars such as "Chinese Spring" which carry recessive alleles,

crossed with rye more readily than cultivars such as "Hope" which carry dominant alleles. This type of genetically controlled mechanism of pre-fertilisation barriers, however, has not yet been investigated in *Trifolium* interspecific crosses.

Interspecific incompatibility may be unilateral; that is, fertilisation may be attainable if the direction of the cross is reversed (Lewis and Crowe, 1958; Hovin, 1962b). Hovin (1962b) found that diploid pollen of *T. hybridum* did not germinate on stigmas of autotetraploid *T. nigrescens* but the reciprocal cross appeared compatible. Studies with *Prunus* species reported by Perez and Moore (1985) have shown correlations between pollen tube growth rate, pollen size and pistil length such that pollen tubes from a species with short styles and small pollen will be unlikely to achieve fertilisation of a longer styled species.

2.1.2 Post-fertilisation Barriers

Post-fertilisation barriers to interspecific hybridisation have been reviewed by Raghavan (1977). These include zygotic or early embryonic inviability, and disturbances of the normal embryo-endosperm-maternal tissue nutritional balance within the ovule which leads to endosperm failure. Hybrid seedling death, physiological abnormality and hybrid sterility are also, in the broad sense, post-fertilisation barriers to gene flow between different species (Williams, E. G.1987).

Failure of the endosperm leading to the death of a potentially viable embryo is a common result of crosses between *Trifolium repens* and other related species. The sequence of events leading to seed abortion after interspecific pollination has been described for crosses of *T. repens* with *T. semipilosum* (White and Williams, 1976), *T. ambiguum* (Williams and White, 1976), *T. uniflorum* (Chen and Gibson, 1971) and for crosses of *T. nigrescens* with *T. occidentale* (Chen and Gibson, 1974).

In normal seed development the endosperm begins nuclear division before the zygote, giving an initial free nuclear stage. At about the time the embryo reaches the "heart" stage of early cotyledonary development the endosperm is becoming cellular around the embryo. This cellular tissue then proliferates, ahead of the growing cotyledons, towards the chalazal end of the embryo leaving only a thin membranous endosperm layer around the embryo at

seed maturation. In interspecific *Trifolium* crosses the first evidence of abnormality leading towards aborted seed is slow development of the endosperm. Sequentially the endosperm fails to develop signs of specialisation such as the formation of an haustorium. Establishment of the haustorium in the first few days after pollination seems to be important to continuing normal development of the endosperm, since abortion is associated either with failure of the haustorium to form, or its failure to remain effectively attached to adjacent maternal tissues (Williams and White, 1976). The embryo starts degeneration when the endosperm is in a highly collapsed state.

Embryo abortion may not only be related to nutrient starvation in the absence of a functional endosperm, but also to disturbances of the normal water potential gradients of the seed. Smith (1973) showed that the endosperm of *Phaseolus vulgaris* L. had a high osmolarity (0.7 osmolar) at the heart stage and that this decreased to a lower value (0.5 osmolar) at the late cotyledonary stage. Yeung and Brown (1982) also working with *Phaseolus vulgaris* found that during early embryogeny the liquid endosperm had the lowest osmotic value, followed by the embryo and then maternal tissues. Later changes in osmotic potential were associated with accumulation of endogenous abscisic acid in the embryo. These workers pointed out the probable basic roles in nutrient partitioning of tissue interrelationships and the osmotic gradient within the developing seed.

Cooper and Brink (1942) attributed endosperm abortion in the cross *Nicotiana rustica* L. x *N. glutinosa* L. to unsuccessful competition with the maternal tissues for nutrients. Occasionally well developed hybrid endosperm is observed following *T. nigrescens* x *T. occidentale* crosses (Chen and Gibson, 1974) when either the egg has not been fertilised or has failed to develop further. This suggests either that the hybrid embryo may contribute to the endosperm failure or that fusion of both sperm with the polar nuclei produces a viable endosperm.

There is accumulating evidence to support the hypothesis that endosperm success or failure depends on the internal balance of maternal and paternal chromosome sets (Lin, 1984). Johnston *et al.* (1980) proposed an Endosperm Balance Number (EBN) hypothesis to explain endosperm development in interploidy-intraspecific or interspecific crosses. According to this hypothesis, normal endosperm development requires two maternally

derived EBN to one paternally derived EBN i.e. an EBN ratio of 2 maternal:1 paternal. The EBN or "effective ploidies" can be manipulated so as to overcome crossing barriers between different species (Johnston and Hanneman, 1982). An EBN system of this type seems to operate in *Trifolium* (Parrott and Smith, 1986a).

Williams, E. G. (1987) concluded that nutrient and water transfer patterns within the developing seed are controlled by a delicate genetic and physiological interdependence of maternal tissues, endosperm and embryo. Hybridity of the endosperm and embryo, representing the entry of a foreign genome into the system, can apparently alter this balance in one of several ways, with the ultimate result of preventing normal maturation of the embryo.

2.2 CYTOGENETIC STUDIES OF *Trifolium repens*

T. repens has a somatic chromosome number of $2n=4x=32$, representing the tetraploid level. However, cytologically it behaves as a diploid with regular bivalent pairing at meiosis and disomic inheritance of genetic markers (Davies, 1970). Diploid behaviour was suggested by Atwood and Hill (1940) to indicate an amphidiploid origin for the species. However, the two basic sets of $x=8$ were subsequently found to have the potential to pair with each other. This was shown by the occurrence of up to 8 multivalent associations in hybrids of *T. repens* with *T. nigrescens* and *T. occidentale* ($2x$ and $4x$) (Chen and Gibson, 1970a, b). Pairing between the basic genomes of *T. repens* reported by Chen and Gibson supports an autotetraploid origin of the species followed by the development of a genetic system for imposing strict bivalent pairing which could be broken down by changing the genetic background after interspecific hybridisation. However, as pointed out by Williams *et al.* (1982), the genetic suppression of homoeologous pairing might have evolved in an amphidiploid carrying closely related genomes, as appears to have occurred in wheat (Sears, 1976) and tall fescue (Jauhar, 1975). Thus the occurrence of multivalent chromosome associations in triploid or tetraploid F_1 hybrids of *T. repens* does not actually answer the question of auto- or allopolyploid origin of the species. It does, however, provide supporting evidence for the genetic control of preferential bivalent pairing. Some autosyndesis of *T. repens* chromosomes (pairing of homoeologous genomes) has been reported in a hybrid with *T. ambiguum* ($4x$ H-435) by Williams *et al.* (1982). This is consistent with the presence

of a genetic mechanism to control preferential bivalent pairing which breaks down after interspecific hybridisation. Although pairing of homoeologous *T. repens* chromosomes (autosyndesis) in hybrids provides an opportunity for release of new variation by novel recombinational events not possible in the parent species, introgression of genes between two genomes, however, requires allosyndetic pairing (pairing between genomes of different species; Williams *et al.*, 1982). Anderson *et al.* (1991c) reported meiotic studies of plants from the backcross of 4*x* H-435 (4*x* *T. ambiguum* x *T. repens*) to white clover. Chromosomal associations were observed as predominantly bivalents, indicating the presence of allosyndetic pairing. The high frequency of allosyndetic pairing in these backcrosses should allow the recombination of genes from both species.

2.3 THE SELF-INCOMPATIBILITY SYSTEM IN *Trifolium*

Trifolium species have been reported to have a gametophytic incompatibility system based on multiple oppositional alleles of the *S* locus. Among *Trifolium* species white clover has frequently been studied for self-incompatibility. Although usually described as self-sterile, this species shows pseudo self-fertility, i.e. breakdown of the strict *S* allele system (Williams, W. M. 1987b). Atwood (1941c) found pseudo self-compatibility in most plants of white clover. He reported great variations not only among plants but also among the ramets of individual clones. This showed that pseudo self-compatibility was modified by the environment. Chen and Gibson (1973) showed that temperature is an important controlling variable in determining success of self pollination in white clover. At 35°C a large percentage of ovules (76%) were self fertilised after 24 hours in genotypes which were highly self-incompatible at 15°C or 25°C.

Atwood (1940) established experimentally that white clover cross and self-fertility were genetically controlled by a gametophytic incompatibility system with multiple alleles at the *S* locus. The system was first established by East and Mangelsdorf (1925) in *Nicotiana*. It is characterised by independent action of *S* alleles in both the pollen and style. Growth of pollen carrying a given *S* allele is arrested in stigmas bearing an identical allele. A typical diagnostic test for this system was used by Atwood (1940) in *Trifolium repens* as shown below.

Two self-incompatible parent plants are crossed. The F_1 progeny are also self incompatible and every F_1 plant is reciprocally fertile with both parents. The F_1 plants comprise four intra-sterile, interfertile groups (designated by roman numerals). By crossing any F_1 group as a female with each of the other three groups and performing backcross progeny tests to the F_1 and parent plants, the above mentioned pattern is found to be diagnostic for this incompatibility system.

Parents: S_1S_2 (I) x S_3S_4 (II)

F_1: S_1S_3 (III) + S_2S_3 (IV) + S_1S_4 (V) + S_2S_4 (VI)

S_1S_3♀ (III) x S_2S_3 (IV) ⟶ S_1S_2 (I) + S_2S_3 (IV)

S_1S_3♀ (III) x S_1S_4 (V) ⟶ S_1S_4 (V) + S_3S_4 (II)

S_1S_3♀ (III) x S_2S_4 (VI) ⟶ S_1S_2 (I) + S_1S_4 (V) + S_2S_3 (IV) + S_3S_4 (II)

2.3.1 The Cytological Basis of Incompatibility

Trifolium species may have two interference zones active in preventing fertilisation by incompatible pollen, one in the stigma and the other about three quarters of the way down the style (Williams, W. M. 1987b).

Atwood (1941b) observed that between 8 and 20 hours after pollination, incompatible pollen showed a lower rate of germination on the stigma and slower rate of pollen tube growth than compatible pollen. Chen and Gibson (1973) did not find any difference in pollen germination between compatible and non compatible crosses. A stigmatic barrier, if it is common, may be of little significance, because the longest pollen tubes from both

compatible and non compatible pollen were found by Atwood (1941b) to grow equally rapidly for the first hour after pollination, i.e. until they had penetrated three quarters of the style length. Beyond that point compatible pollen tubes continued to grow and some entered the ovary. A few incompatible tubes grew slowly into the lower part of the style but none entered the ovary.

The presence of an interference zone about three quarters of the way down the style length was also noted by Pandey (1955). This zone restricts growth of both compatible and incompatible pollen tubes and was interpreted by Pandey (1955) as a physical barrier rather as a point of exhaustion of the food supply, as suggested by Atwood (1941b). However, since incompatible pollen tubes grew little further than this zone, Pandey (1955) considered that the location of the incompatibility reaction was also close to the interference zone.

Chen and Gibson (1973) obtained no evidence for an interference zone in the style. However, the point of stopping of incompatible pollen tubes varied with temperature. After self pollination, incompatible pollen tubes made steady growth comparable to that of compatible pollen tubes. At 15°C the incompatible pollen tubes stopped growth in the upper and middle part of the style length, and at 25°C arrest occurred in the lower part of the style and upper part of the ovary. No pollen entered the ovules. However, at 35°C cessation of pollen tube growth was less common and a large percentage of the ovules (76% after 24 hours) were fertilised. These results indicated that at low and intermediate temperatures, self-incompatibility in *T. repens* is caused by reduced pollen tube growth. Higher temperatures stimulate pollen tube growth and breakdown or circumvent the incompatibility mechanism. There was no evidence of any difference in style structure, suggesting an interference zone.

2.3.2 Large Nnumber of Multiple Alleles at the *S*-locus

Gametophytic systems of incompatibility are characterised by a large number of *S* alleles. Atwood (1942b) tested two series of white clover plants from his breeding stocks. Results of the first series indicated that of the 26 alleles successfully tested, 25 (96%) were different. The second sample of 41 alleles contained 34 (83%) different alleles. Of the others, no more than two were identical. Atwood (1944) extended these studies to wild populations

of white clover. An isolated 0.4 ha pasture was sampled at intervals of 4.5 m and one allele from 49 plants was tested. Thirty six (73%) were different. A larger pasture area of approximately 40 ha was also sampled at intervals of no less than 100 m, and one allele from each of 49 plants was subsequently tested. Thirty nine (80%) were different. These results indicated that relatively small natural populations also carry large number of *S* alleles. Consequently most members of a population are interfertile.

2.4 SELF-FERTILITY IN *T. repens*

Naturally occurring self-fertility in white clover was first reported by Atwood (1941a) when a plant averaged 100 seeds per head upon self-pollination in the field. Extensive genetic tests indicated that self-fertility was conditioned by a self-compatibility (S_f) factor allelic with the *S* series (Atwood, 1941a, 1942a)

Plants carrying the S_f allele set as much seed on selfing as on crossing and this was found to be partially dominant to the ordinary *S* allele in the style i.e. crosses $S_f S_x$ x $S_x S_x$ gave some seed although not as much as fully compatible crosses (Atwood, 1942a, 1945). However, when the cross $S_f S_x$ x $S_x S_y$ was made only $S_x S_y$ progeny occurred among eight self-incompatible plants tested. Apparently, therefore, the normal oppositional effect was not disturbed in crosses where only a portion of the pollen was inhibited. There was an indication that S_f pollen did not compete as well with S_x pollen on an S_f bearing style and that S_f pollen did not function as well as S_x pollen on $S_x S_z$ stigmas (Williams, W. M. 1987b). Williams, W. M. (1987b) reported that S_f on the style did not stimulate S_x pollen from the same male plant e.g. the cross $S_4 S_8$ x $S_4 S_f$ gave no self-incompatible progeny. The S_f allele thus did not violate the rule of independent action of *S* alleles in the pollen.

2.5 CHARACTERISTICS AND POTENTIAL OF *Trifolium* HYBRIDS

The weakness, sterility and inviability of interspecific hybrids are well known characteristics and, are in fact, a major means by which the integrity of related species is maintained in nature (Stebbins, 1958). However, occasional hybridisation and introgressive genetic exchange between different species has played an important role in the evolution of flowering plants. Hybridisation and ploidy together have been involved in the evolution of

several economically important species e.g. bread wheats. The range of species that can be hybridised artificially has been increased by the use of embryo culture to overcome the post-fertilisation barriers. However, reduced fertility of interspecific hybrids is also a constraint to the use of hybrids among *Trifolium* species.

2.5.1 Hybrid Sterility

Interspecific *Trifolium* hybrids range in fertility from highly sterile to partially fertile. Wide variation is observed not only for different interspecific combinations but for different F_1 derivatives and backcrosses of the same combination (Hovin, 1962a; Williams and Verry, 1981; Pandey *et al.*, 1987). Fertility data for different *Trifolium* hybrids have been summarised and discussed by Williams *et al.* (1982), Williams and Williams (1982), Pandey *et al.* (1987), Anderson *et al.* (1991c).

Williams, E. G. (1987) reported that gametic sterility in hybrids may be chromosomal and/or genetic. Chromosomal sterility is associated with gross meiotic abnormality resulting from failure of distantly related genomes to pair, or from the presence of an uneven number of genomes of different basic number. Alternatively sterility may occur even in the presence of effective chromosome pairing as a result of physiological disturbances during gametogenesis, or of cryptic genetic and structural differences between homoeologous chromosomes which normally assort together in allopolyploid parent species but are segregated during hybrid meiosis (Stebbins, 1971). This latter explanation of hybrid sterility has also been suggested to operate in a *T. ambiguum* (4*x*) x *T. repens* hybrid (Williams *et al.*, 1982).

Functional fertility of F_1 hybrids in backcrosses may differ depending on which of the parent species is involved in backcrossing. For example in backcrosses of reciprocal (*Phaseolus vulgaris* x *P. acutifolius*) hybrids as the female parent, Rabakoarihanta *et al.* (1980) recovered a higher frequency of embryos after pollination by *P. acutifolius* (14%) than after pollination by *P. vulgaris* (4%). Asano (1980) found backcrossing of hybrid lilies as the female was more successful if the original male parent of the hybrid was used again as the male parent in backcrossing. In *Trifolium* interspecific hybrids, fertility may decrease in backcross progeny. For example a backcross of (*T. ambiguum* (4*x*) x *T. repens*) x *T. repens*

showed highly irregular meiosis (10.8 univalents per pollen mother cell, PMC) and reduced (5%) pollen stainability in contrast to the almost regular meiosis (0.7 univalents per PMC) and 23% pollen stainability of the F_1 (Williams *et al.*, 1982). Two backcrossed progenies of (*T. repens* x *T. uniflorum*) x *T. uniflorum* showed complete absence of pollen, although the F_1 hybrid parent had 28% pollen stainability (Pandey *et al.*, 1987).

2.5.2 Chromosome Doubling

When sterile intergeneric or interspecific hybrids are obtained, chromosome doubling is often attempted to restore fertility of the hybrids. Chromosome doubling assures that each chromosome will have a homologous partner, resulting in meiotically stable products in the absence of other fertility regulating mechanisms, such as genome incompatibility or genic influences. Dobzhansky (1951) reported that chromosome doubling works best to restore fertility when the initial F_1 hybrid shows little or no meiotic pairing.

A similar result may be achieved by interspecific crosses between induced autotetraploids. Taylor *et al.* (1963) doubled the chromosome number of *T. diffusum* by the use of colchicine and then crossed it with tetraploid *T. pratense*. The amphidiploids produced an average of 89% pollen stainability. At the diploid level (2*x* *T. pratense* x 2*x* *T. diffusum*) the F_1 hybrids were sterile, and colchicine doubling of these diploid hybrids was not successful. Hovin (1962a) obtained a triploid hybrid between *T. repens* and *T. nigrescens* which gave less than 20% pollen stainability. However, the pollen stainability of this hybrid was increased to more than 90% by chromosome doubling (Hovin, 1963). Pollen stainability of the *T. ambiguum* x *T. repens* hybrid 61 (Williams, 1978) was increased from less than 3% to greater than 50% after treatment of a single inflorescence with colchicine (Williams *et al.*, 1982).

Chromosome doubling does not necessarily restore full fertility immediately if genomic incompatibility (genetic sterility) is also present. For example colchicine doubling of the chromosomes of a highly sterile diploid hybrid of *Ornithopus pinnatus* x *O. sativus* restored only 9% pollen fertility which increased to a maximum of only 18% at the F_4 generation (Williams and DeLautour, 1981). In *Phaseolus* the F_1 hybrid *P. vulgaris* x *P.*

coccineus showed a mean fertility of 21% which rose to only 42% after colchicine doubling, and reached a maximum of 66% in the C_5 generation (Smartt and Haq, 1972).

Chromosome doubling of clovers can be readily achieved with aqueous colchicine by immersion of germinating seeds (Evans, 1955; Berthaut, 1965) or the shoot apex of germinating seedlings (Brewbaker, 1952). However, chromosome doubling at the mature vegetative stage is more difficult. Apical and axillary meristems are well protected by sheathing leaf bases, so that immersion of cuttings in colchicine solution has given limited success (Brewbaker, 1952; Evans, 1955; Berthaut, 1965). Treatment of the mature flowering plant with nitrous oxide under pressure has given higher percentages of chromosome doubling (Berthaut, 1968; Taylor *et al.*, 1976) as has spontaneous chromosomal non-reduction during meiosis (Taylor and Giri, 1983; Parrott and Smith, 1984) but these techniques are limited to genotypes with the ability to produce seeds. Anderson *et al.* (1991b) applied an *in vitro* chromosome doubling technique developed by Dore (1976) for *Asparagus*, to clover species and their hybrids. This technique combined colchicine treatment with shoot proliferation as done by Goldy and Lyrene (1984) with *Vaccinium* spp. to enhance the frequency of meristematic target cells. Chromosome doubling frequencies were as high as 81 and 44% for initial root tips and mature shoots respectively. The doubling of chromosomes increased the pollen stainability of *T. ambiguum* x *T. repens* (H-435) from 2.5 to 33.6%. By contrast, doubling the chromosome number of *T. alpestre* x *T. pratense* and *T. sarosiense* x *T. pratense* hybrids did not produce any increase in pollen stainability (Anderson *et al.*, 1991b).

2.5.3 Confirmation of Hybridity

General intermediacy of morphological and quantitative characters may be insufficient or inappropriate for confirming hybridity, if the parent species are similar or if one parent carries major dominant or epistatic factors (Williams, E. G. 1987). Reduction in fertility is a useful indicator, but the best confirmation is provided by the transmission of distinctive qualitative characters from the pollen parent. Hybrid plants showing high fertility and a strong resemblance to the female parent should be suspected of being the result of self pollination. Examples of male transmitted characters used to confirm hybridity are simple co-dominant genetic leaf-marks, specific leaf isozyme bands and nucleolus organising

chromosomes of distinctive morphology (Williams, 1978, 1980; Williams and Verry, 1981; Pandey *et al.*, 1987). Where definitive male transmitted characters cannot be used and the parents are highly heterozygous, it may be preferable to compare putative hybrids with intraspecific progeny of parents rather than with the parents themselves (Smartt, 1979).

2.6 INTERSPECIFIC HYBRIDS BETWEEN *T. repens* AND OTHER SPECIES

2.6.1 Hybridisation with *Trifolium nigrescens*

2.6.1.1 *Heterodera trifolii* Resistance in *T. nigrescens*

T. nigrescens, also known as ball clover, is an annual, diploid, non-stoloniferous species, occurring in natural pastures of the Mediterranean area (Gillett, 1985). *T. nigrescens* has a diploid chromosome number of 16 (Britten, 1963; Pritchard, 1969). It is an extremely variable, free seeding winter annual legume (Williams, W. M. 1987b). Although it has not yet been used as a useful source of germplasm for white clover improvement, this Mediterranean species has recently been shown to have resistance to clover cyst nematode (Mercer, 1988) and could be used in hybridisation with white clover for incorporating clover cyst nematode resistance in white clover germplasm.

Clover cyst nematode (*Heterodera trifolii* Goffart) is regarded as a serious pest of white clover (Eriksson, 1972) which depresses yield and rate of nitrogen fixation (Yeates, 1977, Watson *et al.*, 1985). Control measures other than crop rotation and host resistance seem impracticable (Skipp and Gaynor, 1987). In a screening test, Yeates *et al.* (1973) were unable to detect any resistance to *H. trifolii* in seven white clover seed lines growing in naturally infested soil as a source of inoculum. Kuiper (1960) and Dijkstra (1971), however, found a number of resistant clones among those selected for persistence in a pasture infested with *H. trifolii*. Resistance appeared to be controlled by more than one gene (Dijkstra, 1971). As very little resistance has been found in *T. repens* cultivars, consideration has been given to other species of *Trifolium* as a source of resistant germplasm (Litz, 1986).

H. trifolii is variously reported as compatible or incompatible with alsike clover (*Trifolium hybridum* L.). Norton and Isely (1967) found no more than 3 cysts per pot of 3

plants among the 20 entries tested. Later, Singh and Norton (1970) also reported a very low production of cysts on alsike clover. Similarly subterranean clover (*Trifolium subterraneum* L.) has been reported both as a host and non host of clover cyst nematode. Norton and Isely (1967) found 3 out of 7 entries were hosts. Yeates *et al.* (1973) found both of two entries to be non hosts but Sikora (1977) reported cyst production on all 17 lines of *T. subterraneum* tested. Norton and Isely (1967) reported one line of *T. ambiguum* to be a host of clover cyst nematode and another to be a non-host. Holtzman and Aragaki (1963) reported Kenya white clover (*T. semipilosum*) to be very susceptible to clover cyst nematode.

As *T. nigrescens* was reported to be resistant to clover cyst nematode (Mercer, 1988), White (unpublished data), crossed *T. repens* and *T. nigrescens*. The F_1 hybrid plants were inoculated with clover cyst nematodes and tested for resistance. Results indicated varying degrees of resistance and susceptibility. One particular triploid hybrid designated as 3*x* H-6909-5 was found to be highly resistant (White and Mercer, unpublished data).

Interspecific hybrids of *Trifolium* have rarely been evaluated for characters other than agronomic growth and fertility. To date there are no published reports on the evaluation of *Trifolium* interspecific hybrids for resistance to *H. trifolii*. Pederson and Windham (1989), however, evaluated seven interspecific *Trifolium* hybrids for resistance to the southern root-knot nematode (*Meloidogyne incognita* Kofoid and White, Chitwood). Hybrids with mean gall indices < 2 were (*T. repens* x *T. nigrescens*) x *T. repens*, *T. isthmocarpum* x *T. repens* and *T. repens* x *T. uniflorum*. Because of its cross-fertility and high level of resistance, Pederson and Windham (1989) found that *T. nigrescens* may be a valuable source of resistance to *M. incognita* for white clover improvement.

2.6.1.2 Hybridisation

T. repens and *T. nigrescens* cross with some difficulty, although certain combinations of plants produce large number of hybrids. Williams *et al.* (1978) reported several hundred putative hybrids after caging plants of each species with a bumble bee and harvesting normal seeds. A number of progeny were confirmed to be triploid (3*x* = 24) interspecific hybrids showing intermediate morphological characters and pollen sterility (0.5-6% stainable pollen). The cross can be more successful when *T. repens* is used as a female parent than

used as a male. Kazimierski and Kazimierska (1970) obtained 69% of ovules developing into mature flowering hybrid plants with *T. repens* as a female, compared with only 8% for the reciprocal cross. Hovin (1962a) obtained 35 seedlings from crosses where *T. nigrescens* was used as a seed parent but all were albino or chlorophyll deficient. Embryo culture did not greatly improve the success of this cross. On the other hand, a reciprocal cross (*T. repens* ♀) generally produced normal seeds and seedlings. Different *T. nigrescens* accessions vary in their effectiveness as pollen parents. Hovin (1962a) found two Italian accessions to be five times more effective than Turkish accessions in pod set and developing embryos per pod. Italian pollen parents also gave more vigorous progenies than the Turkish parents.

Hybrids between *T. repens* and *T. nigrescens* have also been obtained by Keim (1953a, b), Evans (1962a) and Trimble and Hovin (1960). These authors have reported that hybrid plants were intermediate in morphology between the parent species, and showed low fertility. Hybrids were weakly perennial, rooted infrequently at the nodes and may have been more susceptible to viruses than *T. repens* (Gibson *et al.*, 1971).

Studies of chromosome pairing at meiosis in *T. repens* x *T. nigrescens* hybrids indicate some homology between the chromosomes of the two species. Hovin (1962a) studied diakinesis in three triploid hybrids ($2n=3x=24$) and found an average of 4.2 univalents per pollen mother cell (PMC). Similarly, at metaphase I, smeared and sectioned materials gave on an average 2.9 and 2.5 univalents per PMC respectively. The presence of fewer than 8 univalents per PMC may be interpreted as evidence for pairing of *T. nigrescens* and *T. repens* chromosomes. Chen and Gibson (1970b) studied metaphase I in a triploid hybrid plant and found that the average number of trivalents, bivalents and univalents per PMC were 4.3, 3.7 and 3.7 respectively. Occurrence of a high number of definite trivalents may be interpreted as further evidence of homology between the chromosomes of the two species. Although tetrads in the hybrid appeared normal, stainable pollen from 10 plants averaged only 7% (range 1-16%). These hybrids were self sterile. Approximately 500 backcrosses to *T. nigrescens* failed to produce seed but 6 viable seeds resulted from a similar number of backcrosses to *T. repens* using the triploid hybrid with 16% pollen stainability as the seed parent. These seeds produced 6 plants ($2n=28$) with up to 50% stainable pollen. The plants resembled the recurrent parent but were less prostrate and had fewer roots at the

lower nodes than is typical of *T. repens*. The result suggested that further backcrosses might produce progenies from which monosomic *T. repens* could be derived.

Kazimierski and Kazimierska (1970) compared meiosis in green and albino sectors of a sectoral chlorophyll chimaeral hybrid and showed that meiosis was more regular and pollen viability consequently higher in the green sector.

Genetic studies also suggest homology between the chromosomes of the two species. A detailed study of 2 fertile hybrid plants from reciprocal crosses of tetraploid *T. nigrescens* ($2n=4x=32$) and octoploid *T. repens* ($2n=8x=64$) was made by Brewbaker and Keim (1953). F_1 and I_1 progeny of these hybrids generally had 48 chromosomes. One of the hybrid plants (WN2) resulted from a cross between a doubled self-incompatible *T. nigrescens* plant lacking a leaf mark and a self-compatible *T. repens* plant homozygous for a V-mark. The hybrid proved to be self-compatible and, on selfing, segregated non-marked plants. The homozygous recessive unmarked hybrids and inbred plants of WN2 would not have been expected if the parental chromosomes paired within the species. Brewbaker and Keim (1953), therefore, assumed that the chromosomes which carry the "*V*" loci of the parental species paired essentially at random in hybrid WN2.

In a recent study Marshall *et al.* (1995) reported the production of a large number (approximately 300) of F_1 hybrids between *T. repens* and *T. nigrescens* without embryo culture. Somatic chromosomes were counted for fifty of these F_1 plants which were all found to be triploid ($2n=3x=24$). These F_1's were then backcrossed to *T. repens* which resulted in a large number of BC_1F_1 progeny. Chromosome counts, isoenzymes and leaf markers confirmed the backcross origin of these plants. Marshall *et al.* (1995) concluded that the two species seem to be closely related, as they produced a large numbers of F_1 plants and backcross seeds without the use of the embryo rescue technique.

2.6.2 Hybridisation with *Trifolium ambiguum*

Caucasian clover (*T. ambiguum* Bieb.) (forms also known as Kura, Honey and Pellett clover in USA) (Keim, 1954; Kannenberg and Elliott, 1962) is a rhizomatous perennial species with a potentially wide range of adaptation throughout high mountain environments in middle latitudes including cold, temperate inter montane areas, as well as continental

range lands and steppes (Bryant, 1974). Its habitat ranges from valley to sub-alpine in Caucasian Russia, the Crimea and Asia minor (Kannenberg and Elliott, 1962).

2.6.2.1 Polyploidy in *T. ambiguum*

T. ambiguum is one of a few species in the genus exhibiting natural polyploidy. The species naturally exists in diploid, tetraploid and hexaploid forms with a basic number of x=8 (Hely, 1957; Kannenberg and Elliott, 1962; Evans, 1976).

Kannenberg and Elliott (1962) examined the morphological and physiological characteristics of the three different ploidy levels and found overlapping ranges for most of the measurable characteristics, although the mean values were usually distinct for different ploidy levels. They suggested that cytological examination might be the only positive criterion for establishing the ploidy level of a given plant. Kannenberg and Elliott (1962) also reported the existence of interploidal fertility in *T. ambiguum* . They found that, in general, fertility between ploidy levels increased directly with ploidy, the lowest average seed production occurring in the $2x$ x $4x$ cross. The presence of a few $4x$ and $5x$ plants in a predominantly $6x$ population was suggested as being due to cross pollination of $6x$ by $2x$ and $4x$ *T. ambiguum* respectively.

It has been suggested that the optimum level of ploidy may be higher than $6x$ but such forms had not been found in nature until Khoroshailove *et al.* (1987) reported an octoploid ($2n$=64) *T. ambiguum* for the first time from Armenia, after studying the karyotype of 116 seed samples collected from all areas of distribution (in USSR). The most wide spread cytotypes proved to be hexaploid ($2n$=48). Tetraploids were half and diploids a third as frequent. Diploids have so far been found only in the Caucasus, where the whole range of cytotypes can be found.

Date of flowering, persistence and leaflet shape and size varied between populations of different ploidy levels, while productivity was not closely related to ploidy (Dear and Zorin, 1985) though the two most productive lines were hexaploid.

2.6.2.2 Nodulation in *T. ambiguum*

The lack of efficient nodulation is a severe handicap for the utilisation of *T. ambiguum* as a forage legume (Townsend, 1970; Speer and Allinson, 1985). Parker and Allen (1952) were unable to obtain nodule formation with any of 52 *Rhizobium* strains from 26 ecologically related species contained in 23 genera. Similarly 35 diverse strains of *Rhizobium trifolii* - 19 from other *Trifolium* species and 16 from *T. ambiguum* gave ineffective nodulation. Keim (1954) reported that even though *T. ambiguum* might possess small nodules, they did not possess nitrogen fixing bacteria and further noted that no *Rhizobium* bacterium is known that can react symbiotically with *T. ambiguum.* Erdman and Means (1956) found that when *T. ambiguum* was inoculated with rhizobial strains isolated from three Turkish clovers (*T. ochroleucum* Huds; *T. spadiceum* L. and an unknown species), as well as from Turkish soil where *T. ambiguum* had been grown, and then grown in the glasshouse, both the nitrogen concentration and dry matter yield of *T. ambiguum* were increased. Hely (1957) pointed out that most strains of *Rhizobium trifolii* were unable to nodulate *T. ambiguum* and that the effectiveness of the symbiotic association varied both with the host line and bacterial strains. From 64 Australian isolates, effective on *T. repens*, and a number of Turkish strains, Hely (1957) found that a distinctly different group of *R. trifolii* was involved in effective symbiosis with *T. ambiguum.* Furthermore, there were differences in nodulation among ploidy levels; hexaploids exceeding tetraploids which in turn greatly exceeded the diploids in nodulation susceptibility. Using a diploid line of *T. ambiguum* (CPI 2264) and effective strains of *R. trifolii* (CC 231) obtained indirectly from eastern Turkey, Hely (1963) indicated that nodulation began in the second week after inoculation. By the third week after inoculation about 50% of the plants had effectively nodulated. Plants that nodulated later than 4 weeks after inoculation exhibited poor growth. Hely (1963) concluded that in these latter plants the symbiotic relationship was in delicate balance and sensitive to changes in the environment. Subsequently, Hely (1972) suggested that a major step in the domestication of *T. ambiguum* would involve a genetic shift towards prompt nodulation and the development of effective nodules. The strong association between time of initial nodulation and effective nitrogen fixation subsequently prompted the initiation of a selection programme that, in turn, resulted in the release of the diploid cultivar 'Summit' (Hely, 1972).

Evans and Jones (1966) confirmed Hely's work by recording consistent differences in the response to inoculation of diploid, tetraploid and hexaploid forms of *T. ambiguum*, the diploids being the slowest to nodulate. Zorin *et al.* (1976a) indicated that the rhizobial strain CC 2836 isolated from the roots of hexaploid *T. ambiguum* line CPI 53179, was a highly effective inoculant with a hexaploid caucasian clover. Subsequently commercial inoculants have been developed and are readily available (Speer and Allinson, 1985). Dear and Zorin (1985) found that by using superior inoculants, identified by Zorin *et al.* (1976b, c), they were able to overcome the problem of poor nodulation so that the *T. ambiguum* remained well nodulated in the field and had acceptable herbage nitrogen contents (2.3-3.4% N). Rumbaugh *et al.* (1991) released ARS-2678 *T. ambiguum* germplasm which was selected for drought tolerance and high temperature, and for increased nodulation and N_2 fixation activity when inoculated with *Rhizobium leguminosarum* biovar *trifolii*.

2.6.2.3 Virus Resistance in *T. ambiguum*

T. ambiguum has been extensively studied for its response to viral infection and was found resistant to many of the viruses common to other temperate clovers, such as alfalfa mosaic virus (AMV), bean yellow mosaic virus (BYMV), clover yellow mosaic virus (CYMV), clover yellow vein virus (CYVV), peanut stunt virus (PSV), red clover vein mosaic virus (RCVMV) and white clover mosaic virus (WCMV) (Choopanya and Halpin, 1968; Barnett and Gibson, 1975; Jones *et al.*, 1981).

Alconero (1983) detected a greatly reduced effect of BYMV, CYVV, WCMV and RCVMV on *T. ambiguum* plants. In another study Alconero *et al.* (1986) found that 81 *T. ambiguum* accessions persisted better under field conditions because of the rhizomatous growth habit and reduced effects of viruses relative to *T. repens* and *T. pratense*. Pederson and McLaughlin (1989) studied six *Trifolium* interspecific hybrids, 24 F_2 plants from *T. ambiguum* x *T. repens* hybrid 435 and 48 *T. ambiguum* plant introductions and populations for resistance to peanut stunt virus, clover yellow vein virus and alfalfa mosaic virus. Fifteen F_2 plants from *T. ambiguum* x *T. repens* hybrid 435 were resistant to all three viruses. *T. ambiguum* plants comprising all three ploidy groups had 99 and 100% resistance to PSV and CYVV respectively, and were later released as a source of resistant germplasm to these two viruses (Pederson *et al.*, 1991). Pederson and McLaughlin (1989) also suggested that F_2

T. ambiguum x *T. repens* plants may be used as a valuable source of virus resistance for incorporation into adapted white clover cultivars. Anderson *et al.* (1991a) studied the natural incidence of PSV infection in hybrid populations of *T. ambiguum* x *T. repens* and found no resistance in the F_1 hybrids (H-262 and H-435), though one of the four backcrosses to *T. repens* and two 4x *T. ambiguum* populations exhibited no infection.

2.6.2.4 Cold and Drought Tolerance

The ability of *T. ambiguum* to withstand poorly drained soils appears to be superior to that of red clover and white clover (Speer and Allinson, 1985). Bryant (1974) noted that variations exist among *T. ambiguum* strains with respect to their ability to tolerate free standing water. Three strains exhibited a survival rate in excess of 80% when they were flooded for up to 40 days. The species is also tolerant to summer droughts, although it will become dormant during drought-stress conditions. Its ability to persist under dry conditions is undoubtedly due to its deep, well developed root and rhizome system (Spencer *et al.*, 1975). Some evidence suggests that *T. ambiguum*, while most productive when the water supply is adequate, is capable of producing acceptable yields even when water is limiting. Hence Stewart and Daly (1980) reported that a well established sward of tetraploid *T. ambiguum* growing under fertile irrigated conditions in New Zealand, produced a maximum herbage yield of 13,250 kg/ha/year. Under dryland conditions, herbage yields were 12,100 kg/ha/year. Drought resistance appears to be greater in diploid groups than tetraploids and hexaploids (Bryant, 1974).

T. ambiguum is very winter hardy and has been reported to be resistant to the mechanical effects of freezing and thawing (Speer and Allinson, 1985).

2.6.2.5 Persistence, Productivity and Herbage Quality

Agronomic evaluations, particularly those involving comparisons of *T. ambiguum* with other forage legumes, are very limited. Yields obtained from *T. ambiguum* swards generally have been reported to be relatively low, especially in the establishment period. Hence Spencer *et al.* (1975) indicated that under suitable conditions white clover accumulated 3.6 times as much herbage dry matter as *T. ambiguum*. However, following

prolonged moisture stress, most white clover died while *T. ambiguum* remained productive. Lucas *et al.* (1980) compared the establishment and productivity of "Grasslands Maku" lotus (*Lotus uliginosus* Schk.), white clover and *T. ambiguum* on an acidic infertile grassland site in New Zealand. Initially *T. ambiguum* was substantially less productive than the other two species but after 4 years growth, productivity of the three species was similar. Once established, *T. ambiguum* is capable of producing yields that are comparable to those produced by other legumes (Stewart and Daly, 1980). Dear and Zorin (1985) compared the persistence and productivity of 12 *T. ambiguum* lines encompassing three ploidy levels, with *T. repens* and *T. pratense*. All *T. ambiguum* lines persisted throughout the experimental period, whereas the *T. repens* and *T. pratense* cultivars disappeared by the fourth year. Though the control cultivars of *T. repens* and *T. pratense* out-yielded *T. ambiguum* in the first year they were substantially less productive in latter years as their density declined. On the other hand, Bryant (1974) reported top growth of the shorter lived *T. repens* to be superior to that of the longer lived tetraploid *T. ambiguum*. However, under dry conditions, *T. ambiguum* proved to be more productive than white clover and showed less dependence on phosphorus supply.

Allinson *et al.* (1985) compared the nutritional characteristics of *T. ambiguum* with lucerne (*Medicago sativa* L.), birdsfoot trefoil (*Lotus corniculatus* L.), *T. repens*, crownvetch (*Coronilla varia* L.) and cicer milkvetch (*Astragalus cicer* L.) over a period of two years. Digestibility of *T. ambiguum*, as indicated by *in vitro* dry matter disappearance, ranged from 70-82% in 5 separate harvests. These values were consistently higher than those obtained for other legumes with the exception of white clover (cv. Ladino). Concentrations of crude protein in herbage of *T. ambiguum* ranged from 18.3-21.7%, values typical of good quality legume herbage. Similar concentrations have also been reported by FitzGerald (1980), Lucas *et al.* (1980) and Davis (1981).

2.6.2.6 Hybridisation

T. repens is a stoloniferous perennial, which establishes rapidly and produces well in pasture. However, its productivity and persistence are lowered by several viruses (Alconero, 1983; McLaughlin and Pederson, 1985; Alconero *et al.*, 1986; Ragland *et al.*, 1986; McLaughlin and Boykin, 1988; Anderson *et al.*, 1991a) and a shallow fibrous, short lived

tap root system (Westbrooks and Tesar, 1955) which contributes to its susceptibility to drought and root chewing insects (Williams, E. G. 1987). *T. ambiguum* , as noted before, is a hardy, cold tolerant, more deeply rooting long lived rhizomatous perennial (Speer and Allinson, 1985), resistant to several viruses that attack white clover (Barnett and Gibson, 1975) but slow to establish in pasture. Hybridisation between these two species might ideally combine the seedling vigour of *T. repens* with the virus resistance (Barnett and Gibson, 1975), rhizomatous growth habit and persistence of *T. ambiguum* (Williams, E. G. 1987).

Zohary and Heller (1984) classified *T. repens* and *T. ambiguum* in section *Lotoidea* of the genus *Trifolium*. Interspecific hybridisation has mainly been achieved only between species of the same section (Ferguson *et al.*, 1990). Although *T. repens* and *T. ambiguum* were reported to be readily grafted (Hely *et al.*, 1952) and fertilisation was known to occur when species were hybridised (Guravitch, 1949; Chen and Gibson, 1972; Williams and White, 1976, 1977) no fully developed seeds were obtained because of embryo abortion due to endosperm failure.

Keim (1953b) was able to achieve a hybrid combination of *T. ambiguum* (2*x*) and *T. hybridum* (2*x*). However successful *T. repens* and *T. ambiguum* hybrids were not reported until Williams (1978) obtained one confirmed sterile hybrid and one suspected hybrid at the 4*x* level. These hybrids were produced through embryo culture using transplanted nurse endosperm. Two further confirmed hybrids, one with partial fertility (designated as H-435) and one highly sterile (H-262) were obtained by crossing different genotypes of *T. repens* and 4*x* *T. ambiguum* (Turkish accession) (Williams and Verry, 1981). The partially fertile hybrid 435 (23% pollen stainability) had greater overall resemblance to the *T. ambiguum* female parent, though it showed several characters intermediate between the two parents. Upon selfing, the partially fertile (self-compatible) hybrid produced a low frequency of F_2 progeny showing a wide range of morphological variation.

Analysis of meiosis indicated a high degree of bivalent pairing and regular 16-16 chromosome separation at anaphase I in both the sterile and partially fertile hybrids (Williams *et al.*, 1982). Thus low fertility was apparently not caused by gross meiotic abnormality, but may have been caused by genetic deficiencies resulting from an independent assortment of homoeologous chromosomes which normally assort together (Williams *et al.*,

1982). F_2 plants produced after selfing the partially fertile hybrid (H-435) had 32 chromosomes and most were apparently sterile. However, several had up to 6% stainable pollen and produced seeds on selfing and backcrossing to *T. repens*.

Two backcrosses of a partially fertile F_1 hybrid (used as a female) to *T. repens* were obtained (Williams and Verry, 1981), one with 32 chromosomes (6% stainable pollen) and the other with 42 chromosomes (10% stainable pollen).

An attempt was made to restore fertility to sterile F_1 hybrid 61 (Williams, 1978) by colchicine treatment. The pollen stainability was increased from less than 3% to greater than 50% (Williams *et al.*, 1982) but further studies were not completed. Meiotic studies of F_1 hybrids showed that genetic exchange between *T. repens* and *T. ambiguum* is possible and that the two species are closely related.

Embryo culture from a *T. ambiguum* (6*x*) and *T. repens* cross was unsuccessful (Evans, 1983). However, ovule culture of this combination did result in hybrid plants with 40 chromosomes although the hybrid plants were highly sterile (Yamada and Fukuoka, 1986). In another attempt Yamada *et al.* (1989) were able to produce hybrids at the tetraploid level by using ovule culture, but again the hybrid progeny showed high sterility. Additional hybrids between these two species have also been reported by Rupert *et al.*, (1979) and Rupert and Evans (1980a, b).

2.7 UNREDUCED (2*n*) GAMETES IN *Trifolium*

An unreduced or 2*n* gamete is a meiotic product that bears the sporophytic rather than the gametophytic chromosome number. Such gametes result from abnormality during either microsporogenesis (2*n* pollen, diplandroidy) or megasporogenesis (2n eggs, diplogynoecy) (Veilleux, 1985).

The first example of a 2*n* gamete was reported by Gates (1909) in an interspecific *Oenothera* hybrid and they have since been reported in virtually every genus examined (Veilleux, 1985). The importance of 2*n* gametes in the evolution of polyploid plant species has been explained by Harlan and DeWet (1975). Their potential as a breeding and genetic analysis tool has been reported by McCoy and Bingham (1988), Watanabe and Peloquin

(1991) and Jongedijk *et al.* (1991). Although sometimes implicated in sterility, 2*n* gametes have been demonstrated to function in fertilisation in many plants, resulting in the production of progeny of higher ploidy than normally expected, a process known as sexual polyploidisation (Mendiburu and Peloquin, 1976).

An important implication for plant breeding is that 2*n* gametes found by mechanisms that are genetically equivalent to first division restitution will transmit significant heterotic interactions relatively intact, whereas somatic doubling is effectively an inbreeding process resulting in reduced vigour (Mendiburu and Peloquin, 1977b; McCoy and Rowe, 1986; Watanabe and Peloquin, 1991).

The unexpected occurrence of polyploid progeny from 4*x*-2*x* or 2*x* - 4*x* hybridisation has usually been the first indication of functional 2*n* gametes. Quinn *et al.* (1974) reported that a bimodal distribution of pollen size can often be found, with the larger grains containing 2*n* and the smaller grains 1*n* gametes. The presence of dyads and/or triads at the microspore tetrad stage is generally good evidence for 2*n* gametes (Parrott and Smith, 1984). However, there is no simple method for identification of 2*n* gamete formation in megaspore formation. Ploidy analysis of progeny following interploid crosses is the most commonly used guide (Veilleux, 1985).

The various mechanisms by which polyploid gametes are produced have been listed by Rhoades and Dempsey (1966) and Mendiburu and Peloquin (1976). These include:

a. Pre-meiotic doubling

b. Post-meiotic doubling of the gametic chromosomes

c. First division restitution

d. Second division restitution

e. Chromosomal replication after the first meiotic division

f. Apospory - the development of a 2*n* egg from a somatic cell in higher plants.

Although the production of 2*n* gametes has been reported in many species (Veilleux, 1985) and has been extensively studied in crops such as *Medicago sativa* (McCoy, 1982;

McCoy and Rowe, 1986; McCoy and Bingham, 1988) and *Solanum tuberosum* (Mendiburu and Peloquin, 1977a, b; Mok and Peloquin, 1975a, b; Peloquin *et al.*, 1989), there are few reports on the production of 2*n* gametes in *Trifolium* species.

Maizonnier (1972) reported for the first time a 2*n* functional gamete in *T. alpestre*. *T. pratense* has subsequently been reported to show functional 2*n* gametes. Taylor and Giri (1983) found that the frequency of tetraploids from 2*x*-4*x* crosses of red clover in the field was sufficient to conclude that almost any clone of red clover would produce unreduced gametes and subsequently 4*x* progenies provided that monoploid pollen was excluded. They also reported that chromosome associations at metaphase I in crosses of 2*x*-4*x* and 4*x*-4*x* generally had the same range as those in plants doubled by nitrous oxide. However in both of these methods more bivalent, univalent and fewer quadrivalent associations occurred than in tetraploids induced by colchicine.

Parrott and Smith (1984) isolated several 2*n* pollen producing clones of red clover. One diploid clone, a synaptic mutant with parallel spindles produced a high frequency of 2*n* eggs and 2*n* pollen and was, therefore, functional in both 2*x*-4*x* and 4*x*-2*x* crosses. Parrott *et al.* (1985) reported tetraploid *T. pratense* from 2*x*-2*x* crosses. Such tetraploids are obtained through the union of 2*n* male and female gametes leading to the development of bilateral sexual polyploidisation (BSP). These tetraploids might be desirable as they have maximum parental heterozygosity. Tofte and Smith (1989) found that the frequency of aneuploids (23.3%) in the bilaterally derived tetraploids (Bi4*x*) F_2 population of red clover was less than in the tetraploid population derived from colchicine (35-50%) and N_2O (39-46%) chromosome doubling.

Taylor and Wiseman (1987) obtained 36 hybrid plants from 4*x*-2*x* crosses in *T. pratense* and found 2 tetraploid and one pentaploid, presumably through male genetic restitutions and 2*n* female gametes respectively. Bullita and Smith (1992) studied pollen size differences in *T. nigrescens* and found that 39% of the plants showed big pollen at a very low level, the highest being from only one plant out of 217 which produced 3.4% big pollen. In another study Bullita *et al.* (1994) reported the presence of big pollen in 17 out of 20 *T. nigrescens* populations and found only two plants, one each from two different populations, which had produced over 30% big pollen. These plants were used as pollen parents in

crosses with self-incompatible *T. repens*. Thirteen interspecific plants with an average of 4.3% stainable pollen were evaluated for ploidy levels but all were triploid, indicating the absence of functional 2*n* gametes in *T. nigrescens*.

To date there is no published report on functional 2*n* gametes in *T. repens*. Anderson *et al*. (1991c) however, obtained hexaploid first backcross progeny from the 4*x* H-435 (4*x* *T. ambiguum* x 4*x* *T. repens*) x 4*x* *T. repens* backcross, presumably through the production of functional 2*n* gametes at megaspore level in 4*x* H-435.

CHAPTER 3
MATERIALS AND METHODS

3.1 *IN VITRO* CHROMOSOME DOUBLING

3.1.1 Plant Material

An individual interspecific *T. repens* ($2n$=$4x$=32) x *T. nigrescens* ($2n$=$2x$=16) hybrid plant ($3x$ H-6909-5) obtained through embryo culture was provided by Dr. Derek White (AgResearch, Grasslands, Palmerston North, New Zealand) and utilised for *in vitro* chromosome doubling. The $3x$ H-6909-5 is a sterile triploid ($2n$=$3x$=24) hybrid found to be resistant to clover cyst nematode (Mercer and White, unpublished data). Only one plant was available at the time this project was initiated, and so the hybrid was first propagated vegetatively from stolon tip cuttings, rooted in moist potting mix (Appendix 3.1) in 30 x 42 x 5 cm plastic trays and transplanted to 10 cm diameter plastic pots containing potting mix during October and November, 1991. Plants were grown in the glasshouse at 18-25°C under natural day length (Appendix 3.1).

3.1.2 Chromosome Doubling Method

A modification of the *in vitro* technique reported by Anderson *et al.* (1991b) was used to double the chromosome number of $3x$ H-6909-5:

3.1.2.1 Explant Preparation

When the vegetatively propagated plants of $3x$ H-6909-5 had developed several stolons, stem segments with nodes were removed from plants, trimmed to remove most leaf-material, cut into about 3 cm segments and washed with tap water in a beaker with one or two drops of "Teepol" detergent added to the water. The stem segments were then rinsed four times with running tap water to remove all the detergent and trimmed above and below each axillary meristem to a total length of approximately 5 mm.

3.1.2.2 Surface Sterilisation

A modification of the procedure of Anderson *et al.*, (1991b) was used for surface sterilisation. Segments were surface sterilised by immersion in 70% ethanol for 1 minute, rinsing with sterile water for 1 minute, and again immersion in 70% ethanol for 1 minute and rinsing with sterile water for 1 minute. Segments were then further surface sterilised in 5% sodium hypochlorite (10% available chlorine) for 4 minutes, followed by four rinses of one minute each in sterile water. Subsequent manipulations were carried out under aseptic conditions in a laminar flow hood. The stem segments were further trimmed on a pre-sterilised filter paper to include the axillary meristem and a minimal amount of subtending tissues (about 0.5-1 mm total length).

3.1.2.3 Medium for *In Vitro* Culture

WCSP (white clover shoot proliferation) medium was used for *in vitro* culture of meristems. This medium is a modification of B5 medium (Gamborg *et al.*, 1968) and was used in this experiment on the recommendation of Dr. Derek White (personal communication). The WCSP was prepared by adding 0.15 mg of 6-(γ-γ dimethylallylamino) -purine (2iP, a cytokinin) and 0.15 mg of indole-3-acetic acid (IAA, an auxin) to one litre of medium (Table 3.1). The final pH of the medium was adjusted to 5.5 with 0.2N KOH or 0.2N HCl. The medium was solidified with 0.8% agar.

3.1.2.4 Meristem Culture

Trimmed, surface sterilised meristems were precultured, 10-15 meristems per petri dish, on 25 ml of medium without colchicine in 22 x 85 mm pre-sterilised plastic petri dishes for 7 days. Dishes were incubated at 23-25°C under cool white fluorescent tubes (approx. 10 μ Em^{-2} s^{-1}) except during the colchicine treatment. During the preculture period, meristems with bacterial and fungal contaminations were discarded, and the clean meristems were frequently transferred to fresh medium.

Table 3.1 Composition of WCSP medium for *in vitro* meristem culture.

Compound	Amount l^{-1}	Compound	Amount l^{-1}
a. Macronutrients		**c. Vitamins**	
$NaH_2PO_4.2H_2O$	0.170 g	Nicotinic acid	1.0 mg
KNO_3	2.500 g	Thiamine.HCl	10.0 mg
$(NH)_4SO_4$	0.134 g	Pyridoxine.HCl	1.0 mg
$MgSO_4.7H_2O$	0.250 g		
Ferric EDTA ($C_{10}H_{12}N_2NaFeO_8$)	0.040 g	**d. Growth regulators**	
myo-inositol	0.100 g	IAA	150 µg
$CaCl_2.2H_2O$	0.150 g	2iP	150 µg
Sucrose	20.00 g		
b. Micronutrients			
$MnSO_4.4H_2O$	13.2 mg		
H_3BO_3	3.0 mg		
$ZnSO_4.7H_2O$	2.0 mg		
$Na_2MoO_4.2H_2O$	250 µg		
$CuSO_4.5H_2O$	25 µg		
$CoCl_2.6H_2O$	25 µg		
KI	750 µg		

3.1.2.5 Colchicine Treatments

Initially the precultured meristems were transferred to WCSP medium containing 0.10% colchicine for 48 and 72 hours in the dark at 4°C (Anderson *et al.*, 1991b). This method of colchicine application, however, produced a toxic effect on the growth of the meristems. An experiment was set up to find the optimum concentration and duration of application of colchicine with a minimum toxic effect on the growth of the meristems.

Five treatments, comparing different concentrations and durations of colchicine application were applied to the precultured meristems. These treatments are listed below.

Treatment 1: WCSP + 0.10% colchicine for 48 hours

Treatment 2: WCSP + 0.10% colchicine for 72 hours

Treatment 3: WCSP + 0.05% colchicine for 48 hours

Treatment 4: WCSP + 0.05% colchicine for 60 hours

Treatment 5: WCSP + 0.05% colchicine for 72 hours

Treatment 6: WCSP without colchicine

The WCSP media containing 0.10% and 0.05% colchicine were prepared by mixing 50 ml and 25 ml of filter sterilised 2% aqueous colchicine solution in 950 ml and 975 ml of the medium respectively shortly after autoclaving, thus yielding a final colchicine concentration of 0.10% and 0.05% respectively.

All meristems which had received colchicine treatments and the untreated control were incubated in the dark at 4°C for the respective time periods. Following this, meristems were placed on fresh WCSP medium and the dishes were incubated at 23-25°C under cool white light (approx. 10 μ Em^{-2} S^{-1}) for the remaining period of culture. To minimise bacterial and fungal infections the uncontaminated meristems were periodically transferred to fresh medium.

Meristems remained in the medium for 5-7 weeks during which time they developed 3-5 roots with one or two shoots. They were then transferred to pre-sterilised moist sand in 6.5 cm diameter plastic pots. The pots were covered with a plastic bag and kept in a growth chamber at 16°C with a 16 hours photoperiod for four weeks. The young plants were supplied with a half strength (0.9 g/l) of "Thrive" (N:P:K 27:5.5:9) fortnightly. After four weeks in the growth chamber, plants were transferred to 10 cm diameter plastic pots containing potting mix (Appendix 3.1) and were kept in the glasshouse.

Chromosome doubled plants were identified by root tip squashes from vegetative cuttings, pollen fertility and morphology of dry pollen grains and meiotic chromosomes from pollen mother cells.

3.2 CYTOLOGICAL TECHNIQUES

3.2.1 Dry Pollen Shape

Dry pollen shape was examined for triploid and colchicine-treated hexaploid hybrid (H-6909-5) plants to aid ploidy determinations (Taylor *et al.*, 1976). Flowers at anthesis were tripped over a glass slide and dry pollen grains were examined at magnifications of x200 and x 400.

3.2.2 Pollen Stainability

To estimate pollen stainability, which is an assessment of pollen fertility, 2-3 anthers from glasshouse grown plants were dehisced over a glass slide to which a drop of 2% acetocarmine was added. The material was then covered with a cover slip. After 5 minutes of staining, the percentage of plump, fully stained grains was determined. At least 1200 grains from 6 or more flowers and 3 or more inflorescences per plant were examined.

3.2.3 Somatic Chromosome Counts

Somatic chromosome counts were made from root tip squashes by adaptation of the method of Williams (1978). For root tip collection, 10 cm diameter plastic pots containing 3-4 months old plants were placed in another pot of the same size half filled with coarse pre-washed moistened sand. After 7-10 days young root tips emerged through the holes from the bottom of the upper pot and grew in to the sand in the lower pot. Two days after this emergence, 1-2 cm long root tips were cut off and collected in a glass beaker containing distilled water. Root tips were thoroughly washed 3-4 times with distilled water to remove any sand particles and then trimmed to an approximately uniform length of 1 cm. Trimmed root tips were rinsed once with distilled water, pre-treated in 0.004M 8-hydroxyquinoline for 5-7 hours at 4°C and fixed in 3:1 95% ethanol: glacial acetic acid at room temperature. The material was then rinsed twice with distilled water and hydrolysed in 1N HCl at 60°C for 10-12 minutes. Root tips were then stained in Feulgen stain for 15-30 minutes. Stained root tips were squashed in 2% acetocarmine for chromosomal counts at metaphase. At least 10 cells from 5 root tips were examined for each plant.

3.2.4 Meiotic Chromosome Configurations

Meiotic chromosome pairings were studied in pollen mother cells (PMCs) using a revision of the method of Giri *et al.*, (1981). Young inflorescences (about 2 mm in diameter and just after emergence from the stipules) were fixed in Camoy's fluid (6:3:1 95% ethanol: chloroform : glacial acetic acid) for 24 hours at room temperature. Fixed flower buds were rinsed three times with 70% ethanol allowing at least 20 min for each change and stained in alcoholic hydrochloric acid carmine stain (Snow, 1963) for at least 72 h. After rinsing with 70% ethanol, the stained material was stored in 70% ethanol in the refrigerator until used.

Flowers containing pollen mother cells at meiotic stages were removed from the stained inflorescence and the anthers were squashed lightly with a flat needle in a drop of 1% acetocarmine on a glass slide. A cover slip was added and the slide warmed to just below boiling point of the liquid for about 30 seconds. The cover slip was then pressed between two folds of filter paper with progressively increasing pressure. Chromosomal associations were recorded at metaphase I in 15-35 pollen mother cells from at least 10 flower buds from each plant.

Multivalent associations in many *Trifolium* species are often difficult to analyse with certainty (Williams *et al.*, 1982) and so in this study, first the total number of meiotic configurations was counted, then the numbers of definite univalents, bivalents, trivalents and quadrivalents were counted, and finally the uncertain associations, if any, were estimated on the basis of total chromosome complement.

3.3 BACKCROSSES OF 3*x* and 6*x* H-6909-5 TO *T. repens* AND *T. nigrescens*

3.3.1 Plant Material

The following plants were used in backcrosses:

i. *T. repens* x *T. nigrescens* (H-6909-5)

3*x* H-6909-5 (section 3.1.1) and three colchicine derived (CT-1, CT-14 and CT-28)

hexaploid plants of H-6909-5.

ii. *T. nigrescens*

One genotype of *T. nigrescens* (Tn -167, section 3.4.1).

iii. *T. repens*

One genotype of *T. repens* "Grassland Crimson Charm" (CC-1) was obtained from John L. Ford, AgResearch Grasslands, Palmerston North, New Zealand. CC-1 has red and "silver sprite" leaf mark alleles in heterozygous condition ($V^m V^i ; R^l r$). This plant also carried a multifoliolate trait, probably also in heterozygous form. (The expression of this character is variable, J. L. Ford, personal communication). In some backcrosses three other genotypes of *T. repens* (cv. "Grasslands Huia") were used. These plants were obtained from Dr. D. R. Woodfield, AgResearch grasslands palmerston North, New Zealand. These genotypes had no leaf markings.

3.3.2 Pollination

Reciprocal backcrosses of 3*x* H-6909-5 to *T. repens* and *T. nigrescens* were initiated in January, 1992 while reciprocal backcrosses of 6*x* H-6909-5 to *T. repens* and *T. nigrescens* were started in February, 1993. Details of the crossing and selfing procedures used in this and other subsequent experiments are given below.

Crosses were made by hand on potted plants grown in the glasshouse (Appendix 3.1). Before pollination, flowers on the female parent were emasculated by the forceps technique of Williams (1954). The entire corolla, with staminal tube and anthers attached, was gently removed with forceps approximately 2 days before flower opening, leaving the pistil and calyx intact.

Hand pollinations were made by removing the corolla from a pollen-parent flower, and using the group of anthers as a brush held with a pair of forceps, rubbing the anthers on the exposed stigma very gently. Pod development data were recorded as total number of pods developed during the first two weeks after each crossing .

Approximately 4-5 weeks after pollination, mature flower heads were harvested, allowed to dry for 1-2 days and then threshed between two pieces of corrugated rubber. Seeds were placed in packets and stored in a refrigerator at approximately 4°C for about 4 weeks to break the dormancy of the newly harvested seeds.

The backcrossing schemes and terminology in the present studies were adapted from Haghighi and Ascher (1988). The first backcross, termed BC_1F_1, involved the F_1 (3*x* or 6*x*) H-6909-5 as one parent and *T. repens* or *T. nigrescens* as the other parent. The second backcross, termed BC_2F_1, involved one of the BC_1F_1 as one parent and the same parental species used in the first backcross as the second parent.

Another backcross protocol, termed Congruity Backcross (CBC) was also used in the present study. Here the interspecific F_1 (6*x* H-6909-5) hybrid was backcrossed to each of the parental species in alternate generations. For example, 6*x* H-6909-5 was backcrossed with *T. nigrescens*. This BC_1F_1 was then crossed with *T. repens* to yield CBC_2 progeny. No terminology was found in the literature for crossing BC_1F_1 with F_1, and therefore such crosses are referred to as BC_1F_1 x F_1. Progenies from BC_1F_1 x BC_1F_1 intercrosses were termed BC_1F_2.

3.3.3 Self-incompatibility

Self-incompatibility of individual plants in this and all the subsequent experiments was assessed by gently rolling at least 4 bagged flower heads of each plant between the thumb and fingers every day for 3 days after bagging (Williams, W.M. 1987b).

3.3.4 Morphological Characteristics

Morphological characteristics of the 3*x* H-6909-5 and colchicine derived H-6909-5 and backcross progeny in comparison to *T. repens* (CC-1) and *T. nigrescens* (Tn-167) were recorded by visual observations on potted plants growing in the glasshouse. For characters like nodal rooting, leaflet and inflorescence size, and leaf marking, photographs were taken.

3.4 SCREENING *Trifolium repens* x *T. nigrescens* (3*x* and 6*x* H-6909-5) HYBRID FOR CLOVER CYST NEMATODE RESISTANCE

3.4.1 Plant Material

i. *T. repens* x *T. nigrescens* (3*x* H-6909-5) F_1 hybrid (2*n*=3*x*=24, section 3.1.1).

ii. Two chromosome doubled (2*n*=6*x*=48) 6*x* H-6909-5 plants (CT-1 and CT-14) derived from colchicine doubling of 3*x* H-6909-5 (section 4.2)

iii. *Trifolium nigrescens* (line Az 2225)

iv. *T. repens* cv. "Grasslands Huia" (C6484)

Seeds of *T. nigrescens* and *T. repens* cv. "Grasslands Huia" were obtained from the Margot Forde Forage Germplasm Centre, AgResearch Grasslands, Palmerston North, New Zealand. *T. nigrescens* line Az 2225 is an annual diploid (2*n*=2*x*=16) species obtained from Perugia, Italy as exchange germplasm. Grasslands Huia is a commercial white clover cultivar (2*n*=4*x*=32) and a known host to clover cyst nematode (Mercer, 1988).

3.4.2 Initial Screening of *T. nigrescens* and *T. repens*

Seedlings of *T. nigrescens* and *T. repens* cv. "Grasslands Huia" were initially screened for clover cyst nematode resistance/susceptibility to identify resistant and susceptible genotypes in both species for comparison with 3*x* and 6*x* H-6909-5.

3.4.2.1 Seed Germination

Initial screening of *T. nigrescens* was started in the last week of November 1992. Sixty seeds of *T. nigrescens* and 30 seeds of *T. repens* were scarified with P 120 sand paper for 3-5 seconds by hand and placed on moist filter paper in petri dishes containing 1 ppm of the non-systemic fungicide "Captan" (active ingredient captan 800g/kg). The petri dishes were initially kept in a refrigerator for 24 hours and then transferred to a 25°C incubator for germination. Fifty germinating seeds of *T. nigrescens* and 20 germinating seeds of *T. repens*

were transferred to a pasteurised sand/soil mix (Appendix 3.1) in 6.5 cm diameter plastic pots, with one germinating seed per pot. The pots were randomly arranged in 40 x 80 cm steel trays with 35 pots per tray and the trays kept in a glasshouse at a soil temperature of 18-25°C and natural day light for the whole growing period. Water was added to the trays as required so that pots were moistened by capillary action. Nutrients were applied fortnightly using half strength of 0.9g/l "Thrive" (N:P:K 27:5.5:9) in solution form. The trays were drained using capillary wicks.

3.4.2.2 Inoculum Preparation

Glasshouse colonies of clover cyst nematodes were initiated in 1985 by Chris F. Mercer (AgResearch Grasslands Division, Palmerston North, New Zealand) from material collected from pastures at Fitzherbert West, Palmerston North, New Zealand and were maintained on white clover (cv. "Grassland Huia"). Inoculum was prepared from these glasshouse colonies using the method described by Mercer (1990) i.e:

Roots and soil of white clover plants growing in 10 cm diameter plastic pots were washed over nested 2 mm, 600 µm and 180 µm sieves. Cysts were separated from the fine soil on the 180 µm sieve by centrifugation in sugar solution (specific gravity 1.25) at 1150 g for 1 minute. Cysts were placed in a 150 µm sieve, rinsed in running tap water, then broken with a rubber scraper drawn over the mesh to release the eggs which were collected under the sieve in a glass beaker. A 10 ml aliquot of the egg suspension was pipetted into a Doncaster dish and the number of embroynated (transparent, with visible larva, Fig. 3.1) and non-embryonated (brown, with no larva, Fig. 3.1) eggs were counted to obtain an estimate of the total number of eggs in the suspension. The egg suspension was diluted with distilled water to the desired concentration of about 2000 eggs per 3 ml of suspension.

3.4.2.3 Inoculation

Fifty seedlings of *T. nigrescens* (line Az 2225) and twenty seedlings of *T. repens* were inoculated in the second week of December 1992 by syringing 3 ml of suspension containing about 2000 eggs into a pencil hole angled under a two week old seedling. Prior to each inoculation the egg suspension was continuously stirred with a wooden rod to get a

reasonably uniform numbers of eggs for each pot inoculated. The inoculated plants remained in the glasshouse at a soil temperature of 18-25°C and in natural day light.

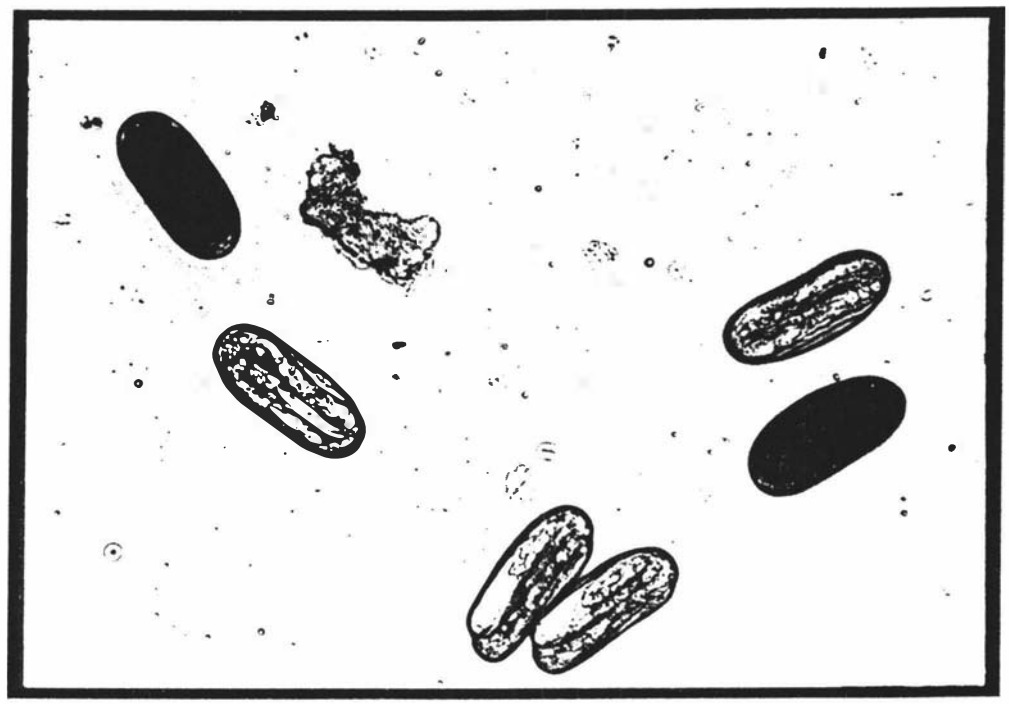


Figure 3.1 Embryonated (transparent with visible larva) and non-embyonated (black with no larva) eggs of clover cyst nematode (*Heterodera trifolii*). x 250.

3.4.2.4 Scoring Plants for Cyst Count

In the second week of February 1993, eight weeks after inoculation, roots of inoculated plants were washed free of soil in an elutriation apparatus (Fig. 3.2) using the method of Wood and Foot (1977), and the cysts were collected in a 180 μm sieve. Cysts were placed in a Doncaster dish and the number of cysts obtained from each plant was counted with the aid of a light microscope. Sieves were washed thoroughly between each plant. Data were recorded as numbers of cysts per plant.

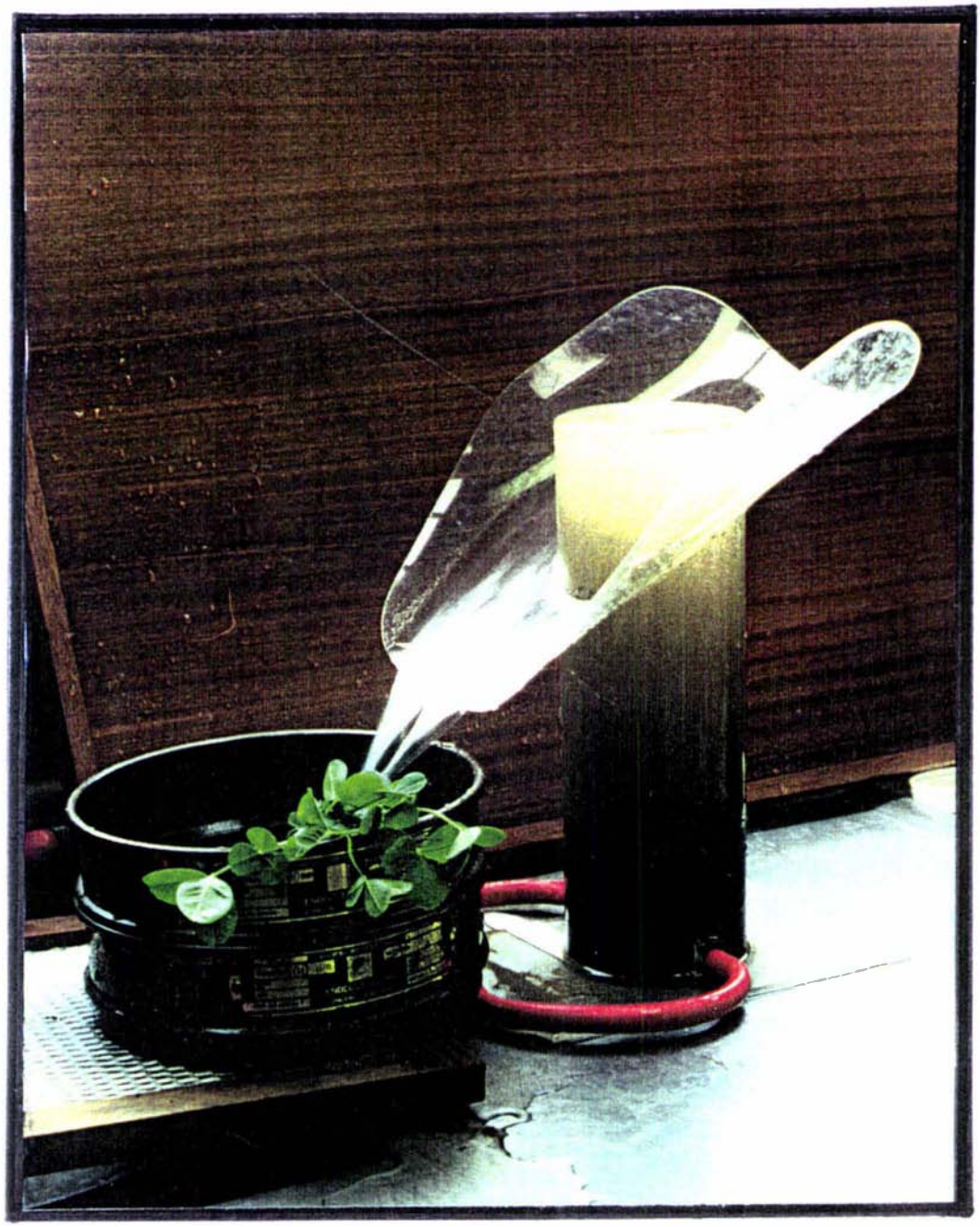


Figure 3.2 Elutriation apparatus for extraction of *Heterodera trifolii* cysts from soil and roots of potted clover plants. Water enters through two opposed inlets at the base of the tower at a rate of 2-3 l/min. At this flow rate, cysts are carried up the tower, spilled on 2 mm sieve (upper) and collected on a 180 μm sieve (lower).

3.4.3 Rescreening

After the initial screening, all the surviving plants of *T. nigrescens* and *T. repens* were trimmed to a uniform root length of 2-3 cm and shoot length of 4-5 cm and were each repotted in a 10 cm diameter plastic pot containing potting mix in the second week of February 1993. Details of the soil mixture and fertilisers used and the glasshouse growing conditions in this and all subsequent experiments are provided in Appendix 3.1.

Because of the annual growth habit of *T. nigrescens*, only 16 plants survived after the initial screening. Ten to 15 stem cuttings of 2-4 cm length from each of the sixteen *T. nigrescens* and five *T. repens* plants were planted in the last week of February 1993 in 30 x 42 x 5 cm plastic trays containing potting mix (Appendix 3.1). Trays were kept in the glasshouse. In the third week of March 1993, three weeks after planting, at least five cuttings (where available) of each plant with 5-10 roots were uprooted from the plastic trays, and the roots were thoroughly washed free of potting mix and trimmed to an approximately uniform size of 2-3 cm. Plants were then planted in 6.5 cm plastic pots containing a pasteurised soil/sand mix (Appendix 3.1). Pots were randomly placed in 40 x 80 cm steel trays with a maximum of 35 pots per tray. The growing conditions were the same as for the initial screening. In the first week of April 1993, ten days after potting, each plant was inoculated with about 2000 eggs of clover cyst nematode, using the procedure described for the initial screening (section 3.4.2.3). Twenty five cuttings, five each from the five initially screened white clover cv. "Grassland Huia" genotypes were included as a test of the inoculum. In the second week of June 1993, eight weeks after inoculation, the number of cysts per plant was counted by the method described for the initial screening (section 3.4.2.4).

3.4.4 Selection of Resistant and Susceptible Genotypes

Based on the comparison of cyst numbers for each of the 16 plants of *T. nigrescens* and 5 plants of *T. repens* cv. "Grasslands Huia" in the two screenings (Table 4.5), two plants of *T. nigrescens* (one resistant and one susceptible) and one plant of *T. repens* (susceptible) were selected for comparison with 3*x* and 6*x* H-6909-5 in the next screening. As *T. nigrescens* is an annual species, the two plants were kept alive by frequent vegetative propagation from stem cuttings in a temperature controlled glasshouse (section 3.4.3).

3.4.5 Screening 3*x* and 6*x* H-6909-5 for Clover Cyst Nematode Resistance

Trifolium repens x *T. nigrescens* interspecific hybrid (H-6909-5) was screened at both the triploid (3*x*) and hexaploid (6*x*) level for clover cyst nematode resistance. Vegetative propagation from stolon/stem cuttings of all the genotypes utilised in this screening was started in the first week of July 1993.

Ten stolon cuttings of 3*x* H-6909-5 and 20 cuttings (10 each from two plants) of colchicine doubled 6*x* H-6909-5 were included for clover cyst counts using the procedure described for rescreening *T. nigrescens* and *T. repens* (section 3.4.3). Twelve stem cuttings from two previously screened *T. nigrescens* genotypes (one with a low and the other with a high number of cysts) and 6 stolon cuttings from one *T. repens* cv. "Grasslands Huia" genotype (with a high number of cysts) were used as controls and tests of inoculum respectively. The pot layout was completely randomised. All the pots were inoculated in the last week of July, 1993. Plants were scored for cyst count in the first week of October, 1993. Data were recorded as numbers of cysts per plant.

3.4.6 Root Dry Weight

Roots of individual plants were placed in 10 x 16.5 cm paper bags and dried in an oven at 80°C for four days before weighing. The number of cysts was then calculated as cysts per gram root dry weight.

3.4.7 Statistical Analysis

Data for cyst number per plant after rescreening of *T. nigrescens* and *T. repens* genotypes were analysed according to a completely randomised design with equal replication by the use of analysis of variance. (SAS Institute, 1988 p.441). Genotype mean comparisons were performed using Fisher's LSD test at the 5% level of probability.

Data for numbers of cysts per gram root dry weight were analysed according to a completely randomised design with unequal replication by the use of analysis of variance. As the genotype means had unequal numbers of observations, the genotype comparisons were performed using Duncan's multiple range test at the 5% probability level.

3.5 BACKCROSSES OF *T. ambiguum* x *T. repens* (4x AND 8x H-435) HYBRID TO *T. ambiguum* AND *T. repens*.

3.5.1 Plant Material

i. 4x and 6x *T. ambiguum*

Eight plants of tetraploid *T. ambiguum* cv. "Treeline" ($2n$=$4x$=32) and 6 plants of hexaploid *T. ambiguum* cv. "Prairie" ($2n$=$6x$=48) were obtained from Allan Nordmeyer, Forestry Research Institute, Christchurch, New Zealand. These were all field grown plants. The ploidy level was confirmed for three genotypes among the tetraploid plants and two genotypes among the hexaploid plants using dry pollen shape and root tip squashes by the method described in sections 3.2.1 and 3.2.3 respectively. These plants were grown in the glasshouse (Appendix 3.1) in 15 cm diameter plastic pots containing potting mix. Both the tetraploid and hexaploid plants utilised in backcrosses, carried a white "V" leaf mark.

ii. *T. repens*

One genotype of *T. repens* "Grasslands Crimson Charm" (CC-1) carrying red leaf and "silver sprite" leaf mark (V^{m} V^{i} ; R^{l} -) and multifoliolate characters was used in the backcrosses (section 3.3.1).

iii. *T. ambiguum* x *T. repens* (4x H-435)

This partially fertile and self-compatible interspecific tetraploid ($2n$=$4x$=32) hybrid was developed by Williams and Verry (1981) in New Zealand with the aid of embryo culture. Pollen stainability of 4x H-435 was initially reported to be as high as 23% (Williams and Verry, 1981) but was later reported to be in the range of 5-10%, depending upon the environmental conditions (Williams *et al.*, 1990). Although the hybrid was initially reported to have overall resemblance to the *T. ambiguum* female parent (Williams and Verry, 1981), it was subsequently reported to be intermediate between the two parents and did not show either the rhizomatous root habit of *T. ambiguum* or the stoloniferous growth habit of *T. repens* (Williams *et al.*, 1990). Three vegetatively propagated plants of 4x H-435 were obtained from J. Van Den Bosch, AgResearch Grasslands, Palmerston North, New Zealand. This hybrid carried no leaf mark.

iv. *T. ambiguum* x *T. repens* (8*x* H-435)

The octoploid clones of 8*x* H-435 utilised in this study were produced by doubling the chromosome number of H-435 (4*x*) by an *in vitro* colchicine method (Anderson *et al.*, 1991b). Pollen stainability of the octoploid C_0 clones averaged 33.6% and the dry pollen shape was tetrahedral (Taylor *et al.*, 1991). 8*x* H-435 is morphologically intermediate between the two parents and carries no leaf mark. C_0 clones of 8*x* H-435 were obtained from N. L. Taylor, Department of Agronomy, University of Kentucky, USA and were grown under glasshouse conditions (Appendix 3.1)

3.5.2 Pollination

All glasshouse grown plants utilised in the backcrosses were self pollinated in order to estimate self compatibility. *T. ambiguum* (cv. "Treeline" and "Prairie"), *T. repens* (CC-1) and the generated backcross progeny were self pollinated by the method described in section 3.3.3. However, because of the poor dehiscence of H-435 (4*x* and 8*x*) the anthers were first ruptured with forceps and the stigma carefully self pollinated with the pollen squeezed from the anthers. The flowers were protected from foreign pollen with paper bags.

3.5.2.1 First Backcross (BC_1F_1)

Reciprocal cross pollinations were made on the potted plants in the glasshouse using the procedure described in section 3.3.2. The backcross schemes and terminology are the same as described in section 3.3.2. H-435 (both 4*x* and 8*x*) was used as one parent and two genotypes of tetraploid *T. ambiguum* (cv."Treeline"), two genotypes of hexaploid *T. ambiguum* (cv. "Prairie") and one genotype of *T. repens* (CC-1) as the other parent for the production of BC_1F_1 seeds. These crosses were initiated in January, 1992.

3.5.2.2 Congruity (CBC) and Second (BC_2F_1) Backcrosses

Where BC_1F_1 seeds were successfully obtained and germinated during June-July, 1992, the resulting plants were again backcrossed to *T. repens* (CC-1) and 4*x* and 6*x* *T. ambiguum* for the production of second backcross (BC_2F_1) and congruity backcross (CBC_2) progeny respectively during December, 1992-February, 1993.

3.5.2.3 BC_1F_1 x BC_1F_1 Intercross (BC_1F_2)

Four BC_1F_1 genotypes were crossed among themselves during December, 1992-February, 1993. The resulting BC_1F_2 progenies were grown in the glasshouse in April 1993. Upon flowering three of the intercrossed BC_1F_2 plants were backcrossed to 6x *T. ambiguum* (cv. Prairie) during December 1993-January, 1994.

The total number of pods developed during the first two weeks after pollination was counted for all crosses. About four to five weeks after pollination, seeds were harvested from mature flower heads and stored for 4-6 weeks in a refrigerator at 4°C to break dormancy.

3.5.3 Seed Surface Sterilisation and Germination

Seeds of the first backcross progeny (BC_1F_1) were surface sterilised by treatment for 30 seconds in 95% (v/v) ethanol which was then drained off and replaced with 0.2% (w/v) $HgCl_2$ acidified with 0.5% (v/v) HCl for 6 min, followed by 5 rinses with sterile water, and then placed in a shallow layer of sterile water overnight in a petri dish at room temperature to imbibe. The seeds were scarified by the action of HCl during surface sterilisation.

Following surface sterilisation, seeds were germinated on 0.8% (w/v) water/agar at 26°C under 10 μ Em^{-2} s^{-1} of light and a 16h photoperiod. Two week old seedlings were then transferred to 10 cm plastic pots containing pasteurised potting mix and grown in the glasshouse (Appendix 3.1).

3.5.4 Chromosome Counts

Mitotic chromosome counts were made for all the material utilised in the crossing, first backcross progeny (BC_1F_1) and backcross derivatives, using the procedure described in section 3.2.3.

Meiotic chromosomal configurations were observed for 4x *T. ambiguum* (cv. "Treeline"), *T. repens* (CC-1) and the backcross progeny (BC_1F_1) by the method given in section 3.2.4.

3.5.5 Pollen Stainability

Pollen stainability was estimated for all the material utilised in backcrosses, first backcross progeny (BC_1F_1), second backcross progeny (BC_2F_1), congruity backcross, and the BC_1F_1 x BC_1F_1 intercross (BC_1F_2) progeny using the procedure described in section 3.2.2. At least 1200 pollen grains were scored from six or more flowers and 3 or more inflorescences per plant.

3.5.6 Morphological Characteristics of BC_1F_1 Progeny

Ten BC_1F_1 genotypes, 4*x* and 8*x* H-435, *T. ambiguum* (cv. Treeline) and *T. repens* (CC-1) plants were evaluated in an experiment to compare different morphological characteristics.

All 14 genotypes were grown from approximately equal sized vegetative cuttings in 30 x 42 x 5 cm plastic trays containing potting mix (Appendix 3.1) in a randomised complete block design during the last week of June 1993. Two cuttings of each genotype were planted in one third of a tray, with three genotypes per tray, and the experiment was replicated three times. Trays were kept outside the glasshouse and were watered as required. Fourteen weeks after planting, during the second week of October 1993, plants were carefully uprooted from the trays and the following measurements were made.

Leaflet width and length- the width and length of three leaflets midway along the stolon or stem.

Stolon or stem number- total number of stolons or stems per plant.

Stolon or stem length- length of longest stolon or stem.

Stolon or stem diameter- diameter of longest stolon or stem.

Tap root diameter- diameter of first tap root just below ground level.

Number of nodes- number of nodes on the longest stolon or stem.

Nodal root primordia- number of nodes on the longest stolon or stem with root primordia.

Nodal rooting- number of nodes on the longest stolon or stem with nodal roots.

Rhizome number- total number of rhizomes per plant.

Rhizome length- length of longest rhizome.

Nodulation- nodulation was recorded by visual observation on a scale from 0 - 5 where, 0 = no nodulation, 1 = 1-5 nodules, 2 = 6-10 nodules, 3 = 11-15 nodules, 4= 16-20 nodules and 5 = 21 or more nodules per plant.

Root dry weight- roots of individual plants were dried in a 10 x 16.5 cm paper bag in the oven at 80°C for 4 days and then weighed.

Shoot dry weight- shoots of individual plants were dried in a 10 x 16.5 cm paper bag in the oven at 80°C for 4 days and then weighed.

3.5.7 Statistical Analysis

Data for each component were subjected to analysis of variance. The GLM procedure of SAS was used for combined analysis of all the 14 lines (SAS Institute, 1988 p.441). The least significant difference (LSD) at $P<0.05$ was calculated from type III sums of squares from the GLM procedure. Data for stolon or stem number, rhizome number and length, root and shoot and total dry weight were not normally distributed; therefore these data were log transformed, re-analysed and a least significant difference (LSD) value for the log scale calculated. In order to present the data in an untransformed state, a least significant ratio (LSR) was calculated by back (exponential) transformation of the LSD from the log scale.

Contingent on a significant F-test, treatment means were significantly different if their ratio (using the largest value as the numerator for a two-tailed test) exceeded the LSR.

CHAPTER 4
RESULTS

4.1 CHROMOSOME DOUBLING FREQUENCY

Responses of the axillary meristems of the triploid *T. repens* x *T. nigrescens* interspecific F_1 hybrid (3*x* H-6909-5) to different colchicine treatments for *in vitro* chromosome doubling are presented in Table 4.1.

Treatments C_1, C_2 and C_5 killed all meristems, as none survived after exposure to colchicine. Treatment C_5 had the same concentration of colchicine as C_3 and C_4, but the time of exposure to colchicine was longer, which suggested that not only the colchicine concentration, but also the duration of its application, had an effect on the toxicity to meristems in culture. Treatments C_3 and C_4 had very similar effects on the growth of the meristems. Apart from bacterial and fungal contamination which was assumed to be random, death of the meristems was partly due to the bleaching effect of sodium hypochlorite used during surface sterilisation of the meristems, and partly because of the toxic effect of colchicine treatment. The bleaching effect was evident during the pre-culturing period prior to colchicine application, during which time between 25.0% and 47.1% (a mean of 39.8%) of the meristems died. The chromosome doubling frequency of meristems was 1.5% (7.1% of the surviving meristems) for treatment C_3 and 2.9% (11.1% of the surviving meristems) for treatment C_4. In total only three plants, one from treatment C_3 and two from treatment C_4, out of 32 (approx. 10%), showed doubled chromosomes. No chromosome doubled plants were obtained from treatment C_6.

4.2 IDENTIFICATION OF CHROMOSOME DOUBLED PLANTS

4.2.1 Dry Pollen Shape

Examination of dry pollen shape was found to be a rapid method for identifying chromosome doubled shoots of colchicine derived plants. The triploid *T. repens* x *T. nigrescens* hybrid (3*x* H-6909-5) with $2n = 3x = 24$, produced cylindrical to oval pollen grains

Table 4.1 Responses of the *T. repens* x *T. nigrescens* (3*x* H-6909-5) F_1 hybrid to protocols for *in vitro* chromosome doubling*.

Treatment**	Total meristems cultured	Meristems bleached No (%)	Meristems contaminated† No (%)	Meristems survived No (%)	Meistems with doubled chromosomes‡ No (%)
C_1 0.1%, 48hr	152	68 (44.6)	27 (17.8)	0 (0.0)	0 (0.0)
C_2 0.1%, 72hr	170	43 (25.3)	48 (28.2)	0 (0.0)	0 (0.0)
C_3 0.05%, 48hr	68	29 (42.6)	19 (27.9)	14 (20.6)	1 (1.5)
C_4 0.05%, 60hr	70	33 (47.1)	15 (21.4)	18 (25.7)	2 (2.9)
C_5 0.05%, 72hr	133	56 (42.1)	23 (17.3)	0 (0.0)	0 (0.0)
C_6 0.0%, ---	57	21 (36.8)	13 (22.8)	22 (38.6)	0 (0.0)
Mean		39.8	22.6		

* Colchicine was applied at 0.1% or 0.05% in solidified WCSP medium.

** Concentration and duration of colchicine application.

† Bacterial and fungal.

‡ Based on meristems that survived from initial culturing until transfer to pots in the glasshouse.

(Fig. 4.1). Pollen grains from the autoallohexaploid 6*x* H-6909-5 were found to be tetrahedral (Fig. 4.1).

4.2.2 Pollen Stainability

Pollen stainability was markedly increased in the colchicine induced autoallohexaploid of H-6909-5. The three colchicine induced hexaploid (6*x*) plants had an average of 89.2% pollen stainability in contrast to only 9.9% for the triploid hybrid (3*x* H-6909-5) (Table 4.3 and Fig. 4.2).

4.2.3 Somatic Chromosome

The doubling of chromosomes in the three plants (designated as CT-1, CT-14 and CT-28) obtained after colchicine treatment was confirmed in at least 10 cells from 5-10 root tips for each plant by the presence of $2n=6x=48$ chromosomes (Fig. 4.3) in contrast to the triploid hybrid (3*x* H-6909-5) which had a $2n$ chromosome number of 24 (Fig. 4.3). Chromosome doubling in the shoot was confirmed by the presence of 48 chromosomes in the pollen mother cells of all three colchicine induced autoallohexaploids (6*x* H-6909-5). Somatic chromosomes from root tip squashes were also counted for three other colchicine derived plants showing low pollen stainability (average 8.9%) and cylindrical or oval dry pollen shape. These plants showed an undoubled number of $2n=24$.

4.3 DESCRIPTION OF 3*x* AND 6*x* H-6909-5

Trifolium repens x *T. nigrescens* H-6909-5 showed similar morphological characters at both the triploid and hexaploid levels except for the leaflet and inflorescence sizes which were comparatively larger for 6*x* H-6909-5 than 3*x* H-6909-5 (Fig. 4.4). The inflorescence size of 3*x* H-6909-5 was intermediate between *T. repens* and *T. nigrescens* while that of the 6*x* H-6909-5 was almost similar to *T. repens* (Fig. 4.8). However both ploidy levels of H-6909-5 exhibited the stoloniferous growth habit of *T. repens* with long internodes, but had infrequent root primordia and nodal rooting which were only observed at the basal 2-3 nodes (Fig. 4.9). The 3*x* and 6*x* plants of H-6909-5 flowered profusely like the *T. nigrescens* parent, and the flowering continued through the year.

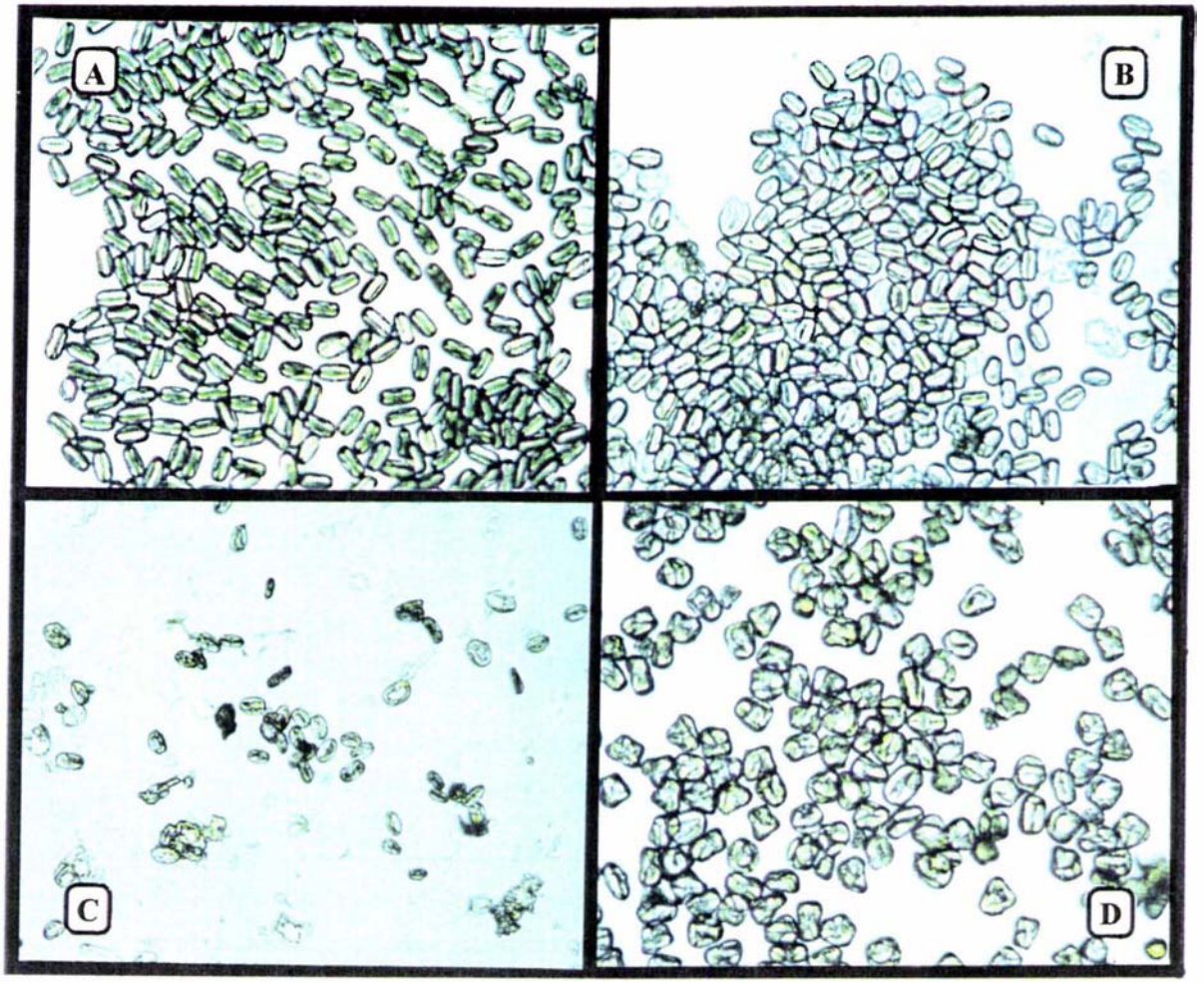


Figure 4.1 Dry pollen shape of **(A)** *T. repens* **(B)** *T. nigrescens*, **(C)** 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (H-6909-5) and **(D)** 6*x* H-6909-5. x 250

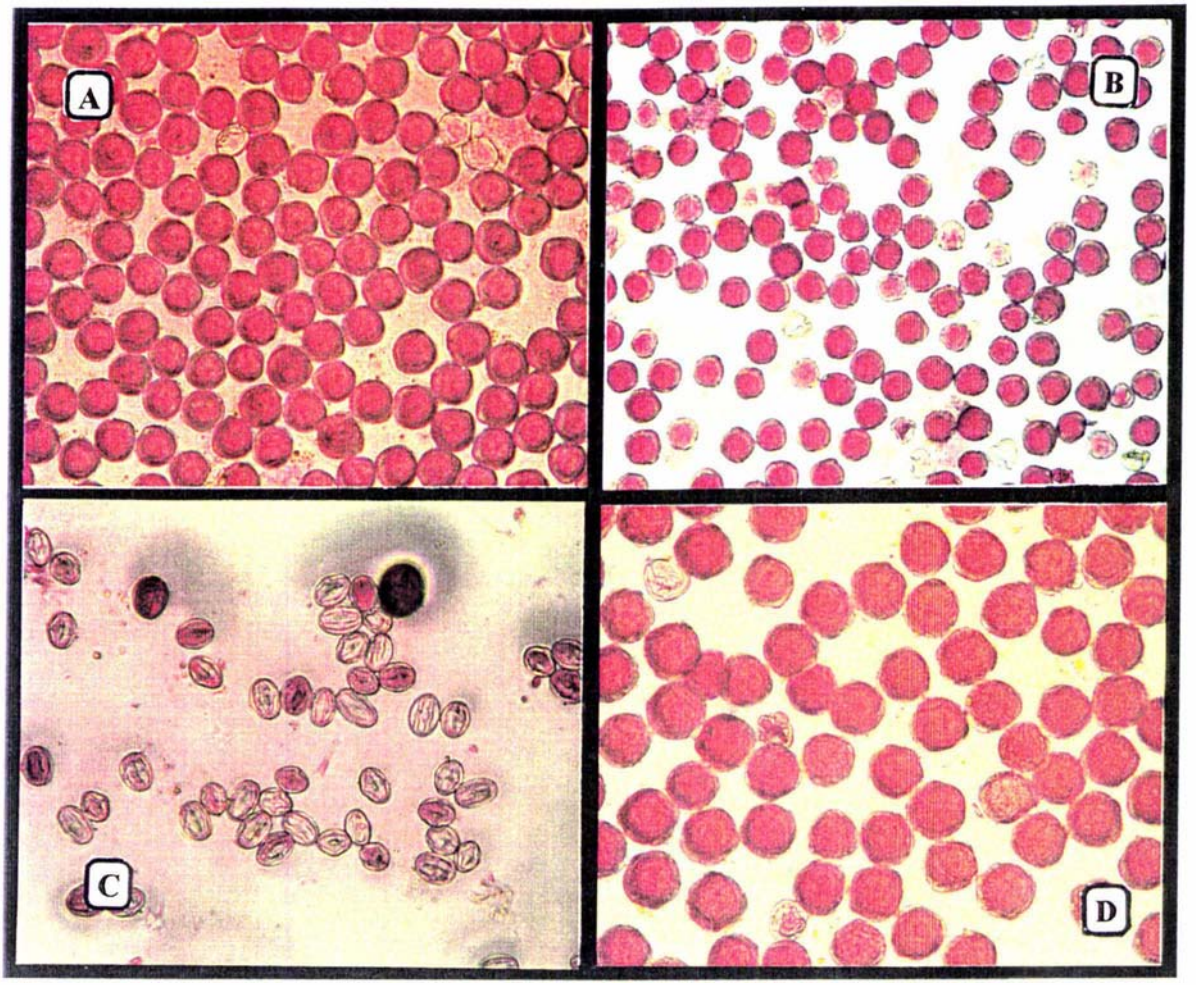


Figure 4.2 Pollen stainability of **(A)** *T. repens* **(B)** *T. nigrescens*, **(C)** 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (H-6909-5) and **(D)** 6*x* H-6909-5. x 250

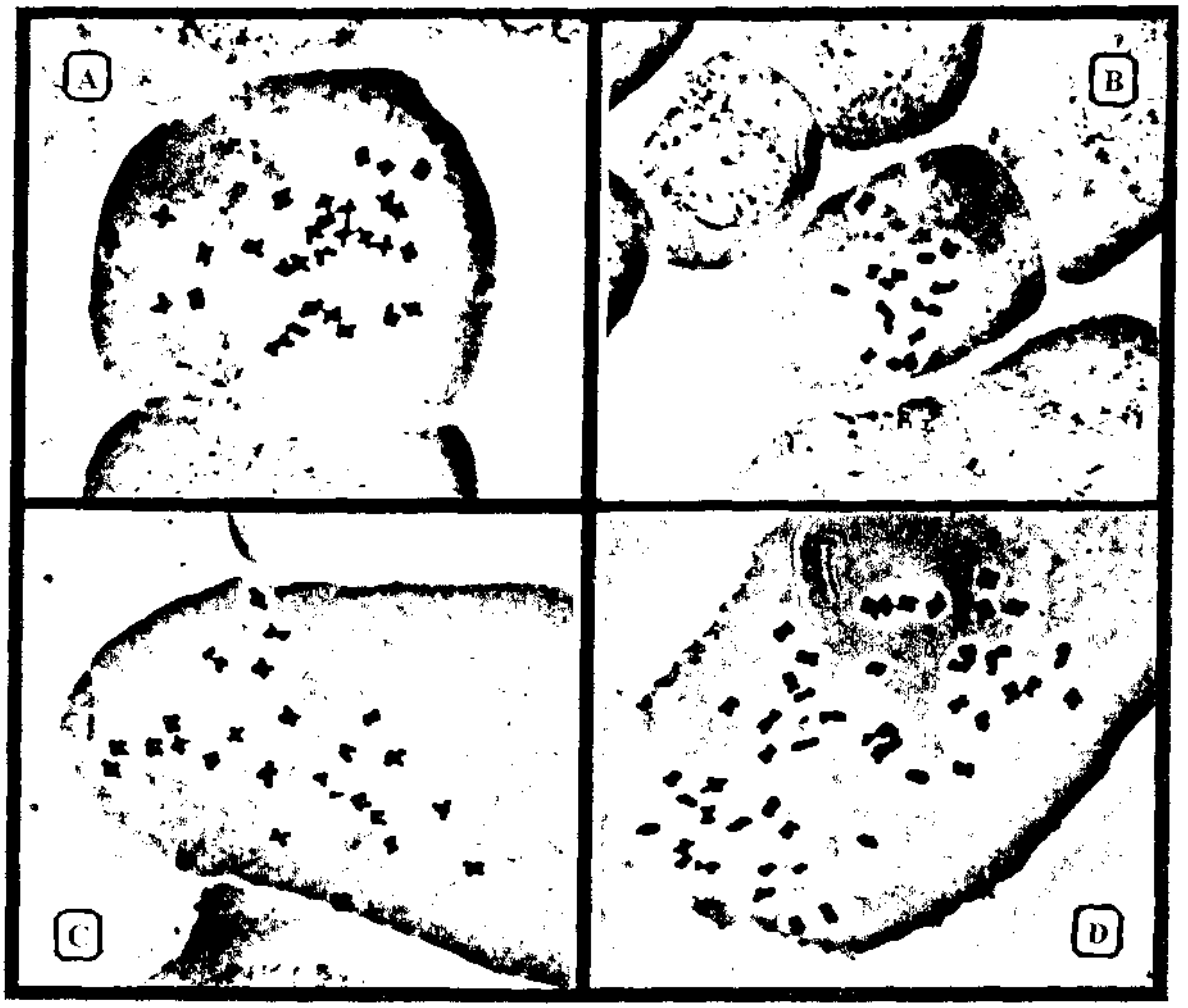


Figure 4.3 Somatic chromosomes of **(A)** *T. repens* (2*n*=4*x*=32), **(B)** *T. nigrescens* (2*n*=2*x*=16), **(C)** 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (H-6909-5) (2*n*=3*x*=24), and **(D)** 6*x* H-6909-5 (2*n*=6*x*=48) A= x 2000, B,C and D= x 1600

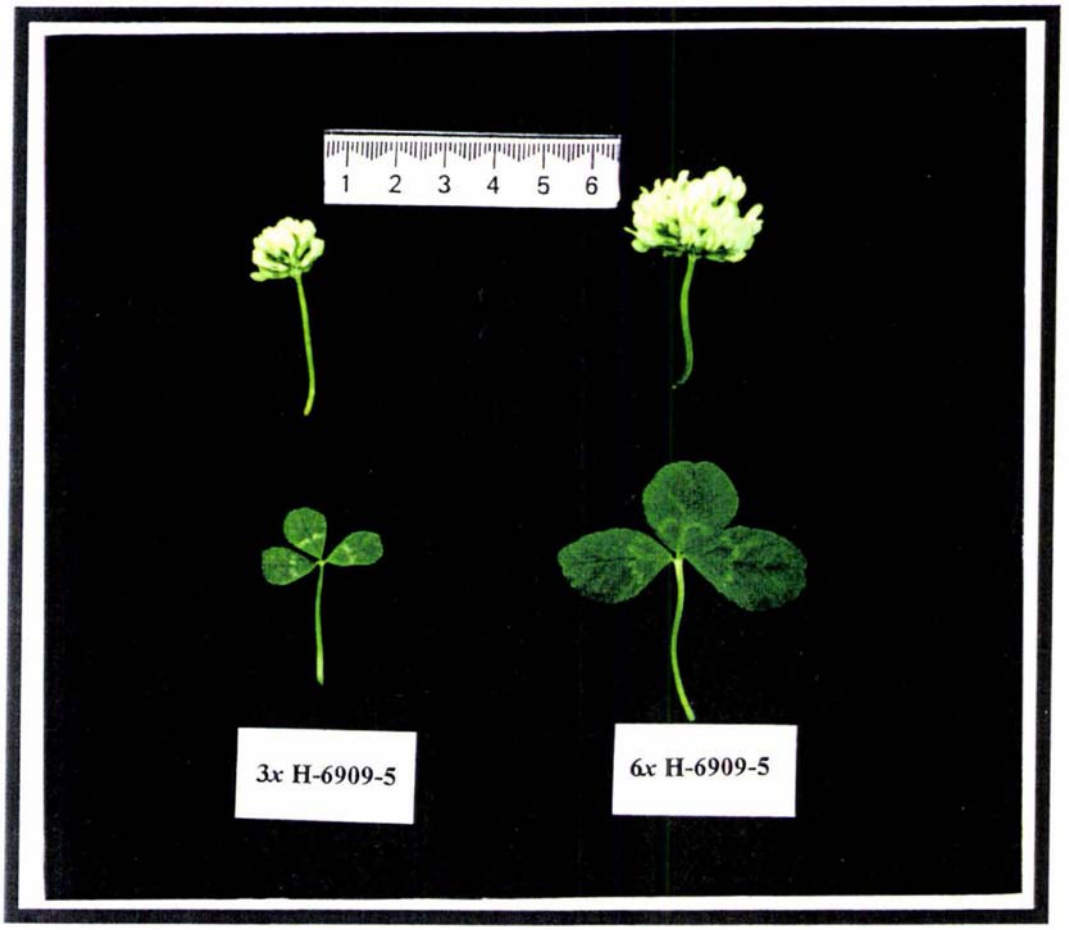


Figure 4.4 Leaflet and inflorescence sizes of the 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (H-6909-5) (left) and 6*x* H-6909-5 (right).

Self pollination of 50 inflorescences of 3*x* H-6909-5 (more than 1200 flowers) and 20 inflorescences (more than 500 flowers) each from the three colchicine induced hexaploid plants of H-6909-5 produced no seed, showing that 3*x* H-6909-5 and colchicine derived 6*x* H-6909-5 plants were self-incompatible.

4.4 BACKCROSSES OF 3*x* AND 6*x* H-6909-5 TO *T. repens* AND *T. nigrescens.*

4.4.1 First Backcross (BC_1F_1)

The plants of both *T. repens* (CC-1) and *T. nigrescens* (Tn-167) used in backcrosses to the 3*x* and the colchicine induced 6*x* *T. repens* x *T. nigrescens* hybrid (H-6909-5) were not the original parents of 3*x* H-6909-5 but had the same chromosome numbers ($2n=4x=32$ and $2n=2x=16$ respectively) (Fig 4.3).

Reciprocal backcrosses of 3*x* H-6909-5 with *T. repens* (CC-1) and *T. nigrescens* (Tn-167) were not successful (Table 4.2). One seed was obtained from 6*x* H-6909-5 used as the female parent after approximately 1,200 pollinations with *T. repens* pollen. The resulting plant carried a "silver sprite" and white "V" (V^m V^i) leaf mark and multifoliolate characters inherited from the *T. repens* parent (CC-1) (Fig. 4.7), thus confirming its backcross origin. From approximately 700 backcrosses using *T. repens* (CC-1) as the female parent and 6*x* H-6909-5 as the male parent, three seeds were obtained. All the three seeds were successfully germinated and grown into mature plants. Only one seed was obtained from about 800 crosses of 6*x* H-6909-5 as the female and *T. nigrescens* as the male parent. This BC_1F_1 seed was successfully germinated and grown into a mature plant. Approximately 1,200 reciprocal crosses of this combination yielded no viable seeds, despite apparently better pod development. In reciprocal backcrosses where 3*x* H-6909-5 was used as one of the parents, pod development was either absent or extremely poor (Table 4.2). In contrast, reciprocal backcrosses which used 6*x* H-6909-5 as one parent showed significant pod development during the first and second weeks after pollination (Table 4.2) but despite this early pod development, most of these backcrosses involving three 6*x* H-6909-5 plants failed to produce seeds.

Table 4.2 Pod development and number of seeds obtained after reciprocal first backcrosses of 3*x* and 6*x* H-6909-5 to *T. repens* and *T. nigrescens*, second backcrosses, congruity backcrosses, BC_1F_1 x BC_1F_1 intercrosses and BC_1F_1 x F_1.

CROSS	Ploidy Level	No. of pollinations	Pods developed No	Pods developed (%)	No. of seeds obtained
First backcross (BC_1F_1)					
3*x* H-6909-5 x CC-1	3*x* x 4*x*	800	19	2.4	0
CC-1 x 3*x* H-6909-5	4*x* x 3*x*	750	0	0.0	0
3*x* H-6909-5 x Tn-167	3*x* x 2*x*	800	29	3.6	0
Tn-167 x 3*x* H-6909-5	2*x* x 3*x*	600	2	0.3	0
6*x* H-6909-5 x CC-1					
CT-1 x CC-1	6*x* x 4*x*	410	90	22.0	0
CT-14 x CC-1 (CBC_1)	6*x* x 4*x*	360	114	31.7	1
CT-28 x CC-1	6*x* x 4*x*	430	154	35.8	0
CC-1 x 6*x* H-6909-5					
CC-1 x CT-1	4*x* x 6*x*	200	83	41.5	0
CC-1 x CT-14	4*x* x 6*x*	260	92	34.4	3
CC-1 x CT-28	4*x* x 6*x*	240	117	48.8	0
6*x* H-6909-5 x Tn-167					
CT-1 x Tn-167	6*x* x 2*x*	190	23	12.1	0
CT-14 x Tn-167 (CBC_1)	6*x* x 2*x*	380	89	23.4	1
CT-28 x Tn-167	6*x* x 2*x*	230	46	20.0	0
Tn-167 x 6*x* H-6909-5					
Tn-167 x CT-1	2*x* x 6*x*	270	117	43.3	0
Tn-167 x CT-14	2*x* x 6*x*	500	227	45.4	0
Tn-167 x CT-28	2*x* x 6*x*	430	134	31.2	0
Second backcross (BC_2F_1)					
(CT-14 x CC-1) x Huia-1	5*x* x 4*x*	160	28	17.5	1
Huia-1x (CT-14 x CC-1)	4*x* x 5*x*	160	97	60.6	8
(CC-1 x CT-14)-1 x Huia-1	7*x* x 4*x*	180	43	23.9	3
Huia-1x (CC-1 x CT-14)-1	4*x* x 7*x*	240	29	12.1	1
Congruity backcross (CBC)					
CT-14 x Tn-167 (CBC_1)	6*x* x 2*x*	380	89	23.4	1
(CT-14 x Tn-167) x CC-1 (CBC_2)	4*x* x 4*x*	170	46	27.1	3
Huia-1x (CT-14 x Tn-167) (CBC_2)	4*x* x 4*x*	130	39	30.0	3
CT-14 x CC-1 (CBC_1)	6*x* x 4*x*	360	114	31.7	1
(CT-14 x CC-1) x Tn-167 (CBC_2)	5*x* x 2*x*	460	17	3.7	0
Tn-167 x (CT-14 x CC-1) (CBC_2)	2*x* x 5*x*	240	69	28.8	0
BC_1F_1 x BC_1F_1 (Intercross)					
(CT-14 x Tn-167) x (CT-14 x CC-1)	4*x* x 5*x*	30	19	63.3	6
(CT-14 x CC-1) x (CT-14 x Tn-167)	5*x* x 4*x*	30	13	43.3	3
(CT-14 x Tn-167) x (CC-1 x CT-14)-1	4*x* x 7*x*	230	26	11.3	1
(CC-1 x CT-14)-1 x (CT-14 x Tn-167)	7*x* x 4*x*	145	21	14.5	1
(CT-14 x CC-1) x (CC-1 x CT-14)-1	5*x* x 7*x*	100	16	16.0	1
(CC-1 x CT-14)-2 x (CT-14 x CC-1)	7*x* x 5*x*	75	36	48.0	0
(CC-1 x CT-14)-1 x (CC-1 x CT-14)-2	7*x* x 7*x*	60	41	68.3	6
BC_1F_1 x F_1 (6*x*)					
(CT-14 x Tn-167) x CT-28	4*x* x 6*x*	80	11	13.8	0
CT-28 x (CT-14 x Tn-167)	6*x* x 4*x*	430	90	69.2	10
(CT-14 x CC-1) x CT-28	5*x* x 6*x*	80	7	8.8	1
CT-28 x (CT-14 x CC-1)	6*x* x 5*x*	100	87	87.0	54
(CC-1 x CT-14)-1 x CT-28	7*x* x 6*x*	80	5	6.3	0
CT-28 x (CC-1 x CT-14)-1	6*x* x 7*x*	100	92	92.0	56
CT-1 x (CT-14 x CC-1)	6*x* x 5*x*	70	54	72.0	8
(CT-14 x CC-1) x CT-1	5*x* x 6*x*	60	17	28.3	0

4..4.1.1 Characterisation of First Backcross (BC_1F_1) Progeny

The backcross hybrid obtained from using 6*x* H-6909-5 (plant CT-14) as the female parent after pollination with *T. nigrescens* (Tn-167) was evaluated cytologically for ploidy level and was, as expected, tetraploid (2*n*=4*x*=32) (Fig. 4.5). This backcross theoretically combines two genomes of *T. repens* and two genomes of *T. nigrescens*. The plant was self-incompatible as it did not set any seed after selfing 10 inflorescences and had 59.6% pollen stainability (Fig. 4.6). This BC_1F_1 (CBC_1) was found to be intermediate in morphology between 6*x* H-6909-5 and *T. nigrescens* but showed more affinity towards *T. nigrescens*. The leaflet size was larger than *T. nigrescens* but was smaller than 6*x* H-6909-5 and was similar to 3*x* H-6909-5 (Fig. 4.7). A similar pattern was observed for inflorescence size (Fig. 4.8). The hybrid was vegetatively propagated from stem cuttings as it showed the annual growth habit of *T. nigrescens*. The hybrid flowered profusely throughout the summer and the parent plant died after the completion of flowering. This backcross had no root primordia at the nodes, and thus had no nodal rooting (Fig. 4.9).

The somatic chromosome number for the opposite backcross obtained from using 6*x* H-6909-5 (plant CT-14) as the seed parent after pollination with *T. repens* (CC-1), was found to be 2*n*=5*x*=40, a pentaploid (Fig. 4.5) with a pollen stainability of 86.7% (Fig 4.6). The backcross origin of this pentaploid hybrid was confirmed by the presence of the "silver sprite" and white "V" ($V^m V^i$) leaf mark and multifoliolate characters derived from its male parent (Fig. 4.7). The hybrid strongly resembled *T. repens* in morphology, having similar leaflet (Fig. 4.7) and inflorescence (Fig. 4.8) size to *T. repens* and a perennial stoloniferous growth habit, and was very easily propagated from stolon cuttings as it had root primordia at each node and also frequent nodal rooting (Fig. 4.9). This hybrid theoretically carries four genomes of *T. repens* and one genome of *T. nigrescens*.

Hand pollination of more than 700 flowers of *T. repens* (CC-1) with 6*x* H-6909-5 pollen resulted in three seeds. All three seeds were germinated and grew into mature plants. The somatic chromosome numbers from root tip squashes of these three backcrossed plants were found to be 2*n*=7*x*=56 (Fig. 4.5) . The pollen stainabilities were 72.7% and 71.8% for plants 1 and 2, respectively (Fig 4.6). Pollen stainability for plant 3 was not recorded, as it started flowering very late. The occurrence of these heptaploid (7*x*) plants using *T. repens*

($2n$=$4x$=32) as the female parent and hexaploid H-6909-5 as the male parent, can only be explained by the union of n pollen (n=$3x$=24) from $6x$ H-6909-5 with a $2n$ egg ($2n$=$4x$=32) from *T. repens*. All three $7x$ BC_1F_1's exhibited red and multifoliolate characters (Fig. 4.7), but these characters were inherited from the *T. repens* (CC-1) female parent and could not be used as genetic markers to identify the backcross origin. The leaflet size did not appear to be larger than *T. repens* (Fig. 4.7), but inflorescence size was larger (Fig. 4.8). These three $7x$ plants strongly resembled the female parent *T. repens* in morphology, having a true stoloniferous growth habit with root primordia at each node and frequent nodal rooting (Fig. 4.9). These $7x$ BC_1F_1 plants theoretically possess six genomes of *T. repens* and one genome of *T. nigrescens*.

4.4.2 Second Backcross (BC_2F_1)

Nine BC_2F_1 seeds were obtained from 320 reciprocal backcrosses between the pentaploid BC_1F_1 (CT-14 x CC-1) and *T. repens* (Huia-1). Four fully developed BC_2F_1 seeds were harvested from 420 reciprocal backcrosses between the heptaploid BC_1F_1 [(CC-1 x CT-14)-1] and *T. repens* (Huia-1) (Table 4.2). The reason for using *T. repens* Huia-1 was that this plant did not carry any leaf mark. The "silver sprite" and white "V" leaf mark (V^m V^i) of the pentaploid BC_1F_1 (CT-14 x CC-1), the red and white "V" leaf mark (V^i-;R^l -) of the heptaploid BC_1F_1 [(CC-1 x CT-14)-1] and the multifoliolate character of both BC_1F_1's were used as genetic markers to confirm the second backcross progeny in crosses involving BC_1F_1's as the male and Huia-1 as the female parents. One BC_2F_1 seed from the cross using pentaploid BC_1F_1 (CT-14 x CC-1) as the male parent and one BC_2F_1 seed from the cross using heptaploid BC_1F_1 as the male parent were germinated. The backcross origins of these two plants were confirmed by the transmission of the leaf mark and multifoliolate characters from the male parents (Fig. 4.10). Somatic chromosome number of the BC_2F_1 plant using $5x$ BC_1F_1 as the male and Huia-1 as the female was $2n$=$4x$=32 (Fig. 4.11), indicating that a $2x$ male gamete (n=16) from $5x$ BC_1F_1 had functioned. The other BC_2F_1 plant obtained from the Huia-1 x $7x$ BC_1F_1 backcross was an aneuploid with $2n$=42 (Fig. 4.11), which suggested that an aneuploid male gamete (n=26) from $7x$ BC_1F_1 had functioned.

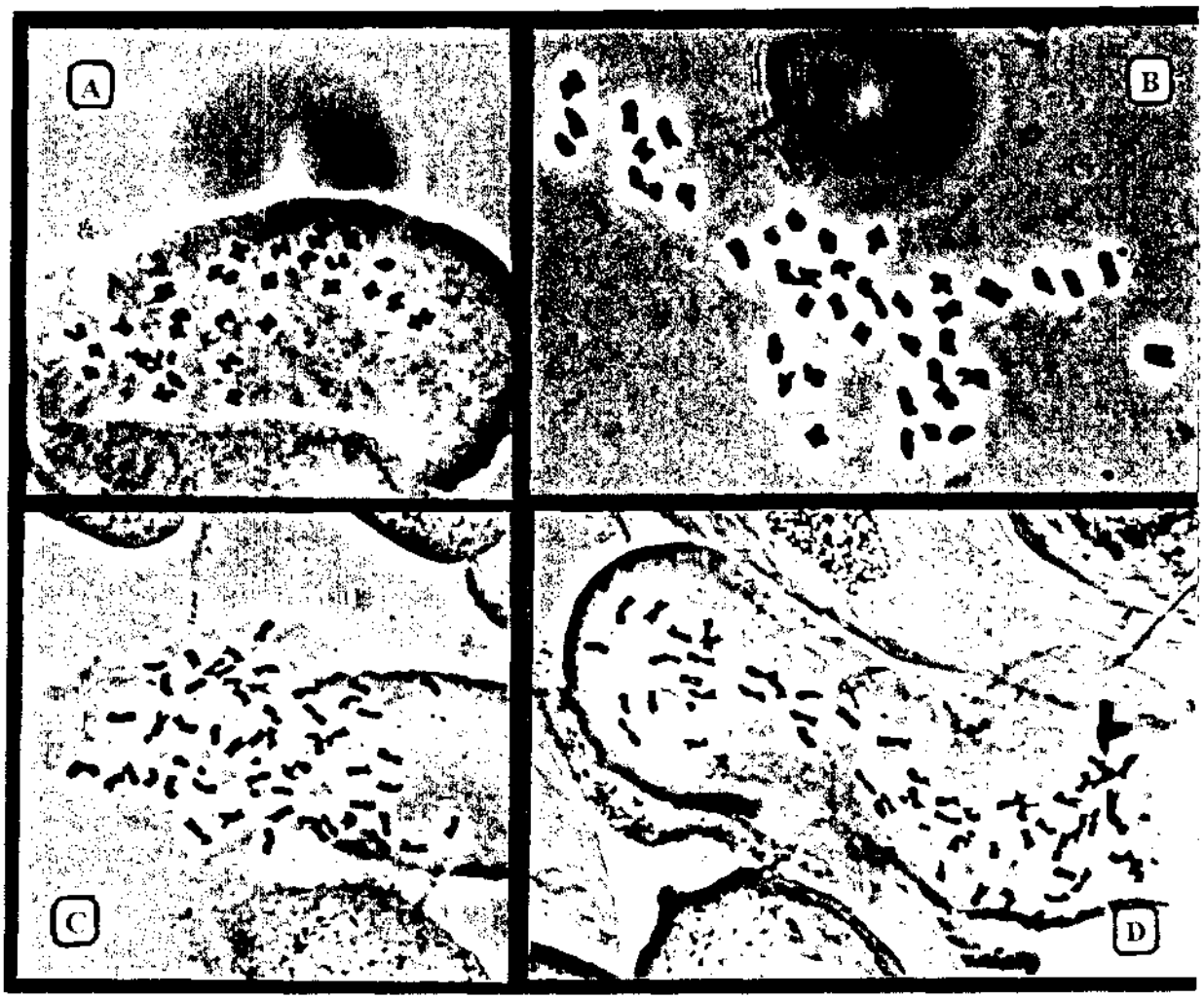


Figure 4.5 Somatic chromosomes of first backcross (BC_1F_1) progeny from crossing 6*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (6*x* H-6909-5) to both parental species. **(A)** 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*, 2*n*=4*x*=32), **(B)** 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*, 2*n*=5*x*=40) and **(C and D)** 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5, 2*n*=7*x*=56) (plant No 1 and 2 respectively). A, C and D = x 1600, B = x 2000.

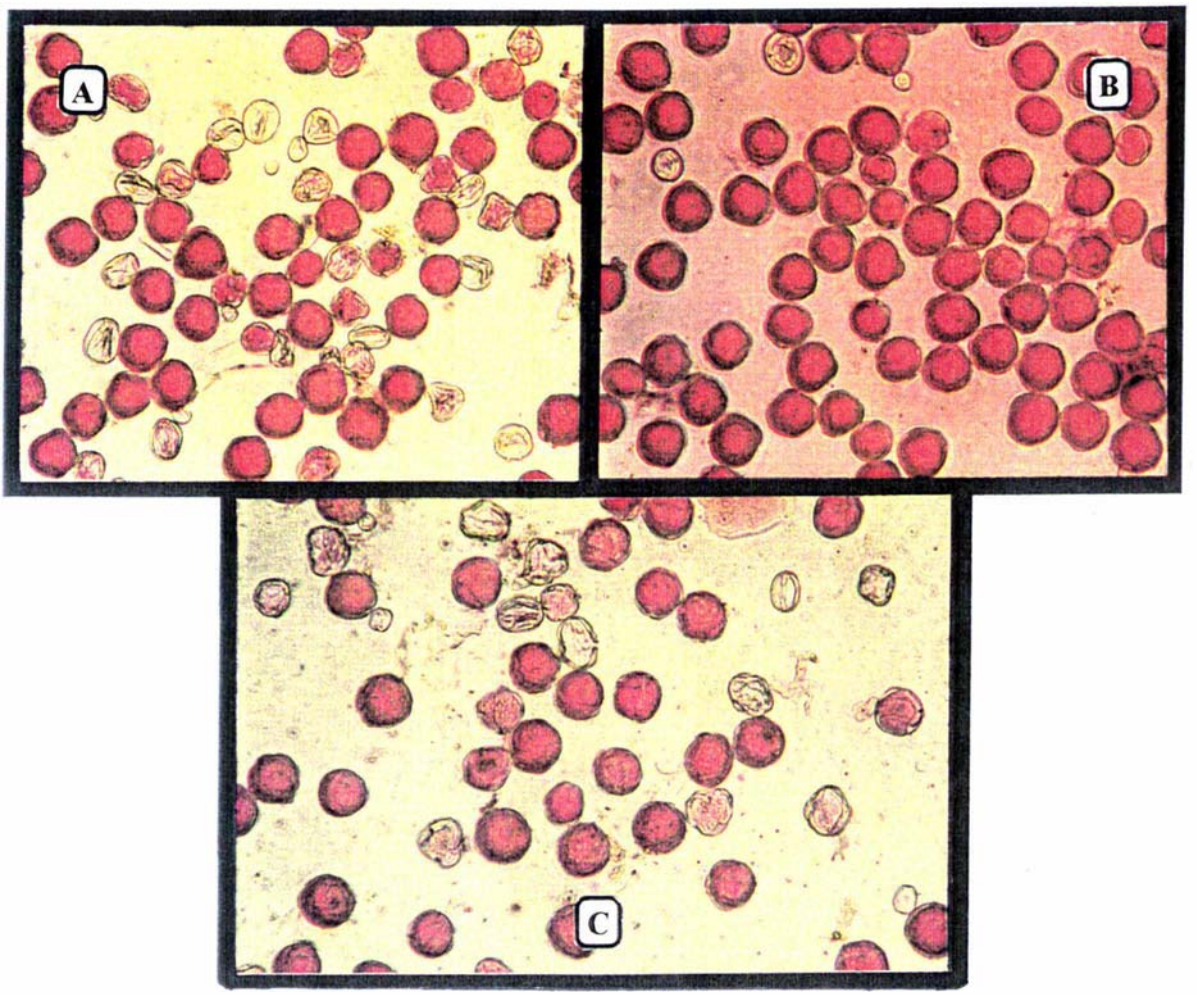


Figure 4.6 Pollen stainability of first backcross (BC_1F_1) progeny from crossing 6*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (6*x* H-6909-5) to both parental species. **(A)** 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*), **(B)** 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*) and **(C)** 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5). x 250.

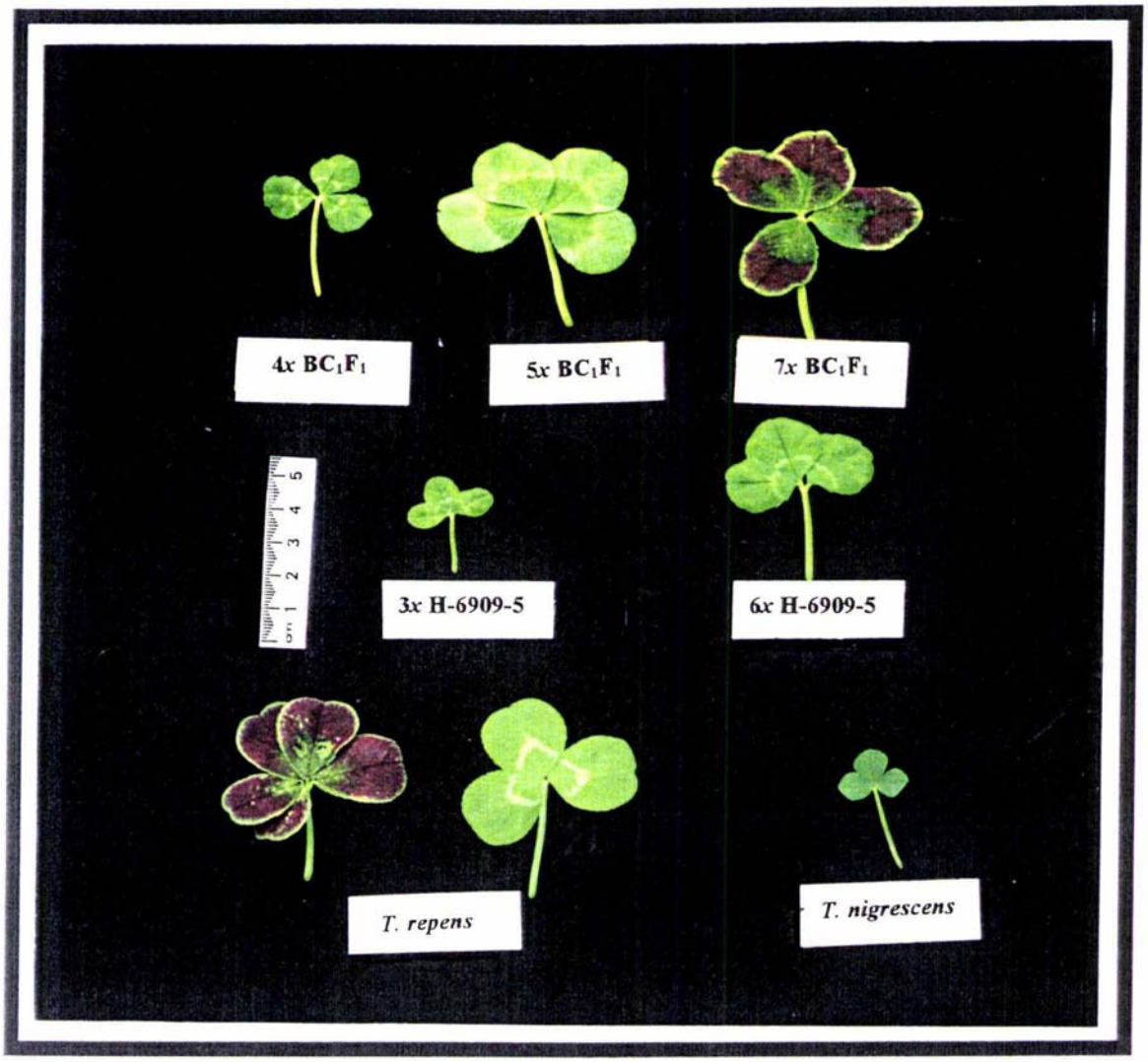


Figure 4.7 Leaf markings and leaflet sizes of *T. repens*, *T. nigrescens*, 3*x* and 6*x* H-6909-5 (*T. repens* x *T. nigrescens*) F_1 hybrids, 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*), 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*) and 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5).

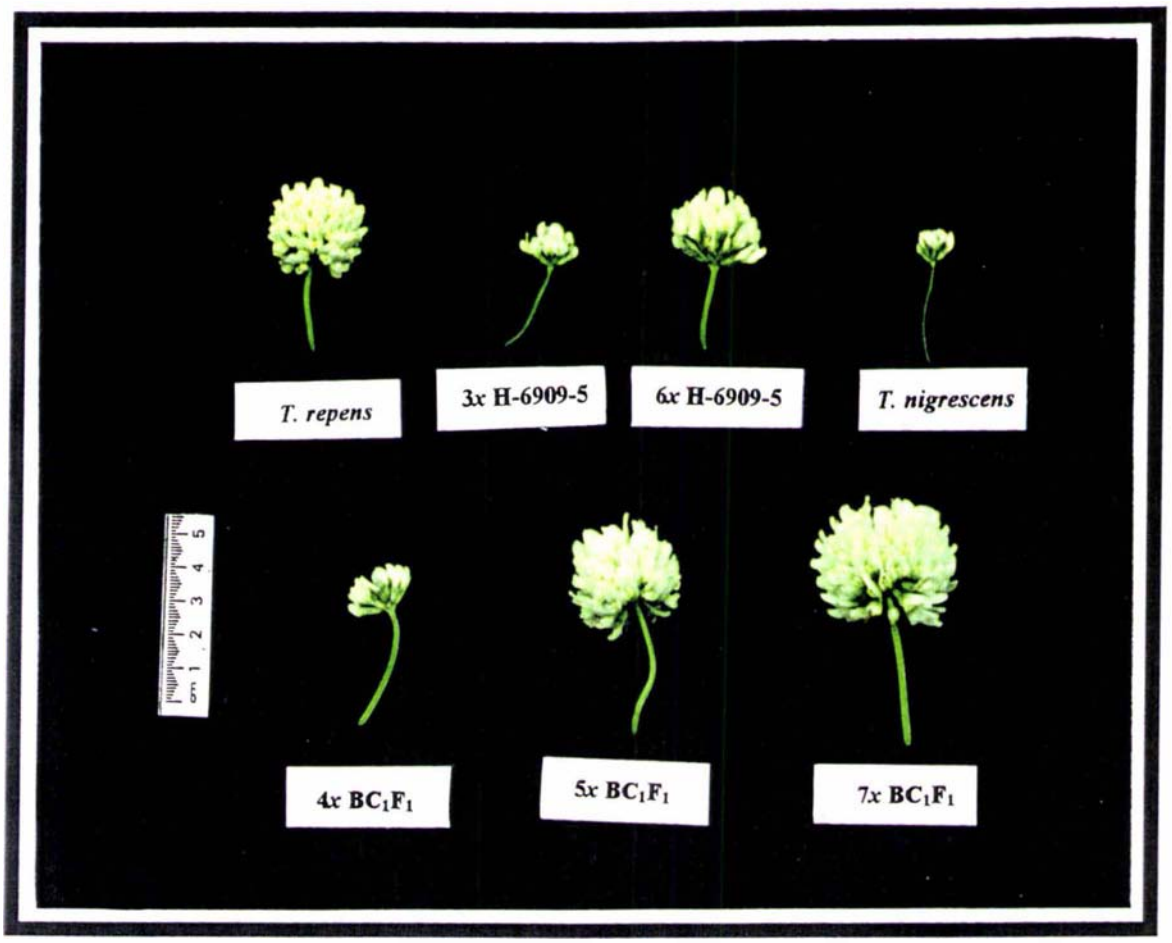


Figure 4.8 Inflorescences sizes of *T. repens*, *T. nigrescens*, 3*x* and 6*x* H-6909-5 (*T. repens* x *T. nigrescens*) F_1 hybrids, 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*), 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*) and 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5).

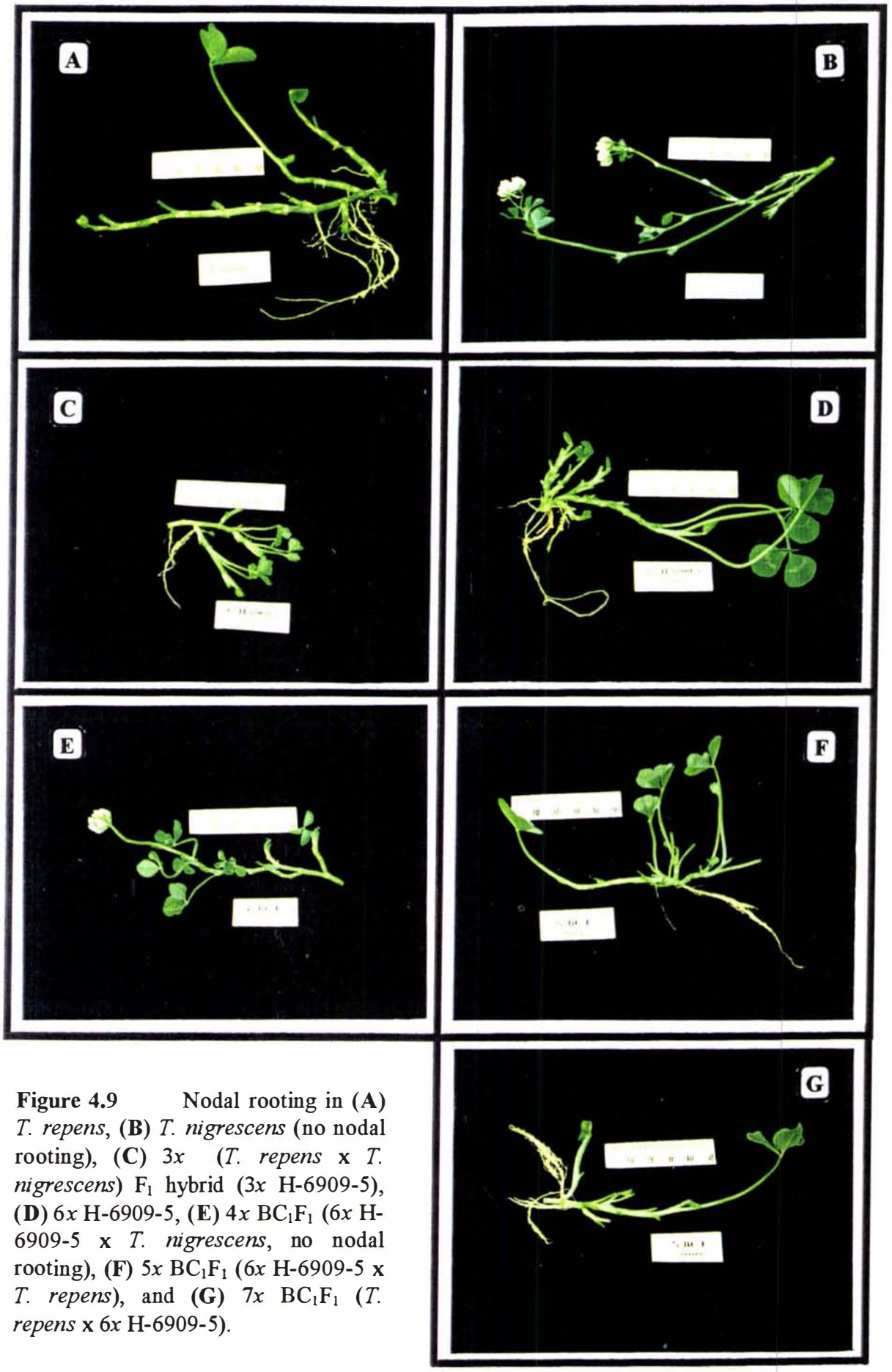


Figure 4.9 Nodal rooting in (**A**) *T. repens*, (**B**) *T. nigrescens* (no nodal rooting), (**C**) 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (3*x* H-6909-5), (**D**) 6*x* H-6909-5, (**E**) 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*, no nodal rooting), (**F**) 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*), and (**G**) 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5).

4.4.3 Congruity Backcross (CBC_2)

The tetraploid BC_1F_1 (CT-14 x Tn-167) was not backcrossed with *T. nigrescens* for a second generation as it already showed strong affinity towards the *T. nigrescens* parent (see section 4.4.1.1). Instead it was crossed reciprocally to *T. repens* (Huia-1 used as the female and CC-1 as the male) to obtain CBC_2 progeny (Table 4.2).

The leaf marking (V^m V^I; R^I -) and multifoliolate characters of *T. repens* (CC-1) were used as genetic markers in CBC_2 crosses involving CT-14 x Tn-167 as the female and *T. repens* (CC-1) as the male, while the white "V" leaf mark (V^i -) of CT-14 x Tn-167 was used as a marker in those crosses involving *T. repens* (Huia-1) as the female parent. One of the CBC_2 seeds from the cross (CT-14 x Tn-167) x CC-1 was germinated. The resultant plant carried the "silver sprite" leaf mark "V^m" (Fig. 4.10) derived from the male parent *T. repens* (CC-1), thus confirming its hybridity.

Another congruity second backcross (CBC_2) was attempted between pentaploid BC_1F_1 (CT-14 x CC-1) and *T. nigrescens* (Tn-167), but no seeds were obtained from 640 reciprocal crosses (Table 4.2).

4.4.4 BC_1F_1 x BC_1F_1 Intercross (BC_1F_2)

The tetraploid, pentaploid and two heptaploid BC_1F_1's were reciprocally crossed amongst themselves. Although the numbers of these intercrosses were comparatively small (Table 4.2), at least one seed was obtained from each cross, with the exception of 7*x* (CC-1 x CT-14)-2 x 5*x* (CT-14 x CC-1) where no viable seeds were obtained from 75 crosses. The two most successful intercrosses were (1) between the tetraploid (♀) and pentaploid (♂) BC_1F_1's and (2) between the two heptaploid BC_1F_1's where 6 well developed seeds were obtained from each intercross after pollinating only 30 and 60 florets respectively (Table 4.2).

Two seeds were geminated successfully from the intercrosses involving tetraploid BC_1F_1 (CT-14 x Tn-167) as the female and pentaploid BC_1F_1 (CT-14 x CC-1) as the male parent. One of these plants showed multifoliolate traits while the other exhibited "silver sprite" (V^m) leaf mark inherited from the male parent. The presence of the multifoliolate

character in one and the silver sprite "*V*^{m}" leaf mark in the other (Fig. 4.10) confirmed their intercross origin. One of these plants with the multifoliolate character was evaluated cytologically for ploidy level and was found to be an aneuploid with $2n$=36 (Fig. 4.11). The occurrence of an aneuploid from the 4*x* BC_1F_1 x 5*x* BC_1F_1 intercross suggested that an aneuploid male gamete with n=20 from the 5*x* BC_1F_1 had functioned as the 4*x* BC_1F_1 produced mostly euploid gametes with $n=2x$=16 (section 4.5).

4.4.5 BC_1F_1 x F_1 (6*x* H-6909-5)

Initially all the colchicine derived hexaploid plants (CT-1, CT-14 and CT-28) of H-6909-5 were used in backcrosses with *T. repens* (CC-1) and *T. nigrescens* (Tn-167). However only plant CT-14 was initially cross fertile in producing BC_1F_1 seeds. Later, plant CT-28 was reciprocally crossed with 4*x*, 5*x* and 7*x* BC_1F_1's to examine its male and female fertility. Results of these crosses indicated that plant CT-28 was more female fertile than male fertile (Table 4.2). Plant CT-1 was not used in crosses with all three BC_1F_1's but produced 8 seeds from 70 florets pollinated with 5*x* BC_1F_1 (CT-14 x CC-1) pollen, while reciprocal crosses were unsuccessful. Seeds from these crosses have not yet been germinated.

4.5 MEIOTIC CHROMOSOME PAIRING IN H-6909-5 AND BC_1F_1 PROGENY

The means and ranges of meiotic chromosome associations at metaphase I in pollen mother cells (PMCs) of *T. repens* (CC-1), *T. nigrescens* (Tn-167), triploid *T. repens* x *T. nigrescens* (3*x* H-6909-5) F_1 hybrid, three colchicine doubled hexaploid *T. repens* x *T. nigrescens* (6*x* H-6909-5) plants, a tetraploid (4*x*) 6*x* H-6909-5 x *T. nigrescens* backcross, a pentaploid (5*x*) 6*x* H-6909-5 x *T. repens* (CC-1) backcross and one heptaploid (7*x*) *T. repens* (CC-1) x 6*x* H-6909-5 backcross are presented in Table 4.3.

Meiosis was highly regular in *T. repens* (CC-1) and *T. nigrescens* (Az 2225-167) with sixteen and eight bivalents for both these species respectively at metaphase I (Fig. 4.12). Four PMCs of *T. repens* were found at anaphase I showing 16-16 normal disjunction of chromosomes (Fig. 4.12). Meiosis also proceeded normally in *T. nigrescens* with 8-8 disjunction at anaphase I (Fig. 4.12).

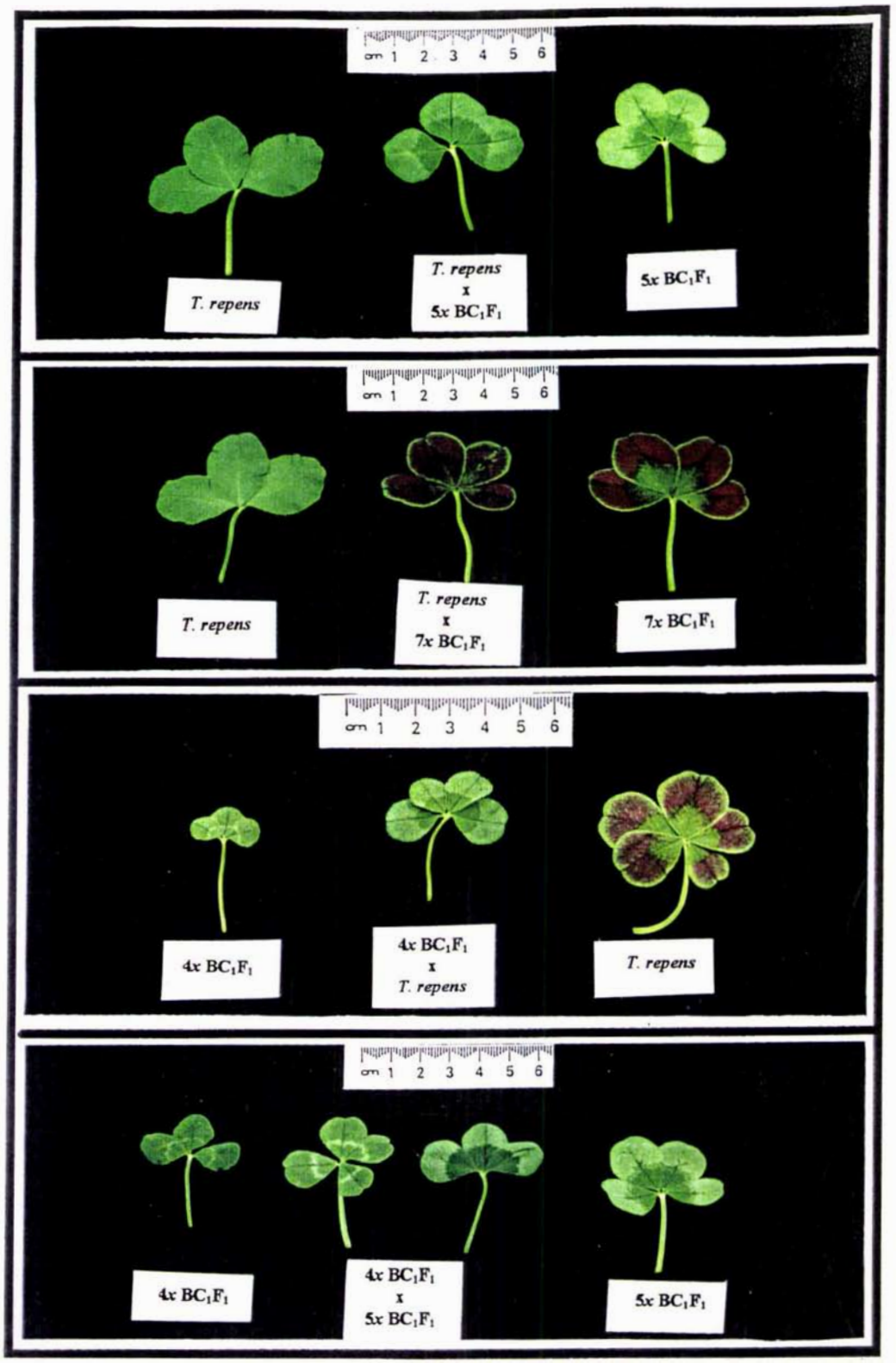


Figure 4.10* Leaf mark inheritance in BC_2F_1, CBC_2 and BC_1F_2 progeny from male parent. **(A)** left, *T. repens* (♀), no leaf marking, *vv*), middle, BC_2F_1 (*T. repens* x 5*x* BC_1F_1) (V^m V^t), right, 5*x* BC_1F_1 (♂) (V^m V^t), **(B)** left, *T. repens* (♀), (no leaf marking, *vv*), middle, BC_2F_1 (*T. repens* x 7*x* BC_1F_1) (V^m V^t; R^l *r* and multifoliolate), right, 7*x* BC_1F_1 (♂) (V^m V^t; R^l *r* and multifoliolate) **(C)** left, 4*x* BC_1F_1 (♀) (V^1 -), middle, CBC_2 (4*x* BC_1F_1x *T. repens*) (V^m V^t), right, *T. repens* (♂)(V^m V^t; R^l *r* and multifoliolate) **(D)** left, 4*x* BC_1F_1 (♀) (V^1 -), middle two leaves BC_1F_2 (4*x* BC_1F_1 x 5*x* BC_1F_1) showing multifoliolate (central left) and "silver sprite" (V^m V^t, central right), right, 5*x* BC_1F_1 (♂) (V^m V^t, and multifoliolate).

*6*x* H-6909-5 = 6*x* (*T. repens* x *T. nigrescens*)
4*x* BC_1F_1 = 6*x* H-6909-5 x *T .nigrescens*
5*x* BC_1F_1 = 6*x* H-6909-5 x *T. repens*
7*x* BC_1F_1 = *T. repens* x 6*x* H-6909-5

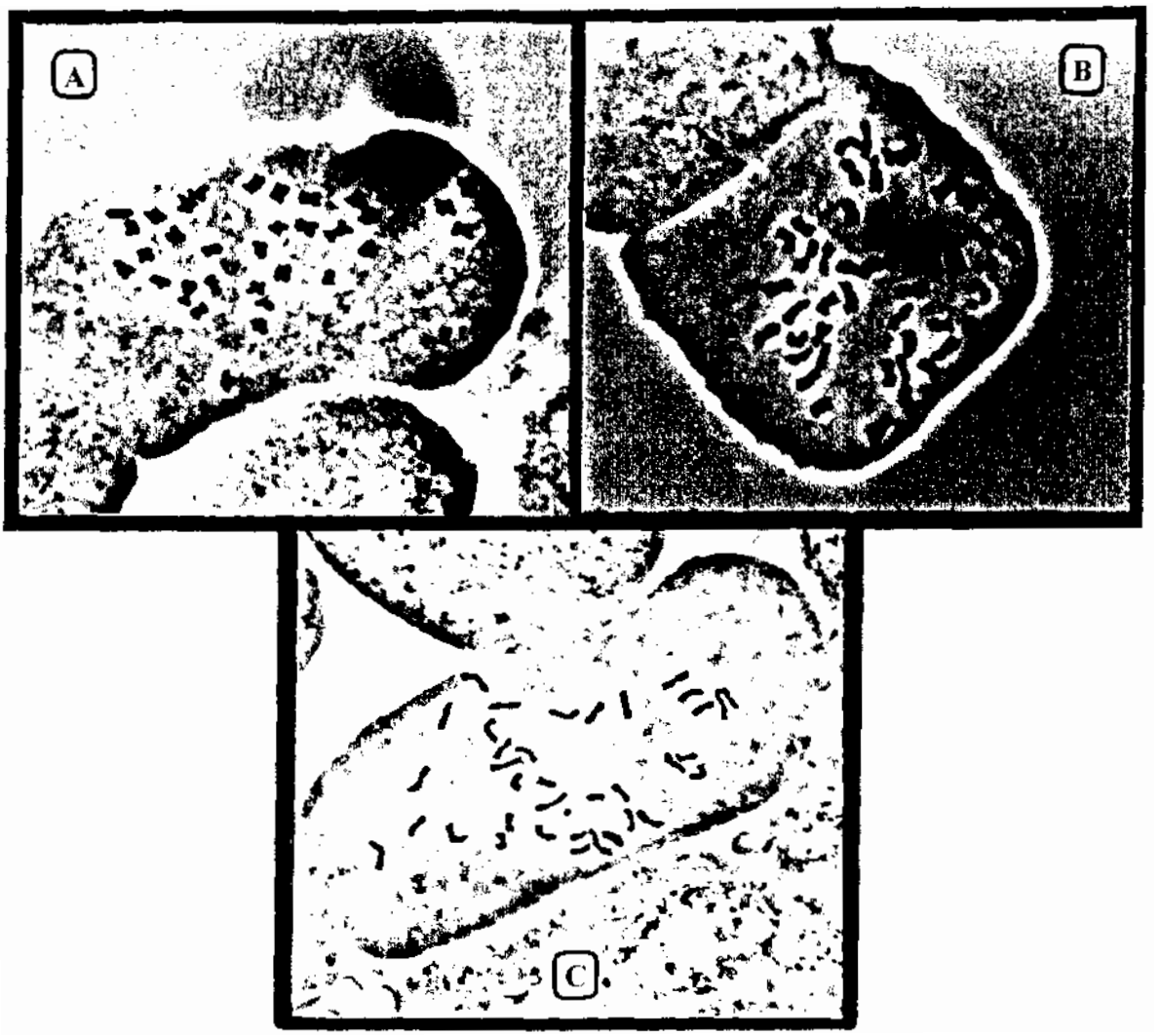


Figure 4.11 Somatic chromosome numbers of BC_2F_1 and BC_1F_1 x BC_1F_1 intercross. **(A)** *T. repens* x 5*x* BC_1F_1 (BC_2F_1, 2*n*=4*x*=32), **(B)** *T. repens* x 7*x* BC_1F_1 (BC_2F_1, 2*n*=42) and **(C)** 4*x* BC_1F_1 x 5*x* BC_1F_1 (BC_1F_2, 2*n*=36). x 1600.

T. repens x *T. nigrescens* F_1 hybrid (3*x* H-6909-5) had the expected somatic chromosome number of $2n=3x=24$ (Fig. 4.3). Meiotic studies of 25 PMCs of this triploid hybrid at metaphase I (Table 4.3) showed that the number of univalents averaged 3.60 per cell with a range of 1-6 and trivalents averaged 2.56 per cell with a range of 0-5. The remaining chromosomal configurations were interpreted as bivalents averaging 6.60 with a range of 3-8 per PMC (Fig. 4.12). Four PMCs were observed at anaphase I showing a 12-12 chromosome disjunction (Fig. 4.12).

The three colchicine derived hexaploid plants (CT-1, CT-14 and CT-28) of the *T. repens* x *T. nigrescens* hybrid (6*x* H-6909-5) had the expected somatic chromosome number of $2n=6x=48$ (Fig. 4.3). Meiotic chromosome configurations were studied in 20 PMCs of plant CT-1, 25 PMCs of plant CT-14, and 30 PMCs of CT-28 at metaphase I. Plants CT-1 and CT-28 had similar meiotic configurations with an average of 0.73 and 0.67 (range 0-2 and 0-5) univalents, 18.0 and 18.44 (range 14-23 and 14-22) bivalents, 0.36 and 0.66 (range 0-1) trivalents and 2.55 and 2.44 (range 0-5 and 0-4) quadrivalents respectively. (Fig. 4.12). A 24-24 chromosome disjunction was observed in at least four PMCs at anaphase I for each plant (Fig. 4.12). Plant CT-14 had a slightly different meiotic chromosome pairing in comparison to CT-1 and CT-28, with an average of 0.82 (range 0-3) univalents, 20.04 (range 15-24) bivalents, 0.39 (range 0-2) trivalents and 1.48 (range 0-4) quadrivalents.

Meiotic chromosome configurations in 25 PMCs of the tetraploid ($2n=4x=32$) CT-14 x Tn-167 BC_1F_1 showed an average of 1.24 (range 0-2) univalents, 8.64 (range 6-14) bivalents, 0.76 (range 0-2) trivalents and 2.80 (range 0-5) quadrivalents per PMC (Table 4.3 and Fig. 4.13). Twenty two cells were found at anaphase I with a 16-16 disjunction (Fig. 4.13) and 16 quadrats were found at telophase II having 16 chromosomes, indicating that the gross meiotic abnormality (univalents and multivalents) had no effect on the meiotic products.

The pentaploid ($2n=5x=40$) BC_1F_1 (CT-14 x CC-1) showed an average of 3.66 (range 1-6) univalents, 10.71 (range 8-18) bivalents, 1.09 (range 0-5) trivalents and 2.91 (range 0-5) quadrivalents per PMC (Table 4.3 and Fig. 4.13). Sixteen PMCs were found to have 20-20 disjunction of the chromosomes at anaphase I while 3 PMCs showed an approximate 24-16 disjunction (Fig. 4.13). These results combined with the results of crossing 5*x* BC_1F_1 with

Table 4.3 Somatic chromosome number, meiotic configurations of pollen mother cells (PMCs) and pollen stainability of *T. repens, T. nigrescens,* F_1 triploid hybrid (3*x* H-6909-5), colchicine induced hexaploid hybrid (6*x* H-6909-5) and backcrosses of 6*x* H-6909-5 to *T. repens* and *T. nigrescens.*

Genotype/Cross	Somatic Chromosome No	Total PMCs scored	Meiotic configuration at metaphase I in pollen mother cells								Pollen Stainability (%)
			I		II		III		IV		
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	
T. repens (CC-1)	2*n*=4*x*=32	15	0.00	(0-0)	16.00	(16-16)	—	—	—	—	90.5
T. nigrescens (Az 2225-167)	2*n*=2*x*=16	20	0.00	(0-0)	8.00	(8-8)	—	—	—	—	93.6
(*T. repens* x *T.nigrescens*)											
3*x* H-6909-5	2*n*=3*x*=24	25	3.60	(1-6)	6.36	(3-8)	2.56	(1-5)	—	—	9.9
6*x* H-6909-5											
Plant No. CT-1	2*n*=6*x*=48	20	0.73	(0-2)	18.00	(14-23)	0.36	(0-1)	2.55	(0-5)	88.8
Plant No. CT-14	2*n*=6*x*=48	25	0.82	(0-3)	20.04	(15-24)	0.39	(0-2)	1.48	(0-4)	90.9
Plant No. CT-28	2*n*=6*x*=48	30	0.67	(0-5)	18.44	(14-22)	0.66	(0-1)	2.44	(0-4)	87.9
6*x* H-6909-5 x *T. nigrescens*	2*n*=4*x*=32	25	1.24	(0-2)	8.64	(6-14)	0.76	(0-2)	2.80	(0-5)	59.6
6*x* H-6909-5 x *T. repens*	2*n*=5*x*=40	35	3.66	(1-6)	10.71	(8-18)	1.09	(0-5)	2.91	(0-5)	86.7
T. repens x 6*x* H-6909-5											
Plant No. 1	2*n*=7*x*=56	15	6.00	(3-11)	9.08	(7-14)	3.83	(1-5)	5.08	(1-9)	72.7
Plant No. 2	2*n*=7*x*=56	—	—	—	—	—	—	—	—	—	71.8

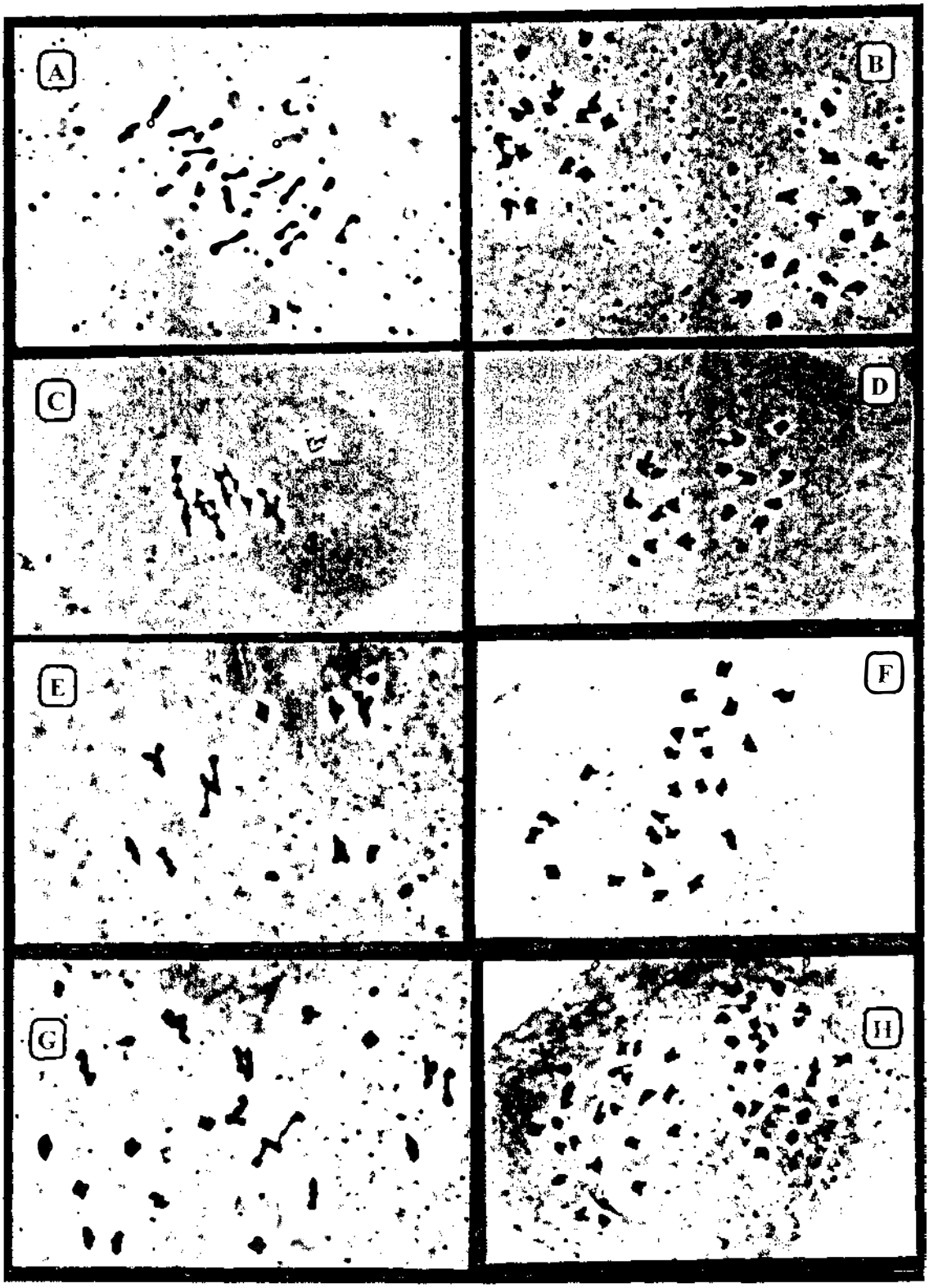


Figure 4.12 Meiotic configurations in *T. repens*, *T. nigrescens* and their F_1 hybrids. **(A)** *T. repens* showing 16 II at metaphase I. **(B)** *T. repens* showing 16-16 disjunction at anaphase I, **(C)** *T. nigrescens* with 8 II at metaphase I, **(D)** *T. nigrescens* showing 8-8 disjunction at anaphase I, **(E)** 3*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (3*x* H-6909-5) with 3 I + 6 II + 3 III at metaphase I, **(F)** 3*x* H-6909-5 showing 12 - 12 disjunction at anaphase I, **(G)** 6*x* H-6909-5 with 2 I + 14 II + 2 III + 3 IV at metaphase I, and **(H)** 6*x* H-6909-5 showing 24 - 24 disjunction at anaphase I. x 1600.

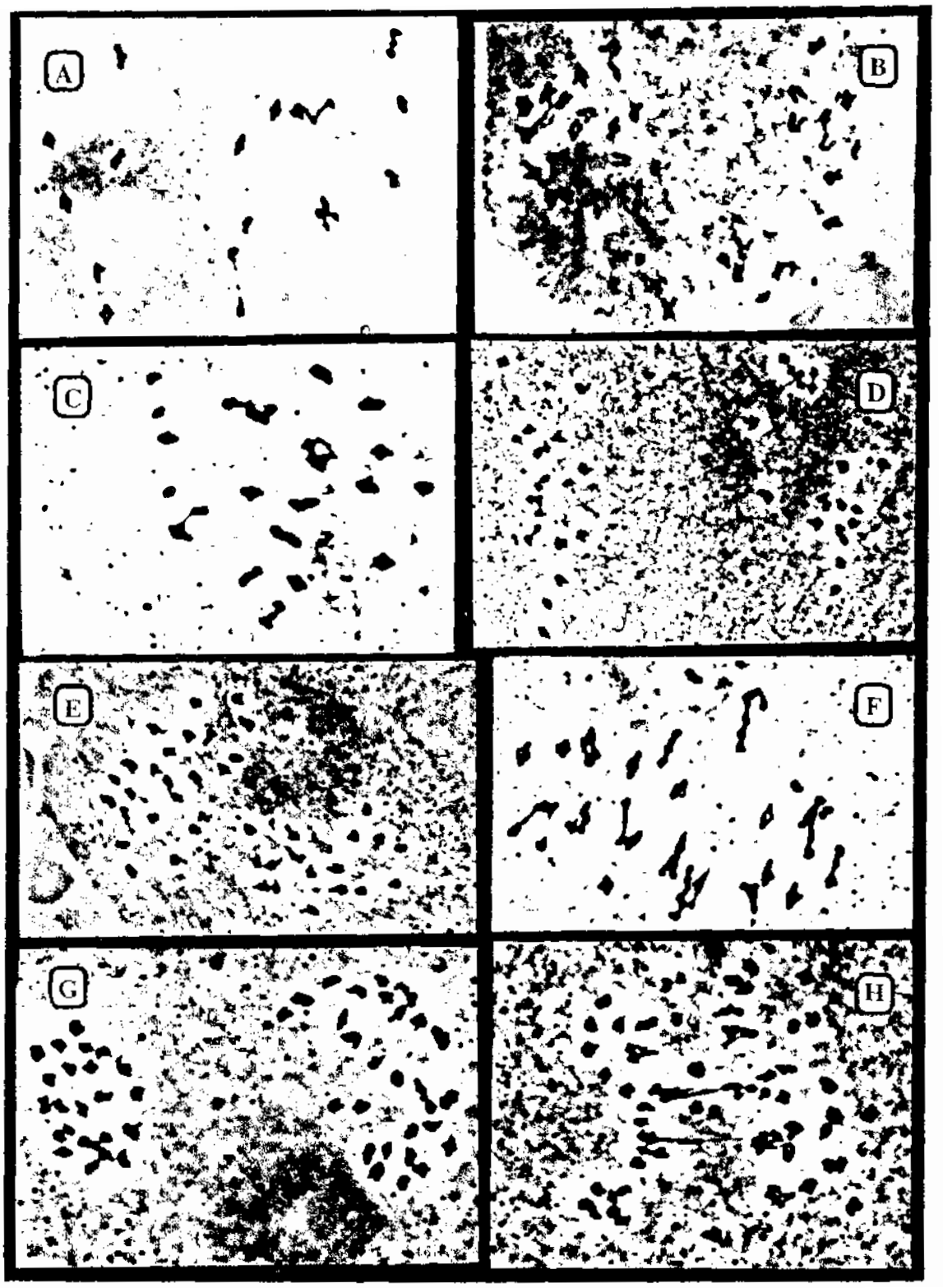


Figure 4.13 Meiotic configurations in first backcross (BC_1F_1) progeny from crossing 6*x* (*T. repens* x *T. nigrescens*) F_1 hybrid (6*x* H-6909-5) with both parental species. **(A)** 4*x* BC_1F_1 (6*x* H-6909-5 x *T. nigrescens*) with, 2 I + 11 II + 2 IV at metaphase I, **(B)** 4*x* BC_1F_1, with 16 - 16 disjunction at anaphase I, **(C)** 5*x* BC_1F_1 (6*x* H-6909-5 x *T. repens*) with, 3 I + 13 II + 1 III + 2 IV at metaphase I, **(D)** 5*x* BC_1F_1, with approximately 20 - 20 disjunction at anaphase I, **(E)** 5*x* BC_1F_1, with approximately 16 - 24 disjunction at anaphase I and lagging chromosome **(F)** 7*x* BC_1F_1 (*T. repens* x 6*x* H-6909-5) with, 3 I + 11 II + 1 III + 7 IV at metaphase I, **(G)** 7*x* BC_1F_1, with approximately 24 - 32 disjunction at anaphase I, and **(H)** 7*x* BC_1F_1, with approximately 28 - 28 disjunction at anaphase I and lagging chromosomes

T. repens (section 4.4.2) and 4*x* BC_1F_1 (section 4.4.4) suggested that 5*x* BC_1F_1 produced three different types of gametes i.e. 2*x*, 3*x* and aneuploid with *n*=20. In some of the PMCs 1-2 lagging chromosomes were also observed at anaphase I (Fig. 4.13). Meiotic configurations were studied in 15 PMCs for one plant of the two heptaploids (2*n*=7*x*=56) *T. repens* (CC-1) x 6*x* H-6909-5 backcross which showed an average of 6.00 (range 3-11) univalents, 9.08 (range 7-14) bivalents, 3.83 (range 1-5) trivalents and 5.08 (range 1-9) quadrivalents (Fig. 4.13). At anaphase I four PMCs were found with a 28-28 disjunction while one PMC at anaphase I was found with an approximate 24-32 disjunction. Two PMCs were observed with 2-4 lagging chromosomes at anaphase I (Fig. 4.13).

4.6 SCREENING 3*x* AND 6*x* H-6909-5 FOR CLOVER CYST NEMATODE RESISTANCE AND SUSCEPTIBILITY

Results of the initial counts of *H. trifolii* cysts on *T. nigrescens* (Az 2225) and *T. repens* cv. "Grassland Huia" (C6484) are presented in Table 4.4.

Numbers of *H. trifolii* cysts were higher on *T. repens* than *T. nigrescens* thus confirming the susceptibility of *T. repens* to clover cyst nematode and the effectiveness of the inoculum. Twenty seven (54%) of the 50 initially screened *T. nigrescens* plants had 0-10 cysts per plant while 80% of *T. repens* plants had more than 100 (a range of 104-239) cysts per plant. The remaining 20% of *T. repens* plants showed a range of 49-69 cysts per plant.

Table 4.4 Mean number and range of *H. trifolii* cysts produced on two species of *Trifolium* after initial screening.

Species	Accession No.	No. of plants tested	*H. trifolii* cyst number per plant	
			Mean	Range
T. nigrescens	Az 2225	50	23.4	0-150
T. repens cv. "Grasslands Huia"	C6484	20	149.7	49-239

The plants initially screened were repotted for a rescreening experiment to test the results of the initial screening. However only 16 out of the 50 *T. nigrescens* plants survived until rescreening because of their annual growth habit. Five cuttings from each of the surviving *T. nigrescens* genotypes and five *T. repens* genotypes were included in the

Table 4.5 Comparison of the number of *H. trifolli* cysts per plant after initial and rescreening of 16 *T. nigrescens* and 5 *T. repens* genotypes

Species	Plant No	Number of cysts after Initial screening*	Rescreening†
***T. nigrescens*‡** (Az 2225)	Tn-4	2	9.2
	Tn-8	38	30.8
	Tn-9	1	3.0
	Tn-14	140	88.8
	Tn-17	2	20.4
	Tn-20	1	28.2
	Tn-26	0	7.8
	Tn-27	150	60.0
	Tn-29	44	33.0
	Tn-30	0	0.6
	Tn-32	0	23.0
	Tn-36	0	3.6
	Tn-39	9	51.0
	Tn-42	12	30.8
	Tn-46	10	47.8
	Tn-48	3	9.2
T. repens (C.6484‡)	Tr-5	54	49.4
	Tr-10	211	199.2
	Tr-12	49	117.0
	Tr-14	236	165.2
	Tr-19	69	126.2
LSD (P<0.05)			30.6

* Number of cysts per plant

† Mean of five cuttings for each genotype.

‡ Plants which survived after the initial screening.

rescreening. Mean numbers of cysts recorded for the 16 *T. nigrescens* genotypes and five *T. repens* genotypes after rescreening are presented in Table 4.5.

Numbers of cysts per plant after rescreening were reasonably consistent with the initial screening (Table 4.5). Significant differences in cyst number occurred among the 16 *T. nigrescens* genotypes. Six *T. nigrescens* genotypes had significantly fewer cysts than the most resistant *T. repens* genotype while four of the five *T. repens* genotypes had significantly more cysts per plant than all 16 *T. nigrescens* genotypes. Based on the results of both screenings (Table 4.5), two genotypes of *T. nigrescens*, Tn-14 and Tn-30, with high and low numbers of cysts, respectively, and one genotype of *T. repens*, Tr-10, with a high number of cysts, were selected and included for comparison in the screening of triploid and hexaploid *T. repens* x *T. nigrescens* F_1 hybrids (H-6909-5).

Six cuttings each from the two *T. nigrescens* (Tn-14 and Tn-30) genotypes, one *T. repens* (Tr-10) genotype and 10 cuttings each from the 3*x* and two 6*x* H-6909-5's (CT-1 and CT-14) were evaluated for cyst nematode resistance/susceptibility (Table 4.6).

Table 4.6 Number of *H. trifolii* cysts produced on *T. nigrescens*, *T. repens* and 3*x* and 6*x* H-6909-5.

Species/hybrid	Plant No	No. of plants tested	Mean number of cysts/g of RDW*
T. repens (C6484)	Tr-10	6	522.8 a†
T. nigrescens (Az 2225)	Tn-14	6	340.3 b
T. nigrescens (Az 2225)	Tn-30	6	9.6 c
3*x* H-6909-5	--	10	6.1 c
6*x* H-6909-5	CT-1	10	3.2 c
6*x* H-6909-5	CT-14	10	2.6 c

* Root dry weight

† Means with a common letter are not significantly different ($P < 0.05$) according to Duncan's multiple range test.

The 3*x* and two 6*x* H-6909-5 plants (CT-1 and CT-14) were as resistant as the resistant *T. nigrescens* (Tn-30) genotype and were significantly lower in cyst number per gram of root dry weight than the susceptible *T. nigrescens* (Tn-14) and *T. repens* (Tr-10) genotypes. The susceptible *T. nigrescens* genotype was also significantly lower in cyst number than *T. repens*.

4.7 BACKCROSSES OF 4*x* AND 8*x* H-435 TO *T. repens* AND *T. ambiguum.*

4.7.1 Self-Pollinations

Self pollination of 8 inflorescences (more than 400 flowers) of *T. repens* (CC-1), 5 inflorescences (more than 250 flowers) from each of the three plants of 4*x* *T. ambiguum* (cv. Treeline) and three plants of 6*x* *T. ambiguum* (cv. Prairie) resulted in no seed, thus confirming the self-incompatibility of these genotypes. Although 4*x* H-435 produced no seed after selfing 10 inflorescences (more than 500 flowers) in the present investigation, its self-compatibility has previously been reported by Williams *et al.* (1982). Self-pollinations of 12 inflorescences (more than 700 flowers) of the colchicine doubled (C_0) 8*x* H-435 resulted in 13 seeds from which 2 seeds were germinated and grown into mature plants.

4.7.2 First Backcross (BC_1F_1)

Results of the reciprocal backcrosses of 4*x* and 8*x* H-435 to one genotype of *T. repens* (CC-1), 3 genotypes of 4*x* *T. ambiguum* (cv. Treeline) and 3 genotypes of 6*x* *T. ambiguum* (cv. Prairie) are presented in Table 4.7. Reciprocal backcrosses of 4*x* and 8*x* H-435 with tetraploid and hexaploid *T. ambiguum* were not successful. Only one seed was harvested from 4*x* H-435 as the female parent after pollination with *T. repens* (CC-1) pollen. The seed was germinated but failed to produce a healthy seedling and died after 10 days.

Fifteen mature plants were grown after germination of 17 of the 124 seeds harvested from 8*x* H-435 pollinated with *T. repens* (CC-1). All 15 BC_1F_1 plants carried leaf markings (Table 4.8, Fig. 4.14) derived from the *T. repens* (CC-1) male parent, thus confirming the backcross origin of the BC_1F_1 progeny. Twenty one fully developed seeds were obtained from *T. repens* (CC-1) after pollination with 8*x* H-435 pollen, from which four seeds were germinated and grown into mature plants. All the nineteen BC_1F_1 plants were self-incompatible as no seed was obtained from any plant after selfing at least 8 inflorescences on each plant.

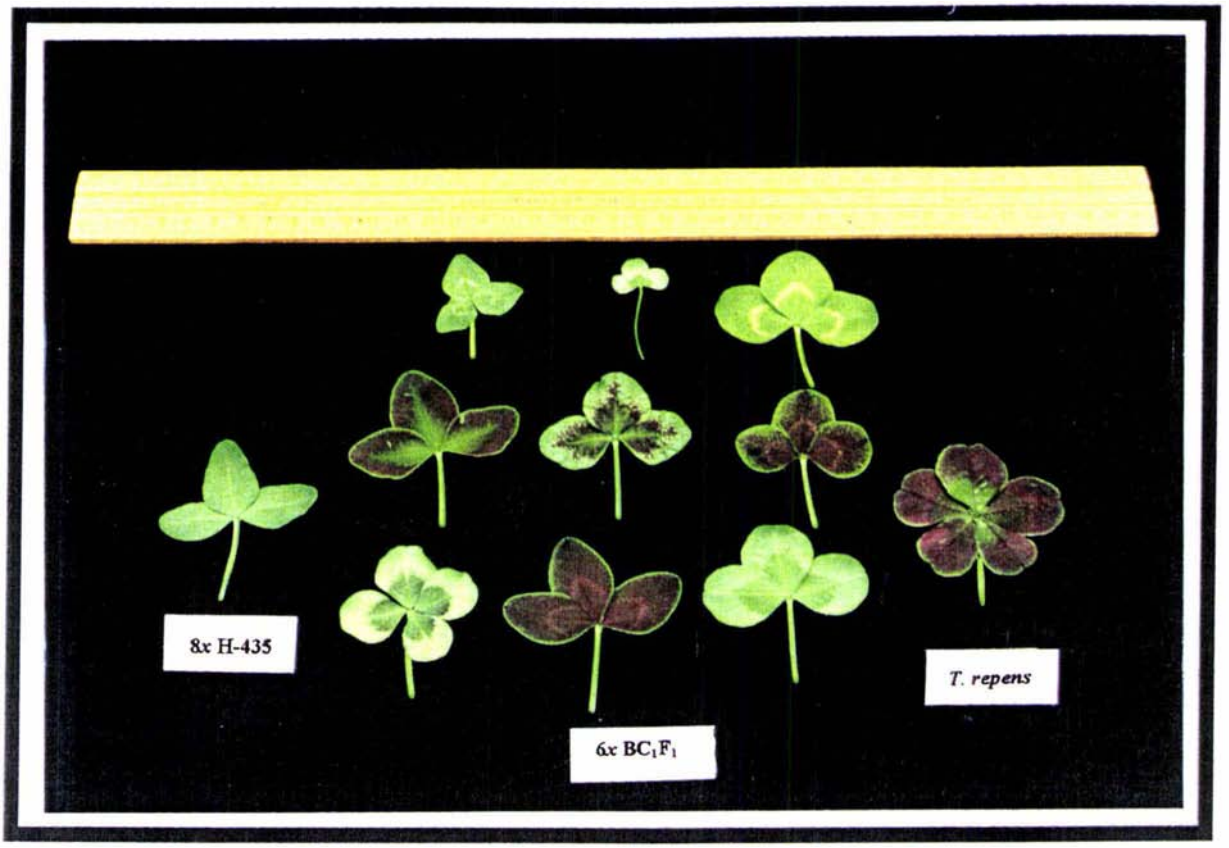


Figure 4.14 Leaf markings in 6*x* BC_1F_1 (8*x* H-435 x *T. repens*) derived from *T. repens* male parent. Extreme left, 8*x* H-435 (*T. ambiguum* x *T. repens*) with no leaf mark, central three columns, 6*x* BC_1F_1 plants, all with leaf markings and one showing the multi-leaflet character, extreme right *T. repens* (CC-1) with red leaflets and multifoliolate traits.

4.7.3 Congruity (CBC) and Second (BC_2F_1) Backcrosses

Two genotypes of BC_1F_1, (8*x* H-435 x CC-1)-2 and (CC-1 x 8*x* H-435)-1 were reciprocally backcrossed with one genotype of tetraploid (cv. Treeline) and three genotypes of hexaploid (cv. Prairie) *T. ambiguum*. Significant pod development was observed during the first two weeks after pollination (Table 4.7) for crosses involving BC_1F_1 as the female parent. However, most of the pods failed to develop seeds. Only three seeds were harvested from (8*x* H435 x CC-1)-2 pollinated with 4*x* *T. ambiguum*. In reciprocal crosses of this combination, pod development was either absent or very poor. No seeds were harvested from these reciprocal crosses.

Two genotypes of BC_1F_1, (CC-1 x 8*x* H-435)-1 and (CC-1 x 8*x* H-435)-2 were used in second backcrosses with *T. repens* (CC-1) using *T. repens* as a male parent only. Significant pod development was observed during the first and second weeks following pollination (Table 4.7). Most of the pods contained shrunken non-germinable seeds, although approximately 17% of the developed pods had either one or two fully developed seeds. Reciprocal backcrosses of this combination were not attempted. Seven seeds of BC_1F_1 x CC-1 (BC_2F_1) were germinated and grown into mature plants.

4.7.4 BC_1F_1 x BC_1F_1 Intercrosses (BC_1F_2)

Three BC_1F_1 genotypes, (CC-1 x 8*x* H-435)-1, (CC-1 x 8*x* H-435)-2 and (8*x* H-435 x CC-1)-2 were intercrossed and produced 114 fully developed BC_1F_2 seeds from 663 crosses (Table 4.7). Six of these seeds were germinated and grown into mature plants. Upon flowering, two genotypes of BC_1F_2 were reciprocally backcrossed with one genotype of 6*x* *T. ambiguum* (cv. Prairie) which gave 17 seeds harvested from one BC_1F_2 [(CC-1 x 8*x* H-435)-2 x (CC-1 x 8*x* H-435)-3]-1 female parent. Three of the the 17 seeds have recently been germinated and the resulting plants are growing in the glasshouse for subsequent identification of ploidy level, fertility and morphological characteristics. The reciprocal cross where 6*x* *T. ambiguum* was used as the female parent and BC_1F_2 plants as the male did not produce any seed, despite significant early pod development in comparison to some earlier crosses where BC_1F_1's were used as male parents (Table 4.7). One of the six BC_1F_2 plants, [(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-4 was self-compatible as it produced 161 seeds after self-pollination of 9 inflorescences.

Table 4.7 Results of reciprocal first backcrosses of 4*x* and 8*x* H-435 to *T. ambiguum* (both 4*x* and 6*x*) and *T. repens*, second backcrosses (BC_2F_1), congruity backcrosses, F_1 x F_1, and BC_1F_1 x BC_1F_1 intercrosses (BC_1F_2).

Cross	No. of Crosses	Pod Development No.	Pod Development (%)	Number of Seeds Obtained
First backcross (BC_1F_1)				
8*x* H-435 x CC-1	1320	683	51.7	124
CC-1 x 8*x* H-435	258	169	65.7	21
8*x* H-435 x 4*x* *T. ambiguum*	1054	7	0.7	0
4*x* *T. ambiguum* x 8*x* H-435	280	54	19.3	0
8*x* H-435 x 6*x* *T. ambiguum*	1032	0	0.0	0
6*x* *T. ambiguum* x 8*x* H-435	337	53	15.8	0
4*x* H-435 x CC-1	1079	288	26.6	1
CC-1 x 4*x* H-435	185	2	1.1	0
4*x* H-435 x 4*x* *T. ambiguum*	970	1	0.1	0
Second backcross (BC_2F_1)				
6*x* BC_1F_1 x CC-1	760	484	63.7	134
Congruity backcross				
6*x* BC_1F_1 x 4*x* *T. ambiguum*	974	156	16.0	3
4*x* *T. ambiguum* x 6*x* BC_1F_1	178	0	0.0	0
6*x* BC_1F_1 x 6*x* *T. ambiguum*	830	284	34.2	0
6*x* *T. ambiguum* x 6*x* BC_1F	250	8	3.2	0
6*x* BC_1F_2 x 6*x* *T. ambiguum*†	318	163	51.3	17
6*x* *T. ambiguum* x 6*x* BC_1F_2†	413	185	44.8	0
Intercross (BC_1F_2)				
6*x* BC_1F_1 x 6*x* BC_1F_1	663	328	49.5	114
F_1 x F_1				
4*x* H-435 x 8*x* H-435	1021	324	31.7	3

† Congruity backcross following BC_1F_1 x BC_1F_1 intercross.

Three fully developed seeds were also obtained from a 4*x* H-435 x 8*x* H-435 cross using tetraploid H-435 as the female parent. Two fully developed plants were grown after germinating these seeds. The third seedling died about two weeks after germination.

4.7.5 Somatic Chromosomes

The somatic chromosome number for *T. repens* (CC-1) was confirmed as $2n=4x=32$ (Fig. 4.15). Three genotypes of 4*x* *T. ambiguum* (cv. Treeline) and two genotypes of 6*x* *T. ambiguum* (cv. Prairie) were also evaluated cytologically for somatic chromosome counts and as expected, were found to be $2n=4x=32$ and $2n=6x=48$ (Fig. 4.15).

Eight out of 15 plants of the BC_1F_1 obtained utilising 8*x* H-435 as the female parent and *T. repens* as the male parent were all found to be hexaploids with a somatic chromosome number of $2n=6x=48$ (Fig. 4.15). The remaining 7 plants were not assessed. Three out of 4 BC_1F_1 plants having *T. repens* (CC-1) as the female parent and 8*x* H-435 as a male parent were also studied for somatic chromosome number and were confirmed to be hexaploid with $2n=6x=48$ (Fig. 4.15). All these BC_1F_1 plants theoretically combine four genomes from *T. repens* and two genomes of *T. ambiguum.*

The somatic chromosome numbers for three out of the seven BC_2F_1 (BC_1F_1 x CC-1) plants having BC_1F_1 as the female parent and *T. repens* (CC-1) as the male parent were studied and found to be pentaploid ($2n=5x=40$, Fig. 4.15). These pentaploids are expected to carry 4 genomes from white clover and one genome from caucasian clover. The two plants obtained from the BC_1F_1 x 4*x* *T. ambiguum* (cv. Treeline) cross were also found to be pentaploid ($2n=5x=40$, Fig. 4.15) but with an expectedly different genomic combination of two genomes derived from white clover and 3 genomes from caucasian clover.

Only two BC_1F_2 (BC_1F_1 x BC_1F_1) plants were evaluted for somatic chromosome count and both were found to be hexaploids with $2n=6x=48$ (Fig. 4.15). One of these confirmed hexaploids was later used in crosses with 6*x* *T. ambiguum* (cv. Prairie).

One of the two 4*x* H-435 x 8*x* H-435 plants studied for somatic chromosome count showed more than the expected 48 chromosomes (Fig. 4.15), the number being very close to 64. However, the chromosome number of this plant has not yet been confirmed. It might be an octoploid with $2n=64$ or an aneuploid with definitely more than 48 chromosomes.

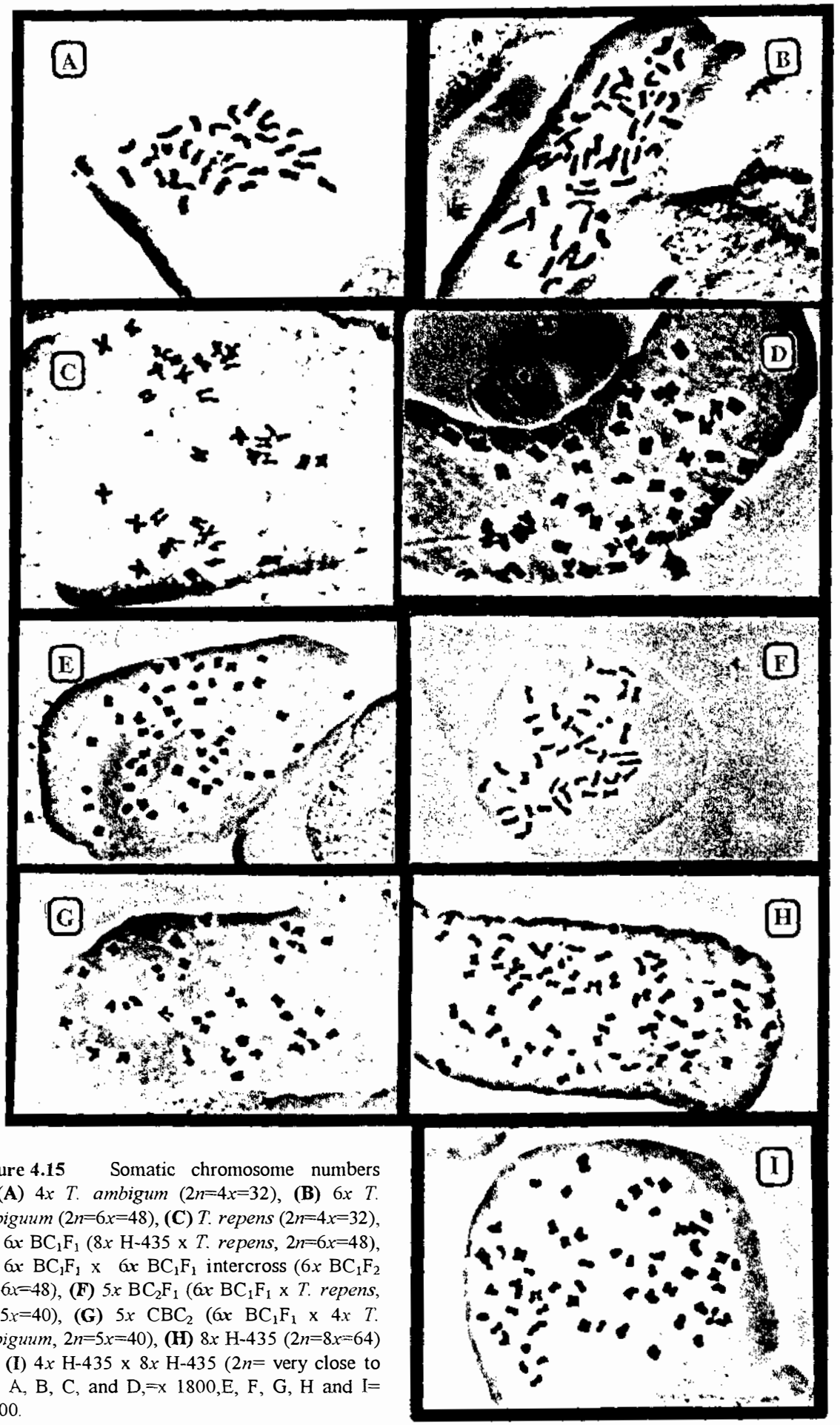


Figure 4.15 Somatic chromosome numbers of **(A)** 4*x* *T. ambigum* (2*n*=4*x*=32), **(B)** 6*x* *T. ambiguum* (2*n*=6*x*=48), **(C)** *T. repens* (2*n*=4*x*=32), **(D)** 6*x* BC_1F_1 (8*x* H-435 x *T. repens*, 2*n*=6*x*=48), **(E)** 6*x* BC_1F_1 x 6*x* BC_1F_1 intercross (6*x* BC_1F_2 2*n*=6*x*=48), **(F)** 5*x* BC_2F_1 (6*x* BC_1F_1 x *T. repens*, 2*n*=5*x*=40), **(G)** 5*x* CBC_2 (6*x* BC_1F_1 x 4*x* *T. ambiguum*, 2*n*=5*x*=40), **(H)** 8*x* H-435 (2*n*=8*x*=64) and **(I)** 4*x* H-435 x 8*x* H-435 (2*n*= very close to 64). A, B, C, and D,=x 1800,E, F, G, H and I= x1600.

4.7.6 Pollen Stainability

Pollen stainability data for all the material utilised in backcrosses, first backcross progeny (BC_1F_1), second backcross progeny (BC_2F_1), congruity backcross and BC_1F_1 x BC_1F_1 intercrosses (BC_1F_2) are presented in Table 4.8.

Pollen stainabilities of six BC_1F_1 plants where 8*x* H-435 was used as the female parent averaged 37.9% with a low of 21.8% for genotype (8*x* H-435 x CC-1)-1 and a high of 64.9% for genotype (8*x* H-435 x CC-1)-3 (Fig. 4.16). Three reciprocal BC_1F_1 plants where *T. repens* (CC-1) was used as the female parent and 8*x* H-435 as the male parent gave an average pollen stainability of 22.8% with a low of 19.1% for genotype (CC-1 x 8*x* H-435)-1 and a high of 26.9% for genotype (CC-1 x 8*x* H-435)-3 (Fig. 4.16)

Average pollen stainability for the four pentaploid second backcross (BC_2F_1) derived using *T. repens* as the male parent was 59.3%, with a low of 44.4% for genotype [(CC-1 x 8*x* H-435)-2 X CC-1]-1 and a high of 70.1% for genotype [(CC-1 x 8*x* H-435)-2 X CC-1]-7 (Fig. 4.16). One of the two 5*x* BC_2F_1 plants having 4*x* *T. ambiguum* (cv. Treeline) as the male parent showed 17.6% stainable pollen (Fig. 4.16). The other plant did not flower.

Pollen stainability of the six BC_1F_2 (BC_1F_1 x BC_1F_1) plants was highly variable, ranging from about 1.0% for genotype [(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-2 to a high of 74.3% for genotype [(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-3 (Fig. 4.16). The average was 40.8%.

4.8 MEIOTIC CONFIGURATIONS OF 6*x* BC_1F_1 PROGENY

Results of meiotic chromosome pairing in pollen mother cells (PMCs) at metaphase I for one genotype of *T. ambiguum* (cv. Treeline), *T. repens* (CC-1) and hexaploid BC_1F_1 are presented in Table 4.9. Meiotic chromosome pairings in 4*x* H-435 and 8*x* H-435 were not examined in the present study. The data of Anderson *et al.*, (1991c) showed an average of 1.19 and 2.64 univalents, 14.34 and 27.62 bivalents, 0.25 and 0.74 trivalents and 0.34 and 0.84 quadrivalents for 4*x* and 8*x* H-435 respectively. Observation of 15 PMCs at metaphase I in *T. repens* (CC-1) revealed only 16 bivalents per PMC, suggesting that meiosis was highly regular in this species. On the other hand meiosis in one of the *T. ambiguum* (cv.

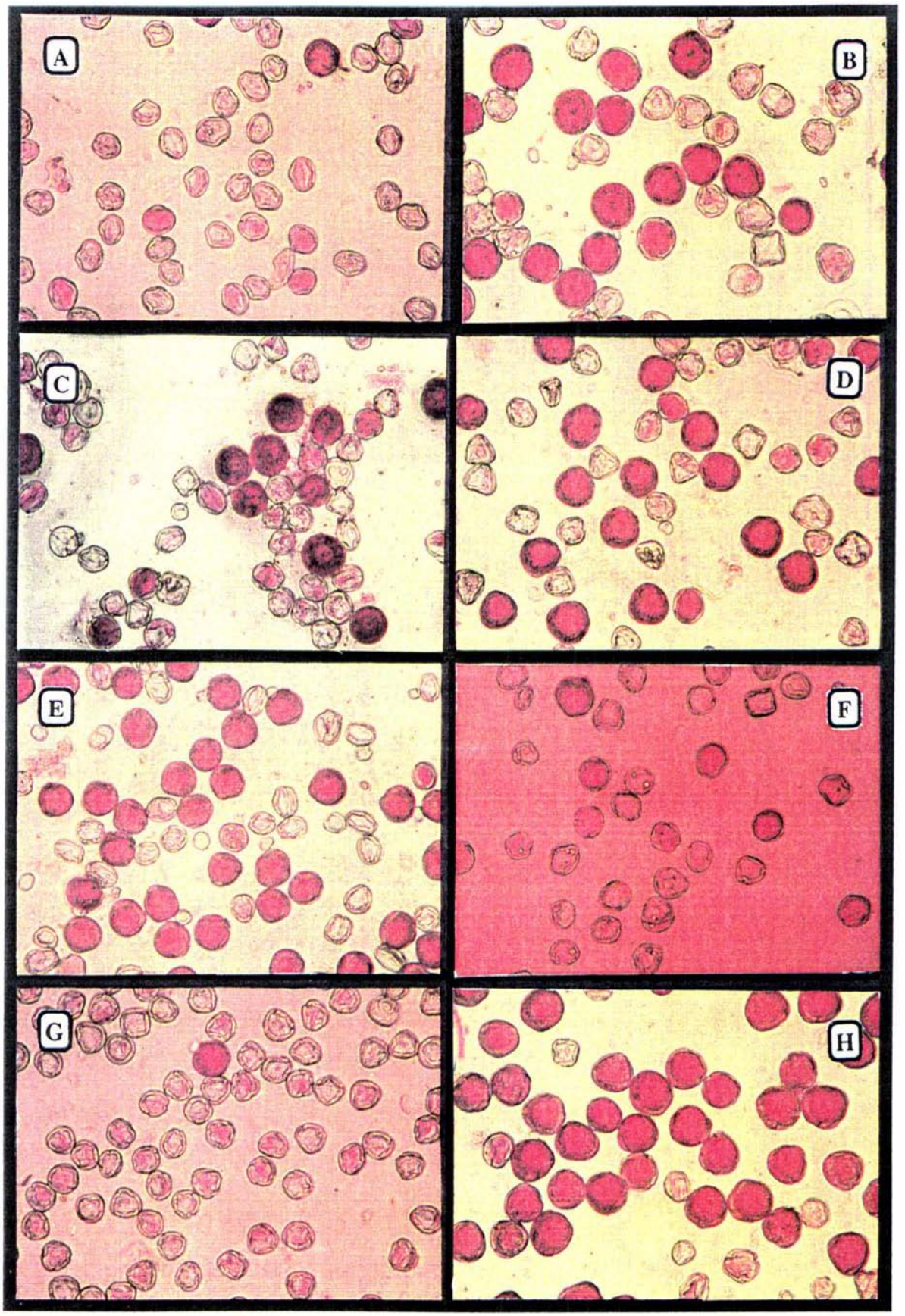


Figure 4.16 Pollen stainability of (**A**) 4*x* H-435 (4*x* *T. ambiguum* x *T. repens*), (**B**) 8*x* H-435, (**C** and **D**) 6*x* BC_1F_1's (8*x* H-435 x *T. repens*), (**E**) 5*x* BC_2F_1 (6*x* BC_1F_1 x *T. repens*), (**F**) 5*x* CBC_2 (6*x* BC_1F_1 x 4*x* *T. ambiguum*), (**G** and **H**) 6*x* BC_1F_2 (6*x* BC_1F_1 x 6*x* BC_1F_1) with very low and high pollen stainability respectively. x = 250

Table 4.8 Percent pollen stainability of 4*x* and 8*x* H-435, first backcrosses (BC_1F_1), second backcrosses (BC_2F_1), congruity backcross (CBC_2), and BC_1F_1 x BC_1F_1 intercrosses, and leaf markings in BC_1F_1 plants.

Genotype	% pollen stainability	Leaf marking*
Parents		
T. ambiguum (cv. Treeline)	96.8	V^h -
T. ambiguum (cv. Prairie)	98.3	V^h -
T. repens (CC-1)	90.5	$V^m V^l$; R^l -
4*x* H-435	13.3	*vv*
8*x* H-435	34.4	*vv vv*
BC_1F_1		
(8*x* H-435 x CC-1)-1	21.8	V^m -; *r* -
(8*x* H-435 x CC-1)-2	46.2	V^l -; R^l -
(8*x* H-435 x CC-1)-3	64.9	V^l -; *r* -
(8*x* H-435 x CC-1)-4	34.1	V^m -; R^l -
(8*x* H-435 x CC-1)-5	28.4	V^l -; R^l -
(8*x* H-435 x CC-1)-6	32.0	V^m -; R^l -
(CC-1 x 8*x* H-435)-1	19.1	V^m -; R^l -
(CC-1 x 8*x* H-435)-2	22.5	V^m -; *r* -
(CC-1 x 8*x* H-435)-3	26.9	V^l -; R^l -
BC_2F_1		
[(CC-1 x 8*x* H-435)-2 X CC-1]-1	44.4	
[(CC-1 x 8*x* H-435)-2 X CC-1]-2	59.8	
[(CC-1 x 8*x* H-435)-2 X CC-1]-6	62.8	
[(CC-1 x 8*x* H-435)-2 X CC-1]-7	70.1	
Congruity backcross		
(8*x* H-435 x CC-1)-2 X 4*x T. ambiguum*	17.6	
BC_1F_2 (BC_1F_1 x BC_1F_1)		
[(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-1	55.7	
[(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-2	0.8	
[(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-3	74.3	
[(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-3]-4	44.6	
[(CC-1 x 8*x* H-435)-1 X (CC-1 x 8*x* H-435)-2]-1	53.4	
[(CC-1 x 8*x* H-435)-1 X (8*x* H-435 x CC-1)-2]-1	16.1	

* Leaf marking references: V^l (Full V, intermediate), Brewbaker and Carnahan (1955); V^m (White tissue distal to the V marking), Lenoble and Papineau (1970); R^l (The upper leaf surface is dark purple and the lower surface is slightly coloured), Carnahan *et al.* (1955).

Treeline) plants was irregular in comparison to *T. repens*, forming on average 0.39 univalents, 0.06 trivalents, and 0.17 quadrivalents per PMC. However, 11 PMCs showed normal 16-16 chromosome separation at anaphase I, indicating that meiosis proceeded normally at the later stages.

Meiotic configurations were examined for three out of 15 hexaploid BC_1F_1 having 8*x* H-435 as the female parent and one out of four hexaploid BC_1F_1 having *T. repens* (CC-1) as the female parent. Pairing was found to be essentially similar in the four BC_1F_1 with frequent univalent and multivalent formation (Table 4.9, Fig. 4.17). However, from the meiotic configurations of these four BC_1F_1 plants, it was impossible to distinguish the chromosomes of *T. repens* and *T. ambiguum*. Therefore, it was not possible from these observations to determine whether the bivalent and multivalent pairing was intra or interspecific. At least four PMCs in each of the BC_1F_1 plants observed at anaphase I showed 24-24 chromosome disjunction (Fig. 4.17).

4.9 MORPHOLOGICAL CHARACTERISTICS OF 6*x* BC_1F_1 PROGENY

Data for different morphological characteristics of ten 6*x* BC_1F_1 plants, *T. repens* (CC-1), one genotype of *T. ambiguum* (cv. "Treeline"), and the 4*x* and 8*x* H-435 plants are presented in Table 4.10. Broad variation was evident for almost every character studied among the 6*x* BC_1F_1 plants, and between the 6*x* BC_1F_1's and the parental genotypes.

Most of the 6*x* BC_1F_1 plants combined the rhizomatous growth habit of *T. ambiguum* with the stoloniferous growth habit of *T. repens*, but showed significantly fewer rhizomes than *T. ambiguum* (cv. Treeline) and 4*x* and 8*x* H-435. Only two out of ten 6*x* BC_1F_1's did not have significantly fewer rhizomes than *T. ambiguum* (cv. Treeline). The greatest rhizome length was recorded for *T. ambiguum* (cv. Treeline) followed by 8*x* H-435 and 4*x* H-435. All of the 6*x* BC_1F_1's had significantly lower rhizome length than *T. ambiguum* and only one approached the rhizome length of 8*x* H-435.

Table 4.9 Somatic chromosome numbers, meiotic configurations of pollen mother cells (PMCs) and pollen stainability of *T. ambiguum* (cv. Treeline), *T. repens* and first backcrosses of 8*x* H-435 to *T. repens*.

Genotype/Cross	Somatic Chromosome No	Total PMCs scored	Meiotic configuration at metaphase I in pollen mother cells								Pollen Stainability (%)
			I		II		III		IV		
			Mean	Range	Mean	Range	Mean	Range	Mean	Range	
T. ambiguum (cv. Treeline)	2*n*=4*x*=32	18	0.39	(0-2)	15.39	(14-16)	0.06	(0-1)	0.17	(0-1)	96.8
T. repens (CC-1)	2*n*=4*x*=32	15	0.00	(0-0)	16.00	(16-16)	0.00	(0-0)	0.00	(0-0)	90.5
(8*x* H-435 x CC-1)-2	2*n*=6*x*=48	26	1.88	(0-3)	17.40	(14-21)	1.48	(0-3)	1.72	(0-3)	46.2
(8*x* H-435 x CC-1)-4	2*n*=6*x*=48	28	1.40	(0-3)	17.13	(12-21)	1.00	(0-2)	2.33	(0-4)	34.1
(8*x* H-435 x CC-1)-5	2*n*=6*x*=48	25	1.62	(0-4)	20.08	(16-22)	0.50	(0-2)	1.15	(0-2)	28.4
(CC-1 x 8*x* H-435)-2	2*n*=6*x*=48	27	3.13	(1-6)	17.70	(12-21)	1.46	(0-3)	1.29	(0-4)	22.5

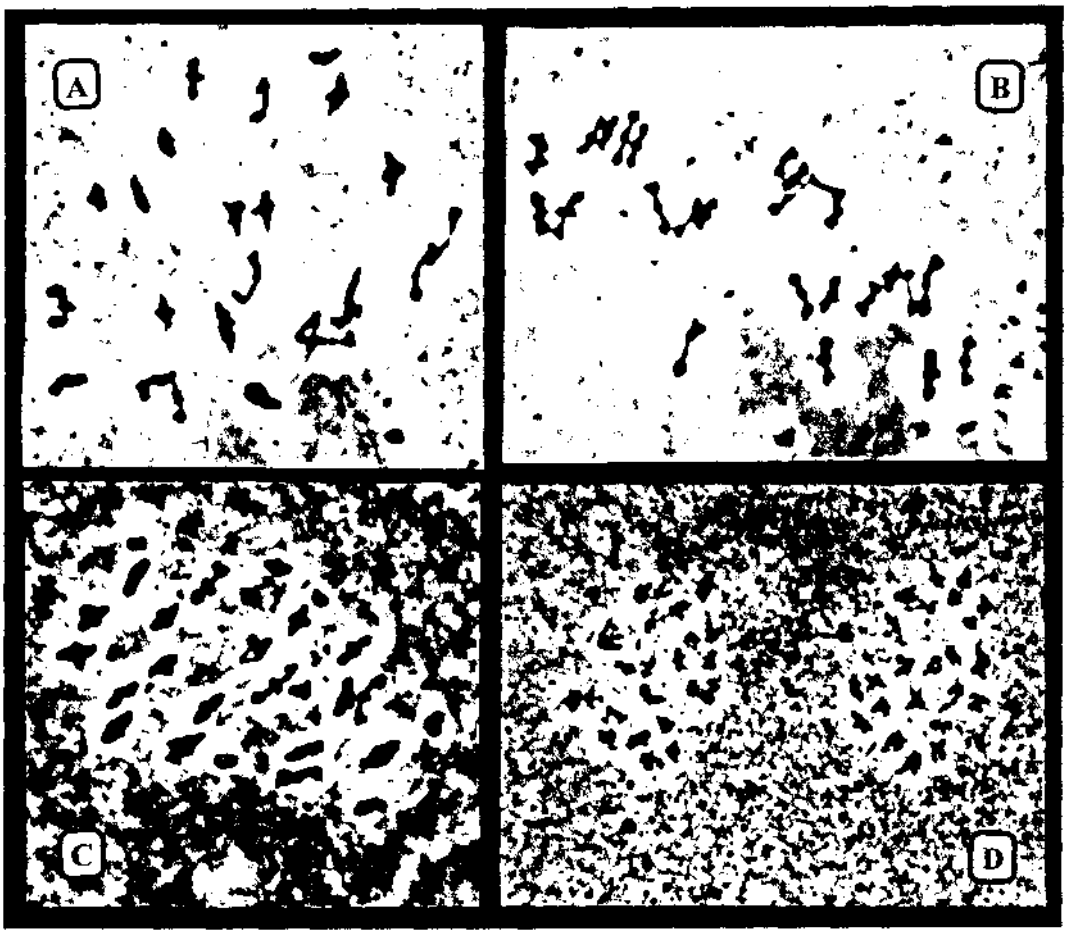


Figure 4.17 Meiotic configurations in 6*x* BC_1F_1 (8*x* H-435 x *T. repens*) progeny. **(A)** 1 I + 16 II + 1 III + 3 IV at metaphase I, **(B)** 20 II + 2 IV at metaphase I, **(C)** 4 I + 22 II at metaphase I, and **(D)** 6*x* BC_1F_1 showing 24 - 24 disjunction at anaphase I. x 1600.

Nine out of ten 6*x* BC_1F_1 plants had significantly fewer stolons than *T. repens* and only one significantly exceeded 8*x* H-435 in stolon number. Four of the ten 6*x* BC_1F_1's approached *T. repens* in stolon length whereas 4*x* and 8*x* H-435 had a significantly shorter stolon length than *T. repens.*

Stolon diameter of *T. repens* was significantly less than 8*x* H-435. A considerable range of stolon diameters was observed among the ten 6*x* BC_1F_1 genotypes and a number of genotypes had similar stolon diameters to 8*x* H-435.

The 4*x* and 8*x* H-435 plants showed significantly fewer root primordia on the nodes of the longest stolon than *T. repens* and four of the 6*x* BC_1F_1 plants did not differ significantly from white clover in the number of root primordia. A similar trend was observed for nodal rooting.

The highest shoot dry weight was recorded for *T. repens* followed by six genotypes of 6*x* BC_1F_1. All 6*x* BC_1F_1's showed significantly less shoot dry weight than *T. repens* but most of them were significantly higher than *T. ambiguum* and 4*x* and 8*x* H-435.

Considerable variations in leaflet length and width were observed among the ten 6*x* BC_1F_1 plants ranging from 16.3-37.5 mm and 12.2-24.3 mm respectively. The parental species *T. repens* (CC-1) and *T. ambiguum* and the 4*x* and 8*x* H-435 did not differ significantly in leaflet length or width. None of the 6*x* BC_1F_1's had significantly longer leaflets than *T. repens* and two had significantly shorter leaflets. One 6*x* BC_1F_1 showed significantly wider leaflets than *T. repens* and one of the pair with shorter leaflets also showed significantly narrower leaflets than *T. repens.*

The highest root dry weight was shown by one of the 6*x* BC_1F_1 plants followed by *T. repens* . However six out of ten 6*x* BC_1F_1's were significantly lower in root dry weight than *T. repens* . None of the 6*x* BC_1F_1's significantly exceeded the 8*x* H-435 in root dry weight, and only one had significantly higher root dry weight than *T. ambiguum.*

T. repens (CC-1) had significantly more nodules than *T. ambiguum* and 4*x* and 8*x* H-435. Six out of ten 6*x* BC_1F_1's did not differ significantly in nodule number from *T. repens*, and were significantly better than 8*x* H-435.

In general two of the ten 6*x* BC_1F_1 genotypes, (8*x* H-435 x CC-1)-5 and (8*x* H-435 x CC-1)-8 showed a significant improvement in stolon number, stolon length, shoot dry weight and nodulation over 8*x* H-435.

Although data for different morphological characteristics of the 6*x* BC_1F_2 (BC_1F_1 x BC_1F_1) plants have not been recorded, four of the 6 initially grown 6*x* BC_1F_2 plants exhibited a combined stoloniferous and rhizomatous growth with frequent nodal rooting and nodulation (Fig. 4.18).

Table 4.10 Morphoogical characterristics of *T. repens*, 4*x* *T. ambiguum*, 4*x* and 8*x* H-435 and 6*x* BC_1F_1 progeny.

Genotype	Leaf length (mm)	Leaf width (mm)	Stolon or stem No/plant	Longest stolon or stem					Tap root diameter (cm)	No of rhizomes/ plant	Rhizome length. (cm)	Nodula-† tion/plant	Root Dry weight (g)	Shoot Dry weight (g)	Total Dry weight (g)	Internode length (mm)
				Length (cm)	Diameter (cm)	No of nodes	No of root Primordia	No of nodes with roots								
T. repens (CC-1)	30.7	18.3	23.0	22.9	3.0	6.7	5.0	3.3	3.2	0.0	0.0	4.8	2.3	8.3	10.6	35.1
4*x* H-435	34.0	16.5	2.15	3.7	2.7	4.3	0.8	0.8	1.6	1.6	12.8	1.8	0.7	0.4	1.1	8.4
8*x* H-435	27.8	19.2	3.3	9.2	4.0	5.3	2.8	1.5	2.0	2.4	26.8	2.6	0.8	0.8	1.6	17.7
4*xT. ambiguum* cv. Treeline	25.0	14.5	4.8	3.5	2.1	3.7	0.0	0.0	2.0	2.7	36.8	1.2	0.4	0.4	0.8	9.6
(8*x* H-435 x CC-1)-1	16.3	12.2	1.6	2.0	1.9	4.3	1.0	1.0	0.7	0.5	2.2	1.5	0.1	0.1	0.2	4.7
(8*x* H-435 x CC-1)-2	33.2	22.7	4.3	18.7	4.2	5.5	2.8	1.7	2.4	0.3	2.0	4.3	0.5	1.7	2.2	33.4
(8*x* H-435 x CC-1)-3	23.7	17.7	3.6	3.9	2.7	4.8	1.7	1.2	1.3	2.1	3.2	2.0	0.1	0.3	0.4	8.3
(8*x* H-435 x CC-1)-4	18.0	16.3	6.0	3.3	2.7	4.0	1.2	1.0	1.8	0.0	1.0	3.5	0.2	0.6	0.8	8.1
(8*x* H-435 x CC-1)-5	34.7	24.3	6.7	22.5	3.9	5.8	3.2	2.2	2.6	0.8	3.8	3.8	1.2	3.5	4.7	40.3
(8*x* H-435 x CC-1)-6	25.5	23.3	3.1	2.8	3.0	4.3	0.2	0.0	1.7	5.4	8.2	3.0	0.4	0.7	1.0	7.2
(8*x* H-435 x CC-1)-8	33.3	24.2	10.2	19.4	3.7	5.7	3.3	2.2	2.5	0.3	2.4	4.0	2.5	3.9	6.5	36.1
(CC-1 x 8*x* H-435)-1	31.8	23.0	8.7	10.4	3.2	5.0	3.3	2.0	2.2	0.6	6.6	4.0	1.1	3.2	4.3	21.2
(CC-1 x 8*x* H435)-2	24.2	21.8	5.2	10.3	3.8	4.8	0.3	0.3	2.4	0.1	1.5	2.8	0.4	1.3	1.7	20.7
(CC-1 x 8*x* H-435)-3	37.5	24.0	8.5	19.1	3.8	6.0	3.5	2.7	2.9	0.8	3.9	3.7	1.4	2.3	3.9	31.7
P	***	***	***	***	***	**	***	***	***	**	**	***	***	***	***	***
$LSD_{(0.05)}$	9.49	5.83	---	6.78	0.84	1.76	1.97	1.74	1.14	---	---	1.55	---	---	---	14.1
$LSR_{(0.05)}$			x2.71	---	---	---	---	---	---	x2.97	x3.31	---	x3.94	x1.25	x1.27	

† On a scale of 0 - 5, where 0=no nodulation, 1=1-5 nodules, 2=6-10 nodules, 3=11-15 nodules, 4=16-20 nodules and 5=21 or more nodules per plant.

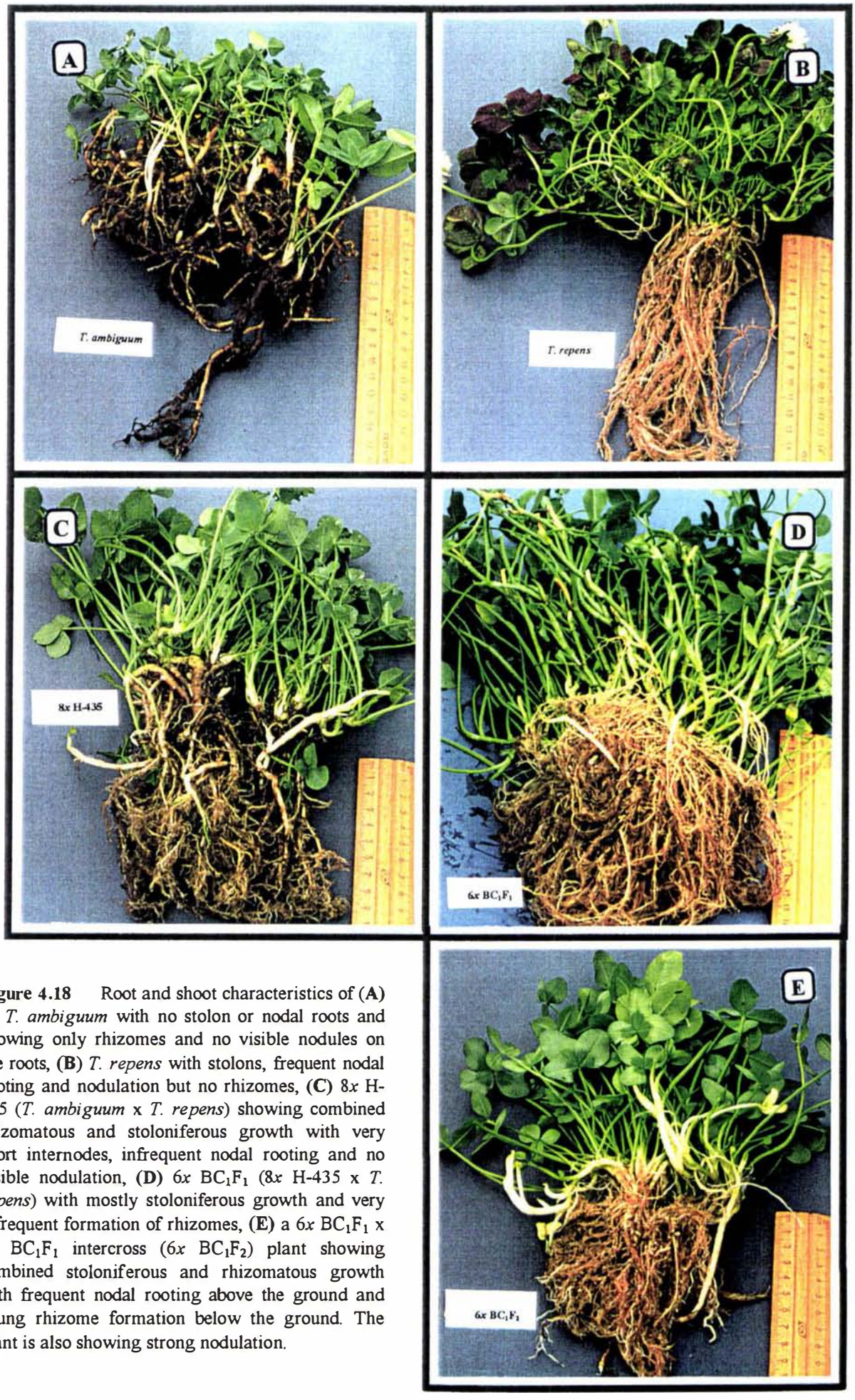


Figure 4.18 Root and shoot characteristics of **(A)** 4*x* *T. ambiguum* with no stolon or nodal roots and showing only rhizomes and no visible nodules on the roots, **(B)** *T. repens* with stolons, frequent nodal rooting and nodulation but no rhizomes, **(C)** 8*x* H-435 (*T. ambiguum* x *T. repens*) showing combined rhizomatous and stoloniferous growth with very short internodes, infrequent nodal rooting and no visible nodulation, **(D)** 6*x* BC_1F_1 (8*x* H-435 x *T. repens*) with mostly stoloniferous growth and very infrequent formation of rhizomes, **(E)** a 6*x* BC_1F_1 x 6*x* BC_1F_1 intercross (6*x* BC_1F_2) plant showing combined stoloniferous and rhizomatous growth with frequent nodal rooting above the ground and young rhizome formation below the ground. The plant is also showing strong nodulation.

CHAPTER 5
DISCUSSION

5.1 *IN VITRO* CHROMOSOME DOUBLING

5.1.1 Chromosome Doubling Frequency

The production of chromosome doubled plants in the present study indicates the effectiveness of *in vitro* colchicine application for chromosome doubling. However, the chromosome doubling rates of 7.1% and 11.1% of the surviving meristems for treatments C_3 and C_4 respectively were lower than the 44.0% obtained by Anderson *et al.* (1991b) for interspecific *Trifolium* hybrids.

The low frequency of chromosome doubled plants in the present experiment could have been due to the low colchicine concentration (0.05% as against 0.10% used by Anderson *et al.*, 1991b) used for treatments C_3 and C_4. However increasing the concentration and/or time of application of colchicine had a severe toxic effect on the growth of meristems in culture, as was evident from treatments C_1, C_2 and C_5. These results are in contrast to those obtained by Anderson *et al.* (1991b) who did not observe significant differences for two durations of 48 and 72 hours with 0.10% *in vitro* colchicine application to *Trifolium* interspecific hybrids. The present results suggest that *T. nigrescens* based hybrids may be more sensitive to colchicine, both with respect to the concentration and duration of application (Table 4.1), than the *T. ambiguum* hybrids used by Anderson *et al.* (1991b). This emphasises the need to determine specific culture conditions for each new set of material before *in vitro* meristem culture is carried out.

The high meristem mortality rates (25.3-47.1%) during the preculture period in all the six treatments were in large part due to the bleaching effect of the sodium hypochlorite used for surface sterilisation. However reducing the concentration of sodium hypochlorite from 5% to 4% resulted in almost 100% microbial contamination. Some of the high number of bacterial and fungal contaminations observed in the present investigation may have been internal in origin, and presumably could have been minimised by the addition of antibiotics such as sodium cefolaxime (Anderson *et al.*, 1991b).

Although the bleaching effect of sodium hypochlorite on most of the meristems in culture was quite obvious after 10-12 hours of preculturing, it was uncertain whether the meristem mortality rate of between 25.3 and 47.1% in all the six treatments during preculture was only due to the bleaching effect, or a combination of bleaching and sensitivity of the meristems to preculturing conditions. Anderson *et al.*, (1991b) also observed a significant reduction in meristem survival during preculturing. It is likely that the surface sterilisation technique and the preculturing periods could be further refined to an optimum level in order to increase the meristem survival rate, which should eventually result in a higher frequency of chromosome doubled plants. This requires further investigation.

Anderson *et al.* (1991b) reported that chromosome doubling frequency was substantially higher in meristems that were precultured, suggesting that preculture had improved targeting of colchicine to receptive meristematic cells. The frequency of chromosome doubled plants in the present investigation might have been improved by lengthening the duration of the preculturing period, as suggested by Anderson *et al.* (1991b). However it remains uncertain whether the increased sensitivity as a result of prolonged preculturing would increase mortality or increase chromosome doubling of the meristems. It is, however, certain that with a refined surface sterilisation technique, the meristem mortality could be reduced, which in turn would most likely result in a comparatively higher frequency of chromosome doubled plants.

5.1.2 Identification of Chromosome Doubled Plants

The chromosome doubled plants did not exhibit marked morphological differences from 3*x* H-6909-5, with the exception of leaflet and inflorescence size. Although data for leaflet and inflorescence size of 3*x* H-6909-5 and 6*x* H-6909-5 were not obtained, the leaflet and inflorescence sizes of 6*x* H-6909-5 appeared to be more than double the size of 3*x* H-6909-5 (Fig. 4.4). Alteration in leaflet size and shape as a result of polyploidy were also observed by Anderson *et al.* (1991b) for *T. ambiguum* x *T. repens* hybrids, and by DeRoo (1975) for red clover, where colchicine doubled plants in both cases had larger and more rounded leaflets than the undoubled plants.

Dry pollen shape was found to be a rapid method for identifying the chromosome doubled plants. Taylor *et al.* (1976) in red clover and Anderson *et al.* (1991b) in *T. ambiguum* x *T. repens* hybrids also observed altered pollen shape and size as a result of chromosome doubling. The present results are consistent with these reports, the dry pollen shape of the three chromosome doubled plants being tetrahedral as compared to cylindrical or oval for 3*x* H-6909-5.

When certain intergeneric or interspecific crosses are made, the first generation hybrid is usually highly sterile because the genomes are so different that each kind of chromosome lacks a homologue to act as its pairing partner at meiosis. Chromosome doubling of such sterile hybrids is often attempted to restore fertility, since each chromosome would now have a pairing partner, resulting in meiotically stable products in the absence of other fertility regulating mechanisms such as genomic incompatibility or genic influences (Anderson *et al.*, 1991b).

The classical example of the effect of chromosome doubling on fertility was obtained by Karpechenko in 1928 (Srb *et al.*, 1965). Karpechenko developed an intergeneric hybrid between *Brassica oleraceae* and *Raphanus sativus*. Both the parental species have a diploid chromosome number of $2n=18$. F_1 plants from this cross also had 18 chromosomes, 9 from each parent, but were highly sterile because of a failure in pairing at meiosis. They did produce a few seeds, however, and some of the plants arising from these seeds were fertile. Cytological examination of these F_2 plants revealed a somatic chromosome number of $2n=36$ and 18 bivalents were formed at meiosis. It is apparent that the fertile F_2 plants arose from fusion of unreduced gametes from F_1 individuals, and that these F_2 plants contained the full diploid complements of the parental species.

Of the several agents which have been tried for the production of polyploids in several plant species, the alkaloid colchicine found in the corms and seeds of *Colchicum autumnale* is known to be a highly potent polyploidising agent (Brewbaker, 1952, Frankze and Ross, 1952; Williams, 1964). Certain aspects of the action of colchicine on dividing nuclei, especially in plants, are well understood. At critical concentrations, colchicine inhibits the formation of spindle fibres and the normal process of mitosis is

modified to a sequence of events called C-mitosis (Srb *et al.*, 1965). In the absence of functioning spindle fibres, chromosomes fail to move into an equatorial plate but remain scattered in the cytoplasm (a stage called C-metaphase). However the chromosomes eventually separate at the centromere, and a C-anaphase is initiated. Following this, a membrane finally develops around the nucleus that has the doubled chromosome number, which during the next division in the absence of the alkaloid, undergoes normal mitosis and gives rise to polyploid nuclei identical to their immediate progenitor. Ever since the effect of colchicine was reported by Blakeslee and Avery (1937), Eigsti (1938) and Nebel and Ruttle (1938), many plants species have been subjected to treatment and the colchicine effect has been shown to be general in all higher plants.

The chromosome doubled plants of H-6909-5 in the present study showed marked improvement in fertility, as pollen stainability averaged 89.2% for the three chromosome doubled plants and only 9.9% for 3*x* H-6909-5. These results are consistent with those reported by Brewbaker and Keim (1953) who recorded 90% pollen stainability for the hexaploid *T. nigrescens* x *T. repens* hybrid WN2 obtained after doubling the chromosomes of the parental species before hybridisation, and Hovin (1963) who reported greater than 90% pollen stainability for a colchicine doubled hexaploid *T. repens* x *T. nigrescens* hybrid as against less than 20% for the triploid hybrids. Other examples of enhanced fertility from chromosome doubling of *Trifolium* interspecific hybrids include *T. ambiguum* x *T. repens* hybrid 61 (Williams, 1978) where pollen stainability was increased from less than 3% to more than 50% after treatment of a single inflorescence with colchicine (Williams *et al.*, 1982), and H-435 where pollen stainability was increased to 33.6% in chromosome doubled 8*x* H-435 as against 2.5% for 4*x* H-435 (Anderson *et al.*, 1991c).

Chromosome doubling did not change the self-incompatibility of H-6909-5. A hexaploid hybrid (WN2) between self compatible *T. repens* ($2n=8x=64$) and self-incompatible *T. nigrescens* ($2n=4x=32$) was self compatible (Brewbaker and Keim, 1953). Self-compatibility of WN2 was proposed to arise from competition interaction in heterogenic pollen grains, suggesting homology of the *S*-loci in the two species. Hovin (1963) obtained four autoallohexaploid hybrids by chromosome doubling of triploid ($3x=24$) hybrids between self-compatible *T. repens* ($2n=32$) and a self-incompatible *T.*

nigrescens ($2n$=16). The *T. repens* parent was heterozygous for the S_f allele. One hexaploid (RN1) was self-compatible, and segregated in I_1 and I_2 into self-compatible and self-incompatible classes. Apparently the S_f allele behaved normally in the hybrid. The remaining three were self-incompatible, although one was pseudoself-compatible and produced on average 1 seed per 10 florets. Cross fertility studies suggested that independent action of *S* alleles and (possibly) dominance occurred, but there was no interaction of *S* alleles as proposed by Brewbaker and Keim (1953).

Doubling the chromosome number of self-incompatible plants could result in some self-fertilisation through competitive interaction of gametophytically determined incompatibility alleles in the pollen (Atwood and Brewbaker, 1953, quoted by Williams, E. G. 1987). However chromosome doubling did not change the self-incompatibility of H-6909-5 in the present study.

Consistent results were obtained when pollen stainability and shape were checked in colchicine derived plants. The three plants with altered pollen also had doubled chromosomes, while the three plants with unaltered pollen that were checked were unchanged in chromosome number. These results suggest that alteration in dry pollen shape and increased pollen stainability are two rapid methods for assessing chromosome doubling after colchicine treatment. Such methods should, however, be supported by chromosome counts because aneuploidy is a common phenomenon after chemical treatment for chromosome doubling (Taylor *et al.*, 1976; Anderson *et al.*, 1991b). However, no aneuploidy was found in the present study, and so it is uncertain whether aneuploids would show altered pollen shape and stainability in this material.

5.2 BACKCROSSES OF H-6909-5 TO *T. repens* AND *T. nigrescens*.

The triploid *T. repens* x *T. nigrescens* F_1 hybrid (3*x* H-6909-5) was male and female sterile in backcrosses to both of its parental species, as no seeds were obtained from a large number of crosses. These results are in agreement with the previous results of Trimble and Hovin (1960) but contrary to those reported by Hovin (1962a) where the partially sterile triploid F_1 hybrid between *T. repens* x *T. nigrescens* produced 6

seeds from 500 florets pollinated with *T. repens*. Differences in cross compatibility due to the genotypes of individual plants or strains that are crossed were reported by Evans (1962b) and Hovin (1962a). Marshall *et al.* (1995) also observed considerable variations in cross compatibility of different *T. repens* and *T. nigrescens* accessions in their interspecific crosses and reported that two accessions of these species consistently produced higher seed set than the others. It is therefore possible that the failure to generate backcross progeny between 3*x* H-6909-5 and *T. repens* in the present study and the success from a similar backcross reported by Hovin (1962a) might be due to different genotypes involved in the backcrosses.

The objectives of *in vitro* chromosome doubling of 3*x* H-6909-5 in the present investigation were to study the effectiveness of the technique, and to evaluate the effect of chromosome doubling on backcross seed set. The three hexaploid colchicine derived plants (CT-1, CT-14 and CT-28) had almost similar pollen stainability, but only CT-14 was cross fertile in backcrosses to *T. repens* (CC-1) and *T. nigrescens* (Tn-167). The other two plants (CT-1 and CT-28) failed to produce any backcross progeny with either of the parental species from similar numbers of crosses. It has been reported that gamete sterility in hybrids may be chromosomal and/or genetic (Williams, E. G. 1987). Chromosomal sterility is caused by gross meiotic abnormalities resulting from failure of distantly related genomes to pair. Genetic sterility, on the other hand, may occur as a result of physiological disturbances or cryptic genetic changes during reproductive development. Colchicine has been reported as a mutagenic agent, causing various types of true and non true-breeding mutations in sorghum (*Sorghum vulgare* Pers.) and barley (*Hordeum vulgare* L.) (Franzke and Ross, 1952; Ross *et al.*, 1954; Foster *et al.*, 1961; Sanders *et al.*, 1962; Gilbert and Patterson, 1965). Gilbert and Patterson (1965) found six out of 26 mutant barley plants showing obvious partial sterility after colchicine treatments. Based on these observations, it is possible that one of the three colchicine derived plants (CT-14) in the present experiment might be different in fertility from the other two (CT-1 and CT-28) plants and that these differences in fertility might have been caused by colchicine. However, some crosses involving CT-1 and CT-28 (Table 4.2) produced fully developed seeds. Thus failure of first backcrosses involving CT-1 and CT-28 may not have been due to differences in fertility but could have occurred by chance.

Brewbaker and Keim (1953) showed that a hexaploid F_1 hybrid (WN2) from a *T. nigrescens* x *T. repens* cross obtained after doubling the chromosome numbers of both parents was cross fertile as the male with doubled *T. repens* (i.e. 8*x*) and doubled *T. nigrescens* (i.e. 4*x*), but was cross sterile as the male to undoubled plants of the parental species. The hybrid was also cross fertile as the female to doubled *T. repens*. However they did not report female fertility to the undoubled parental species. In contrast to the results of Brewbaker and Keim (1953) the CT-14 plant of 6*x* H-6909-5 was both male and female fertile in crosses with 4*x* *T. repens*. Differences in the results of the present experiment and those of Brewbaker and Keim (1953) may once again be related to the use of different genotypes in these backcrosses.

In the present investigation no seed was produced by crossing 6*x* H-6909-5 as the male with 2*n* *T. nigrescens*, while only one seed was harvested from 6*x* H-6909-5 (CT-14) as the female parent pollinated with *T. nigrescens* (Tn-167) pollen. Brewbaker and Keim (1953) also obtained no seed from this cross. The success and failure of different crosses in this experiment has been described in detail in section 5.4.3.

It has previously been reported that functional fertility of F_1 hybrids in backcrosses may differ depending on which of the parent species is involved. For example in backcrosses of reciprocal F_1 hybrids between *Phaseolus vulgaris* L. and *P. acutifolius* A. Gray. as female parents, Rabakoarihanta *et al.* (1980) observed a higher frequency of dividing embryos (14%) after pollination by *P. acutifolius* than after pollination by *P. vulgaris* (4%). Asano (1980) reported that backcrossing of hybrid lilies as female parents was more successful if the original male parent of the hybrid was used again as the male parent in backcrosses. This suggested that the relative dosages of genetic material from the two species may be important in the regulation of normal embryo development or that there are persistent effects of transmission through male or female gametes.

5.2.1 Unreduced (2*n*) Gametes in *Trifolium repens*

Results of the backcross of 6*x* H-6909-5 to *T. repens* (CC-1) were successful. Here the 6*x* H-6909-5 (CT-14) was found to be both male and female fertile. The backcross involving the 6*x* H-6909-5 (CT-14) as the female and *T. repens* as the male

resulted in one BC_1F_1 which was, as expected, a pentaploid ($2n$=5x=40). However using 6x H-6909-5 (CT-14) as the male and *T. repens* (CC-1) as the female resulted in three BC_1F_1 seeds. The three plants grown from this backcross were unexpectedly found to be heptaploids ($2n$=7x=56). The occurrence of heptaploids from a 4x-6x cross can only be explained by the union of n (=3x=24) pollen from 6x H-6909-5 (CT-14) with $2n$ (=4x=32) eggs from white clover (CC-1).

Although the production of $2n$ gametes has been extensively studied in many crop species such as *Medicago sativa, Pisum sativum,* and *Solanum tuberosum* (Veilux, 1985), there are only a few reports describing $2n$ gametes in *Trifolium* species. Mazonnier (1972) reported for the first time $2n$ functional gametes in *T. alpestre.* Later, *T. pratense* was identified by Taylor and Giri (1983), Parrott and Smith (1984) and Taylor and Wiseman (1987) as producing $2n$ gametes. *T. nigrescens* was found to be another species in the genus with the potential for producing $2n$ gametes (Bullita and Smith, 1992; Bullita *et al.*, 1994). However, the latter study related the presence of $2n$ gametes to differences in pollen size which does not necessarily support the occurrence of functional $2n$ pollen in *T. nigrescens.*

To date there has been no published report on the production of functional $2n$ gametes in white clover, although a hybrid between *T. ambiguum* x *T. repens* (4x H-435, Williams and Verry, 1981) was found to produce $2n$ eggs in backcrosses with *T. repens* (Anderson *et al.*, 1991c). The occurrence of heptaploid BC_1F_1 from a 4x *T. repens* x 6x H-6909-5 cross in the present investigation is the first evidence of functional $2n$ gametes (in megaspores) in *T. repens.* It is evident that germplasm can be transferred from the tetraploid level to at least the hexaploid level in white clover using $2n$ eggs, or even to octoploid level, presumably through bilateral sexual polyploidisation (Parrott *et al.*, 1985), if the species also forms $2n$ pollen. Since $2n$ gametes can transmit parental heterozygosity to the polyploid progeny (Smith *et al.*, 1985), the resulting progeny may be more heterozygous and presumably more vigorous and highly fertile in comparison to plants obtained through chemical chromosome doubling as reported for red clover (Parrott and Smith, 1986b).

The other potentially important use of 2*n* gametes in white clover relevant to the present study would be to develop hexaploids through unreduced egg formation, and use them in backcrosses with hexaploid F_1 hybrids between *T. repens* and *T. nigrescens* and hexaploid BC_1F_1 or BC_1F_2 progeny between *T. repens* and *T. ambiguum*. This should result in hexaploid backcross progeny in contrast to an odd ploidy level of 5*x* obtained by using 4*x* *T. repens*. However, at this stage because of the very low frequency of 2*n* gametes (only in megaspores) in white clover, the chemical doubling of chromosomes for polyploidisation seems more efficient than the unreduced gamete method, because of the higher frequency of polyploid production.

5.2.2 Characterisation of 3*x* and 6*x* H-6909-5 and Backcross Progeny

The H-6909-5 at both triploid and hexaploid levels did not show the true stoloniferous growth habit of *T. repens*, or the erect to sub-erect and branching growth habit of *T. nigrescens*. The presence of nodal root primordia, nodal rooting at the basal 2-3 nodes and perennial growth habit on one hand, and the smaller leaflet and inflorescence size (for 3*x* H-6909-5) and profuse flowering on the other hand showed its affinity towards both parental species. These observations are in contrast to those reported by Brewbaker and Keim (1953) where the hexaploid hybrids WN2 and WN3, obtained after crossing chromosome doubled plants of *T. repens* and *T. nigrescens*, showed greater affinity towards the *T. nigrescens* parent. This difference in the morphological features between hexaploid WN2, WN3 and 6*x* H-6909-5 might be due to the different genotypes of the parental species used in crosses.

From the observations recorded for F_1 (both 3*x* and 6*x*) and BC_1F_1 progeny it was evident that morphological features of the parental species in F_1's and BC_1F_1's were expressed according to the parental genomic ratios. The 3*x* and 6*x* F_1 hybrids having *T. repens* and *T. nigrescens* genomes in the ratio of 2:1 had an intermediate expression of parental morphology. The tetraploid BC_1F_1 (CT-14 x Tn-167) with parental genomic ratio of 1:1 i.e. two genomes of *T. repens* and two of *T. nigrescens*, had more affinity towards *T. nigrescens* as it showed an annual growth habit, no root primordia at the nodes, no nodal rooting, small leaflet and inflorescence size and profuse flowering. However, in comparison to *T. nigrescens* the hybrid was propagated easily from stem cuttings.

In contrast to the tetraploid BC_1F_1, the pentaploid BC_1F_1 (CT-14 x CC-1) with a parental genomic ratio of 4:1 (four genomes of *T. repens* and one genome of *T. nigrescens*), exhibited the true stoloniferous perennial growth habit of *T. repens* with root primordia at each node and frequent nodal rooting. This BC_1F_1 has shown no obvious *T. nigrescens* features and close similarity to *T. repens*. The same morphological features were observed for the three heptaploid BC_1F_1's which theoretically combine 6 genomes of *T. repens* and only one genome of *T. nigrescens*.

These observations suggest that the recovery of strong perennial stoloniferous backcrosses with frequent nodal rooting depends on the genomic ratio of the two species. This was evident in the CBC_2 plant obtained after crossing the tetraploid BC_1F_1 (CT-14 x Tn-167) with *T. repens*. This plant is expected to carry 3 genomes of *T. repens* and one genome of *T. nigrescens*, and was found to be more like *T. repens*.

The first backcross progenies provided the material for generating second backcross progenies which were successfully obtained by crossing the pentaploid and one of the heptaploid BC_1F_1's to *T. repens*. The tetraploid BC_1F_1 (CT-14 x Tn-167) was not crossed with *T. nigrescens* for the second backcross as it already showed strong affinity towards *T. nigrescens*, but was used as the reciprocal parent in congruity backcrossing, a backcross scheme reported by Haghighi and Ascher (1988). All the congruity backcrosses (CBC_2) and second backcross (BC_2F_1) will be characterised both cytologically and morphologically at a later stage, although two BC_2F_1 have been studied for somatic chromosome counts (section 4.4.2).

5.3 MEIOTIC CONFIGURATIONS IN 3*x* AND 6*x* H-6909-5 AND FIRST BACKCROSS (BC_1F_1) PROGENY

The regular bivalent pairing in *T. repens* with a somatic chromosome number of $2n=4x=32$ observed by Atwood and Hill (1940) and the disomic inheritance of genetic markers (Davies, 1970) suggested a diploid behaviour of the species. The strict bivalent pairing in *T. repens* led Atwood and Hill (1940) to the conclusion of an amphidiploid origin of *T. repens*. However, the two homoeologous genomes with a basic set of $x=8$ chromosomes were subsequently found to have the potential to pair with each other after interspecific hybridisation with *T. nigrescens*, *T. occidentale*, (Chen and Gibson, 1970 a, b) and *T. ambiguum* (Williams *et al.*, 1982; Anderson *et al.*, 1992b).

Chen and Gibson (1970 a, b) found up to 8 trivalent associations in triploid F_1 hybrids of *T. repens* with 2*x* *T. nigrescens* and 2*x* *T. occidentale*. Pairing of the two basic homoeologous genomes of *T. repens* was interpreted by Chen and Gibson to support an autotetraploid origin of the species, followed by the development of a genetic system allowing only bivalent pairing which could be altered after interspecific hybridisation. However, the amphidiploid origin of *T. repens* cannot be eliminated. According to Williams *et al.* (1982) the genetic suppression of homoeologous pairing may have evolved in an allotetraploid carrying closely related genomes, as occurred in wheat (Sears, 1976) and tall fescue (Jauhar, 1975). Thus the multivalent formation in triploid hybrids of *T. repens* with *T. nigrescens* and *T. occidentale* does not necessarily support the auto or allotetraploid origin of *T. repens*, but does provide evidence for a genetic control mechanism to prevent pairing of homoeologous genomes in *T. repens*.

The cytological observations recorded for the 3*x* and 6*x* F_1 hybrid (H-6909-5) between *T. repens* and *T. nigrescens* and the first backcross (BC_1F_1) progeny provided further supporting evidence for (1) genetic control of strict bivalent pairing in *T. repens* which breaks down after interspecific hybridisation and (2) pairing between the chromosomes of *T. repens* and *T. nigrescens*.

Results obtained for meiotic configurations in triploid H-6909-5 in the present investigation are in contrast to those reported by Hovin (1962a) but consistent with the results of Chen and Gibson (1970b). Hovin (1962a) reported predominantly bivalent formation i.e. an average of 9.6 and a range of 8-11 bivalents in 14 PMCs of the triploid *T. repens* x *T. nigrescens* hybrid. The presence of more than 8 bivalents in the triploid F_1 hybrid between *T. repens* and *T. nigrescens* would suggest that, apart from autosyndetic pairing (between the homoeologous chromosomes of *T. repens*) or allosyndetic pairing (between the *T. repens* and *T. nigrescens* chromosomes), pairing of non-homologous chromosomes within the genomes of both species had occurred. However, as evident from the present investigation (Table 4.3) and the data of Chen and Gibson (1970b) very strict bivalent pairing has been observed for the parental species. In the present investigation up to 8 (with an average of 6.36) bivalents were recorded for the triploid H-6909-5 indicating both auto- and allosyndetic pairing of the parental genomes, but no non-homologous pairing.

The three chromosome doubled hexaploid plants of H-6909-5 (CT-1, CT-14 and CT-28) showed very similar meiotic configurations with slightly more bivalents in plant CT-14 (Table 4.3). The slightly greater pollen stainability of plant CT-14 (90.9%) than plant CT-1 (88.8%) and CT-28 (87.9%) might be due to comparatively more bivalents. This was the only plant which produced first backcross (BC_1F_1) progeny with both parental species. Comparatively greater numbers of bivalents (more than 18 on average) in the three hexaploid plants of H-6909-5 suggested that homologous chromosomes of each species even in hybrids had more pairing affinity and so auto- or allosyndetic pairings were reduced. This has been shown by the less than one univalent and trivalent in 6*x* H-6909-5, although quadrivalents in 6*x* H-6909-5 were as frequent as trivalents in 3*x* H-6909-5 and demonstrate the occurrence of some auto- and allosyndesis.

The meiotic configurations in the tetraploid BC_1F_1 (CBC_1) also suggested both auto and allosyndetic pairing. This BC_1F_1 presumably carries two homoeologous genomes of *T. repens* and two homologous genomes of *T. nigrescens*. Assuming again that non-homologous chromosomes within or between the parental genomes do not pair, the formation of up to 5 quadrivalents and 2 trivalents in the PMCs of this 4*x* BC_1F_1 is a strong indication of allosyndetic pairing between *T. repens* and *T. nigrescens* chromosomes.

The occurrence of an average of 3.66 univalents in the pentaploid BC_1F_1 with four genomes of *T. repens* and one genome of *T. nigrescens* is consistent with the results obtained for the 3*x* H-6909-5. Increase in the number of univalents and trivalents in the pentaploid BC_1F_1 suggests that presumed homologous chromosome pairing between *T. nigrescens* genomes in 6*x* H-6909-5 might have been replaced by allosyndetic pairing in the pentaploid BC_1F_1.

Although studied in only 15 PMCs, the meiotic configurations of one of the 7*x* BC_1F_1 plants also showed both auto- and allosyndesis. The backcross carried only one genome of *T. nigrescens* with six genomes of *T. repens*. The occurrence of up to 11 (with an average of 6.00) univalents and 9 (with an average of 5.08) quadrivalents demonstrates the probable occurrence of allosyndetic pairing. The higher pollen stainability of 5*x* and 7*x* BC_1F_1's than the 4*x* BC_1F_1 suggested that gross meiotic abnormalities and odd ploidy levels did not greatly reduce the fertility of the plants.

5.4 ENDOSPERM BALANCE NUMBER IN BACKCROSSES

5.4.1 Endosperm Failure after Interspecific Hybridisation

Failure of endosperm to develop after interspecific hybridisation has been reported to be the major cause of embryo abortion. The sequence of events leading to the death of a viable embryo after interspecific pollination has been reviewed in section 2.1.2; Chapter 2.

The distinguishing feature of the fertilisation process in angiosperms is the involvement of two male nuclei. One of the male nuclei released from the pollen tube fuses with the egg nucleus to restore diploidy in the zygote. The other fuses with the central cell (containing two polar nuclei) to give rise to a triploid nucleus, and subsequently the endosperm (Bewley and Black, 1978). Each of the polar nuclei in the central cell contains a copy of the maternal chromosomes also found in the egg. Therefore as the male nuclei unite with both the egg and polar nuclei in the central cell, the nuclear constitution of the endosperm differs from that of the embryo by possessing an extra set of maternal chromosomes. The endosperm is a unique tissue in a sense that it is a product of fertilisation that does not produce germ cells, but is necessary for the normal development of the embryo in almost all species (Brink and Cooper, 1947).

In many interspecific crosses, particularly those involving a ploidy difference, the endosperm fails to develop normally and consequently leads to the abortion of an embryo (section 2.1.2). Brink and Cooper (1947) reported many intra- and interspecific crosses made at different ploidy levels ($2x$-$4x$) in which seeds aborted early because of the collapse of the endosperm.

Endosperm failure is not entirely due to species differences or differences in ploidy levels. If it were due only to species differences, crosses between diploids and their autotetraploid forms should always be successful. The fact is that crosses between a diploid and its colchicine derived autotetraploid form generally fails because the endosperm aborts (Johnston and Hanneman, 1980). If differences in the ploidy levels were the only cause, this should be overcome by $2n$ gametes or induced chromosome doubling. Also in interspecific interploidy crosses the problem is not necessarily due to

incompatibility between the genomes of the species, since it is often overcome by the functioning of 2*n* gametes or the induced chromosome doubling of one of the species (Hanneman and Peloquin, 1968; Johnston and Hanneman, 1980). It is, however, certain that deterioration of endosperm eventually causes the embryo to degenerate. For example in *Solanum* species the pattern of seed failure was similar in many crosses (mainly due to the collapse of endosperm) but the rate varied with intra- and interspecific crosses (Beamish, 1954).

Several hypotheses and theories have been proposed to explain seed abortion after interspecific and interploidy crosses. All theories involve three tissues i.e. maternal, endosperm and embryonic, as these are in close association within the developing seed and carry different genetic and ploidy constitutions.

5.4.2 Theories

Muntzing (1930) proposed that the genomes in the maternal:endosperm: embryo must be in the ratio of 2:3:2 for normal seed development. Watkins (1932) proposed a 0:3:2 of maternal:endosperm:embryo ratio of genomes, which suggested that maternal tissue was not important. Valentine (1954) suggested that it was a 2:3:0 ratio of maternal:endosperm:embryo genomes which was important. In contrast to Watkins (1930), Valentine's theory emphasised the importance of maternal tissue but entirely disregarded the embryo. Later Nishiyama and Inomata (1966) proposed that the success of endosperm development depended on a 2:1 ratio of the maternal:paternal genomes within the endosperm, regardless of the ploidy level of maternal or embryonic tissues.

Johnston *et al.* (1980) concluded that neither the ploidy of the maternal parent nor the embryo influences the development of the endosperm. Not only must the endosperm have a ploidy level that is a multiple of 3*x* for normal development, but it must also have a two maternal:one paternal genome ratio. However, as reported by Johnston *et al.* (1980), endosperm development does not depend exclusively on any of the hypotheses listed above. Johnston *et al.* (1980) extended the 2:1 hypothesis of Nishiyama and Inomata (1966), and proposed the Endosperm Balance Number hypothesis as explained below.

5.4.3 Endosperm Balance Number Hypothesis

The failure and success of different backcrosses, BC_1F_1 x BC_1F_1 intercrosses, BC_1F_1 x 6*x* F_1 crosses and congruity backcrosses (Table 4.2) in the present investigation can be assessed in relation to the "endosperm balance number" hypothesis of Johnston *et al.* (1980). According to this hypothesis, plants are allocated an endosperm balance number (EBN) based on their crossing behaviour with other plants. Normal endosperm development requires a ratio of two maternally derived EBN to one paternally derived EBN i.e. successful interspecific hybridisation can be achieved between species producing gametes with the same EBN. The following example was given by Johnston *et al.* (1980) to explain the 2:1 EBN hypothesis. *Solanum chacoense* (2*x*) is assigned an arbitrary EBN of 2. When crossed with *S. acaule* (4*x*) viable seeds are produced and result in 3*x* offspring. Therefore *S. acaule* (4*x*) is also assigned an EBN of 2. When *S. chacoense* is treated with colchicine, a 4*x* (4EBN) plant is produced, which crosses with *S. tuberosum* (4*x*) favourably but does not cross with *S. acaule* (4*x*) or *S. chacoense* (2*x*). Therefore *S. tuberosum* (4*x*) is assigned an EBN of 4. When *S. acaule* (4*x*) is crossed with 2*x* (2EBN) *S. tuberosum* haploids, viable seeds are produced, but no seeds are obtained from crosses with 4*x* (4EBN) *S. tuberosum.* However, colchicine doubled *S. acaule* 8*x* (4EBN) results in viable seeds when crossed with 4*x* (4EBN) *S. tuberosum.*

From the work of Johnston and Hanneman (1980), it was clear that no particular female:male genome ratio can be associated with all successful crosses. Their results, however, indicated that the 2:1 EBN ratio generally resulted in viable endosperm in interspecific crosses. EBN is not directly related to the ploidy level. Two species of the same ploidy level can have a different EBN, or two species with a different ploidy level can share the same EBN.

Parrott and Smith (1986a) were the first to support the 2:1 maternal:paternal EBN hypothesis in *Trifolium* interspecific hybrids. They assigned EBNs to some *Trifolium* species on the basis of their crossability with other species, and found that successful interspecific hybridisation has been reported among those *Trifolium* species that match EBNs rather than ploidy levels. According to this scheme both *T. repens*

(2*n*=4*x*=32) and *T. nigrescens* (2*n*=2*x*=16) share the same EBN (i.e. EBN=4). Parrott and Smith (1986a) however, reported evidence in support of the EBN hypothesis in only F_1 interspecific *Trifolium* hybrids. No report has yet been published in support of the 2:1 maternal: paternal EBN ratio in backcrosses of *Trifolium* species.

Results obtained from the first backcrosses (BC_1F_1) of 6*x* H-6909-5 to *T. repens* and *T. nigrescens* were initially used to test conformity with the EBN hypothesis in the present investigation (Fig. 5.1). Based on the report of Parrott and Smith (1986a) an EBN=1 and EBN=2 were assigned, respectively, to each genome of *T. repens* and *T. nigrescens* i.e. an EBN=4 to both species. The chromosome doubled plants of 6*x* H-6909-5 (with 4 genomes of *T. repens* and 2 genomes of *T. nigrescens*) therefore have an EBN=8.

Backcrosses of 6*x* H-6909-5 (EBN=8) to *T. nigrescens* (EBN=4)were of very low frequency, as only one seed was obtained from 2,000 reciprocal backcrosses. Similarly, only one seed was obtained from 1,200 backcrosses of 6*x* H-6909-5 (EBN=8) used as the female parent to *T. repens* (EBN=4). The very low frequency of success in both of these backcrosses might be attributed to differences in EBN of the parents.

Interestingly, when *T. repens* (CC-1) was used as the female parent in backcrosses to 6*x* H-6909-5, three fully developed seeds were obtained from 700 crosses. The three plants grown from these seeds were all heptaploids (7*x*) and were presumably obtained through unreduced gametes (2*n* egg) contributed by the *T. repens* female parent. The production of only heptaploid and the absence of expected pentaploid progeny from this backcross provides support for the gametic EBN hypothesis in backcrosses of 6*x* H-6909-5 to *T. repens* (in this case both female and male gametes have an EBN of 4). Occasional seeds have also been produced in certain inter-EBN crosses in studies of *Solanum* (den Nijs and Peloquin, 1977; Johnston and Hanneman, 1980) and *Trifolium* (Parrott and Smith, 1986a) species. These inter-EBN interspecific hybrids were presumably obtained due to the functioning of 2*n* gametes. For example 2*x* (2EBN) *Solanum chacoense* x 4*x* (4EBN) *Solanum tuberosum* yielded a few plump seeds that gave rise to 4*x* (4EBN) F_1 plants (Johnston *et al.*, 1980).

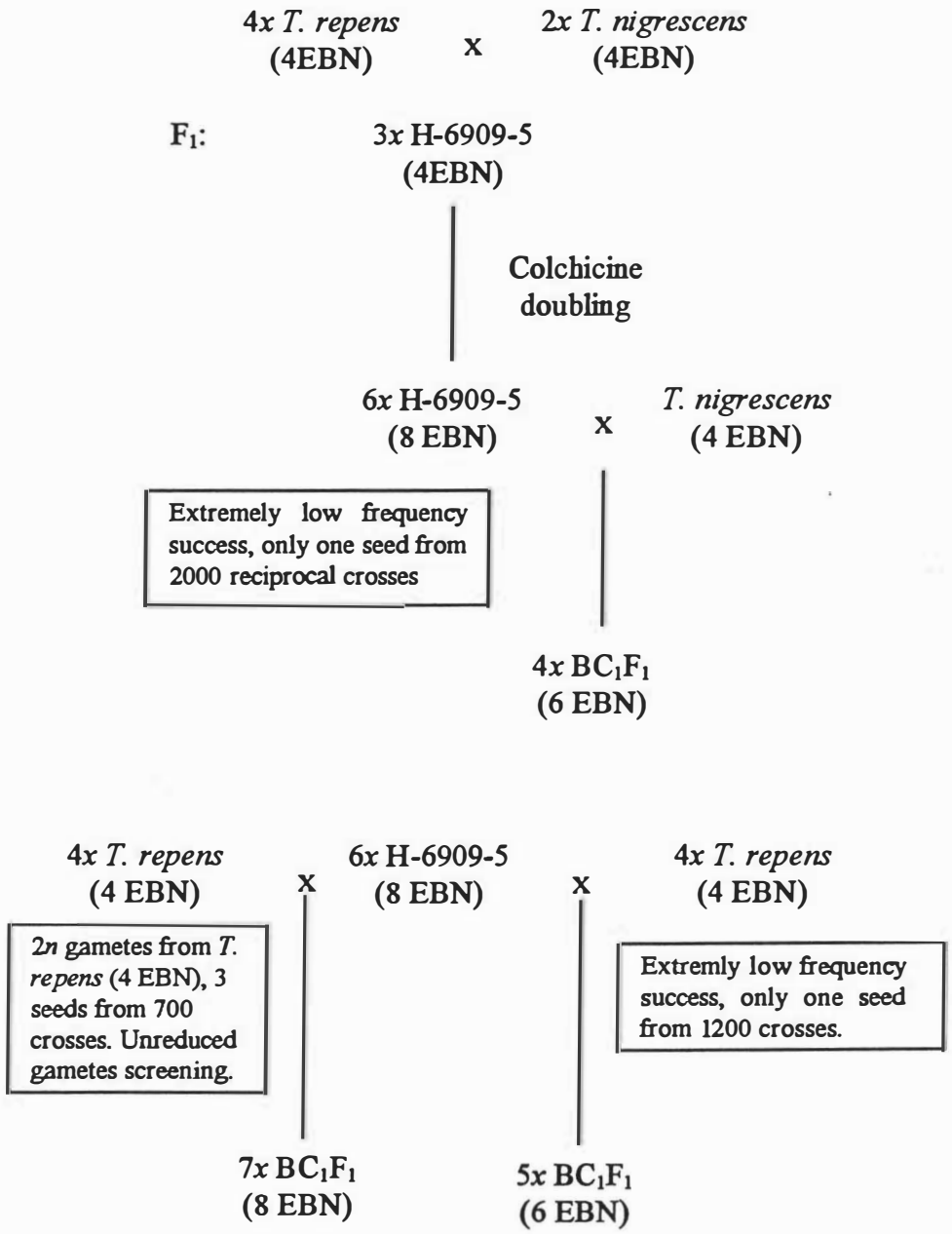


Figure 5.1 Example of using endosperm balance number to predict the success of the first backcross (BC_1F_1) between 6x H-6909-5 and parental species.

Apparently a 2*n* egg in *S. chacoense* was fertilised. Similarly, a 6*x* (8EBN) *Trifolium sarosiense* x 2*x* (2EBN) *T. alpestre* cross (Maizonnier, 1972) resulted in a 5*x* (8EBN) F_1 hybrid (Parrott and Smith, 1986a) through the production of a 2*n* pollen grain.

It is clear that the use of a 2*n* gamete from a 2*x* plant is equivalent (in an EBN sense) to the use of an *n* gamete from a 4*x* plant, as both such gametes would have the diploid (2*x*) complement of chromosomes. Therefore, 2*n* gametes in inter-EBN crosses provide an alternative to chemical doubling of chromosomes of the lower EBN species to overcome the EBN barriers. Thus the 2:1 EBN requirements can serve as a relative screen for functional 2*n* gametes. This is what may have happened in the present study where three 7*x* BC_1F_1's were obtained from a 4*x* (4EBN)-6*x* (8EBN) backcross.

Further evidence supporting the 2:1 EBN hypothesis was obtained from second backcrosses (Table 4.2). Here the 5*x* BC_1F_1 (2*n*=5*x*=40, presumably with 4 genomes of *T. repens* and one genome of *T. nigrescens* and an EBN=6) was reciprocally backcrossed with *T. repens* (EBN=4). Using the 5*x* BC_1F_1 as the female parent, only one seed was obtained from 160 crosses in contrast to 8 seeds obtained from the same number of reciprocal backcrosses. Although meiotic studies of pollen mother cells (PMCs) of 5*x* BC_1F_1 at anaphase I most commonly showed 20-20 chromosome disjunction (i.e. the formation of aneuploid gametes) some of the PMCs had 24-16 disjunction. It is therefore possible that the failure of the cross using 5*x* BC_1F_1 as the female and *T. repens* as the male might be due to a difference in the EBN of the two parents. Conversely, the production of 8 seeds from the reciprocal backcross might have been the result of the union of 2*x* (2EBN) pollen from the 5*x* BC_1F_1 parent, giving a 2:1 EBN ratio in the endosperm. It is expected that the number of 2*x* eggs would be lower than 2*x* pollen grains in 5*x* BC_1F_1 (as pollen grains are produced in abundance), so making the cross more successful when 5*x* BC_1F_1 was used as a male parent. Although only one BC_2F_1 plant was grown from the 8 BC_2F_1 seeds, this was confirmed to be tetraploid, supporting the 2:1 EBN hypothesis in backcrosses.

Four BC_2F_1 seeds were also obtained from 420 reciprocal backcrosses of 7*x* BC_1F_1 with *T. repens*. The production of these four BC_2F_1 plants does not necessarily support the 2:1 EBN hypothesis, as the cross was 7*x* (8EBN) x 4*x* (4EBN). However

the number of seeds obtained from these crosses was very low. The only seed obtained from *T. repens* pollinated with 7*x* BC_1F_1 was an aneuploid with 2*n*=42. The remaining three reciprocal seeds from these crosses have not yet been germinated. It is possible that these three seeds might have been obtained by the union of a 2*n* pollen grain from *T. repens*, which would be expected to work on the basis of the EBN hypothesis.

Further evidence to support the 2:1 EBN hypothesis arises from different BC_1F_1 x BC_1F_1 intercrosses. Here the 4*x*, 5*x* and 7*x* BC_1F_1's were reciprocally inter-crossed. Based on the endosperm balance numbers assigned by Parrott and Smith (1986a) to *T. repens* and *T. nigrescens* genomes, the 4*x*, 5*x* and 7*x* BC_1F_1's would have an EBN =6,6 and 8 respectively. Nine seeds were obtained from 60 reciprocal intercrosses between 4*x* BC_1F_1 (6EBN) and 5*x* BC_1F_1 (6EBN) indicating the success of the cross in both directions (Table 4.2). Here both parents share the same EBN i.e. EBN=6. However, six of the nine seeds were obtained from 30 crosses where the 5*x* BC_1F_1 was used as the male parent. As mentioned earlier, most PMCs studied at anaphase I from 5*x* BC_1F_1 showed a 20-20 chromosome disjunction, indicating the production of aneuploid gametes with half the chromosome complement of the parent plant. The one plant obtained from this set of six seeds (4*x* BC_1F_1 female parent pollinated by 5*x* BC_1F_1) was an aneuploid with 2*n*=36 (Fig. 4.11). To date there has been no published discussion of the operation of EBN in aneuploid gametes. However the success of this cross and one other (to be discussed later) indicate that probably aneuploid gametes with half the chromosome complement of the parent species might also have half EBN i.e. aneuploid pollen from 5*x* BC_1F_1 in the present study with 20 chromosomes (presumably 16 from *T. repens* and 4 from *T. nigrescens*) might have an EBN=3.

Reciprocal intercrosses between 4*x* BC_1F_1 (6EBN) and 7*x* BC_1F_1 (8EBN) were much less successful, and only two seeds were obtained from 375 crosses (Table 4.2). Similarly 7*x* BC_1F_1 (8EBN) x 5*x* BC_1F_1 (6EBN) crosses were also relatively unsuccessful and only one seed was harvested from 175 crosses. On the other hand intercrosses between two heptaploid BC_1F_1 plants (8EBN) were relatively more successful and yielded six seeds from only 60 crosses. The success and failure in all these BC_1F_1 x BC_1F_1 intercrosses gives further evidence to support the endosperm balance number hypothesis.

Evidence consistent with the EBN hypothesis was further obtained from some other crosses involving 6*x* H-6909-5 (8EBN) as one of the parents and 4*x*, 5*x* and 7*x* BC_1F_1's as the other. In first case the 6*x* H-6909-5 (8EBN) was reciprocally crossed with the 5*x* BC_1F_1 (6EBN). Only one seed was obtained from 140 crosses using 5*x* BC_1F_1 as the female parent and two 6*x* H-6909-5 plants (CT-1 and CT-14) as the male parents. In contrast, 170 reciprocal crosses yielded 62 seeds. Ploidy level of the progeny plants has not yet been determined. However the success of this cross in one direction and its failure in the other might be explained by the 2:1 EBN hypothesis. As mentioned before, the 5*x* BC_1F_1 produced aneuploid gametes with *n*=20, with 3*x* and 2*x* gametes as well. It is most probable that 3*x* pollen (EBN=4) would have functioned preferentially through this selective screen bringing a 2:1 EBN ratio in the endosperm. The ploidy level of these seeds, which will be evaluated at some later stage, will provide evidence for or against this assumption.

Similar results were obtained from reciprocal crosses of 6*x* H-6909-5 to 7*x* BC_1F_1. No seeds were obtained from 7*x* BC_1F_1 pollinated with 6*x* H-6909-5. In contrast, the reciprocal cross resulted in 56 seeds from 100 pollinations. Both genotypes involved in the cross have an EBN=8 and if the 2:1 EBN was operating, then the cross should have been successful in both directions. However, meiotic configurations of 7*x* BC_1F_1 showed a large number of univalents (an average of 6, Table 4.3) at metaphase I and some lagging chromosome at anaphase I (Fig. 4.13). Therefore, a 32-24 or 28-28 disjunction of chromosomes during gametogenesis as happened in 5*x* BC_1F_1, may not be as frequent in 7*x* BC_1F_1. Pertinent to this was the ploidy level of a single plant obtained from a *T. repens* x 7*x* BC_1F_1 cross which had 2*n*=42 (Fig. 4.11), indicating that the functional gametes from 7*x* BC_1F_1 had *n*=26. This is consistent with the possibility that 7*x* BC_1F_1 produced high frequencies of aneuploid gametes and not necessarily with *n*=28 chromosomes. These aneuploid gametes might affect the EBN of the endosperm, resulting in the failure of the cross in one direction.

If once again it is assumed that aneuploid gametes from 7*x* BC_1F_1 (8EBN) with *n*=28 have EBN=4, then the greater number of seeds obtained using 7*x* BC_1F_1 as the male parent might be due to the selective screening of functional *n*=28 pollen. Another possible explanation for the failure of 7*x* BC_1F_1 x 6*x* H-6909-5 in one direction and its

success in the other might be due to unilateral interspecific incompatibility. Liedl and Anderson (1993) reported that when SI x SI interspecific crosses are attempted, one of the following outcomes is possible. (1) an SI response in both directions i.e. the SI systems are homologous (Lewis and Crowe, 1958), (2) a unilateral response similar to that described by Harrison and Darby (1955) or (3) compatible pollinations in both directions i.e the SI systems are non homologous, (Lewis and Crowe, 1958). Also backcrosses of 3*x* H-6909-5 (4EBN) to *T. repens* (4EBN) and *T. nigrescens* (4EBN) produced no seed, although the crosses were made presumably at the same EBN level. Failure of these backcrosses, however, does not rule out the operation of the EBN mechanism in these crosses. Although successful backcrossing of the triploid *T. repens* x *T. nigrescens* hybrid to *T. repens* has been reported previously by Hovin (1962a) and Marshall *et al.* (1995), the failure of backcrosses of the 3*x* H-6909-5 to its parental species in the present study was probably due to the sterility of the F_1 hybrid. It is therefore emphasised that successful crosses in species with the same EBN may be prevented by pre- or post-fertilisation barriers unrelated to EBN.

It is evident from virtually all the examples listed in support of the 2:1 EBN hypothesis from the present studies that the EBN hypothesis has the potential to explain differences in reciprocal crosses of genotypes with different EBNs. The examples given in support of the 2:1 EBN hypothesis are summarised in Table 5.1.

Apart from the many examples given in favour of the EBN hypothesis for backcrosses and other intercrosses, there are still two crosses which possibly deviate from the 2:1 EBN hypothesis, albeit at low frequency. When 4*x* BC_1F_1 (6EBN) was reciprocally crossed with *T. repens* (4EBN) in a congruity backcross, six CBC_2 seeds were obtained from 450 crosses. Similarly the 4*x* BC_1F_1 (6EBN) was also reciprocally crossed with 6*x* H-6909-5 (8EBN). Although no seeds were obtained from the cross using 4*x* BC_1F_1 as the female parent, the reciprocal cross yielded 10 seeds from 430 crosses. However, as reported by Parrott and Smith (1986a), crosses between species differing in EBN can be successful, but at a very low rate. The success rates in both these crosses (apparently less than 2 seeds per 100 crosses) were very low, and according to this criterion, may not therefore be considered to depart from the hypothesis.

Table 5.1 Frequency of seed set after intra- and inter EBN crosses.

Cross*	EBN Level	Seed set per 100 florets
a. *n* gametes:		
4*x* BC_1F_1 x 5*x* BC_1F_1	(6 EBN) x (6 EBN)	15
7*x* BC_1F_1 x 7*x* BC_1F_1	(8 EBN) x (8 EBN)	10
6*x* H-6909-5 x 5*x* BC_1F_1	(8 EBN) x (6 EBN)	36†
6*x* H-6909-5 x 7*x* BC_1F_1	(8 EBN) x (8 EBN)	43
4*x* BC_1F_1 x *T. repens*	(6 EBN) x (4 EBN)	1.30
6*x* H-6909-5 x 4*x* BC_1F_1	(8 EBN) x (6 EBN)	1.96
6*x* H-6909-5 x *T. nigrescens*	(8 EBN) x (4 EBN)	0.05
6*x* H-6909-5 x *T. repens*	(8 EBN) x (4 EBN)	0.08
4*x* BC_1F_1 x 7*x* BC_1F_1	(8 EBN) x (6 EBN)	0.53
7*x* BC_1F_1 x 5*x* BC_1F_1	(8 EBN) x (6 EBN)	0.57
b. 2*n* gametes		
T. repens x 6*x* H-6909-5	(4 EBN) x (8 EBN)	0.4

* Reciprocal crosses

† Presumably 3*x* (4 EBN) pollen grains of 5*x* BC_1F_1 would have functioned in this cross.

When crosses were made presumably at the same EBN level, reasonable seed setting occurred i.e. 10-43%. In contrast, for crosses made at different EBN levels, or where 2*n* gametes are involved, seed setting frequency was very low i.e. 0.05-1.96%.

5.5 SCREENING 3*x* AND 6*x* H-6909-5 FOR CLOVER CYST NEMATODE RESISTANCE/ SUSCEPTIBILITY

Interspecific *Trifolium* hybrids have rarely been evaluated for characters other than fertility and agronomic growth. Pederson and Windham (1989) however screened eight *Trifolium* species and seven interspecific *Trifolium* hybrids for resistance to *Meloidogyne incognita* Kofoid and White (Chitwood) (southern root knot nematode) and found varying degrees of resistance among the seven hybrids. They concluded that *T. nigrescens* would be a valuable source of germplasm for the improvement of white clover cultivars.

The present study reports for the first time the screening of interspecific *T. repens* x *T. nigrescens* hybrids for resistance to clover cyst nematode. Results of both the initial and rescreening tests of *T. nigrescens* and *T. repens* showed that *T. nigrescens* was more resistant to clover cyst nematode than *T. repens*. These results are consistent with those reported by Mercer (1988) who found a mean number of 1.6 (range 0-5) cysts per plant on *T. nigrescens* in contrast to a mean number of 500 cysts per plant for white clover. Considering plants with 0-5 cysts per plant as resistant in the present experiment, 34% and 19% of the *T. nigrescens* plants were resistant in the initial and rescreenings tests, respectively. No resistant plant was observed for *T. repens* in either screening. The rescreening results were consistent with the initial screening, indicating the repeatability of the screening technique.

The reason for recording initial data as number of cysts per plant was that screened plants were kept alive to allow for comparison with 3*x* and 6*x* H-6909-5, so the roots of the *T. nigrescens* plants were not destructively harvested. Subsequent data from screening 3*x* and 6*x* H-6909-5 in comparison to *T. nigrescens* and *T. repens* were recorded as cysts per gram of root dry weight.

Both the 3*x* and 6*x* H-6909-5 showed the same degree of resistance to clover cyst nematode and were as resistant as the selected resistant *T. nigrescens* genotype. This high transmission of resistance from parent to hybrid in one generation was consistent with control by one or a small number of dominant genes, and contrasted with white clover where resistance appeared to be controlled by more than one gene (Dijkstra, 1971).

The H-6909-5 hybrids were not evaluated for agronomic traits in replicated trials in comparison to white clover, but at both ploidy levels they appeared to be markedly inferior to white clover in growth, perenniality and fertility. Nevertheless 6*x* H-6909-5 hybrid has provided very useful genetic material for backcrossing to *T. repens*.

Fertile backcross progenies have been produced between 6*x* H-6909-5 and both of its parental species. The pentaploid (5*x*) and heptaploid (7*x*) BC_1F_1 progeny, although possessing odd ploidy levels, have exhibited the vigorous strong stoloniferous habit of *T. repens* as well as reasonable fertility. These backcrossed progeny have not been tested for resistance to the clover cyst nematode but will be evaluated at a later stage.

The present results suggest that *T. nigrescens* is a valuable species to hybridise with *T. repens* to improve its resistance to clover cyst nematode. The species was also reported to be resistant to *Meloidogyne incognita* (Pederson and Windham, 1989). The advantages of using *T. nigrescens* in interspecific hybridisation with *T. repens* are the ease of producing interspecific hybrids (relative to other interspecific *Trifolium* hybrids), fertility of the backcrosses and the level of resistance in *T. nigrescens* and H-6909-5. Gibson *et al.* (1971) concluded that interspecific *T. repens* x *T. nigrescens* was not useful for the improvement of *T. repens* due to poor agronomic performance and lack of virus resistance. However in the present study the 6*x* *T. nigrescens* x *T. repens* hybrid provided useful genetic material for clover cyst nematode resistance which might be exploited in the backcrossed progeny. It is emphasised at this point that further investigations are needed to determine the genetic basis of resistance in *T. nigrescens*.

5.6 POTENTIAL USES OF BC_1F_1 PROGENY

The three different categories of BC_1F_1 i.e. 4*x*, 5*x* and 7*x* have not yet been grown in replicated trials for evaluation of agronomic characters or clover cyst nematode resistance. Instead these BC_1F_1's have so far provided useful genetic material at three different ploidy levels for further backcrosses.

From the meiotic data at anaphase I of the 5*x* and 7*x* BC_1F_1, 20-20 and 28-28 chromosome disjunctions respectively might yield aneuploid gametes, which in crosses

with parental species would presumably produce aneuploid progenies. One BC_2F_1 plant initially grown from seeds obtained from *T. repens* x 7*x* BC_1F_1 and one from a 4*x* BC_1F_1 x 5*x* BC_1F_1 intercross were evaluated cytologically for ploidy levels and were found to be aneuploids with $2n$=42 and 36 respectively. The aneuploid gametes in these crosses are most likely to be contributed by the 5*x* and 7*x* BC_1F_1's, as 4*x* BC_1F_1 and *T. repens* formed normal gametes.

Aneuploid production is also expected from other BC_1F_1 x BC_1F_1 intercrosses and BC_1F_1 x 6*x* H-6909-5 crosses (Table 4.2). Ten seeds of different BC_1F_1 x BC_1F_1 and BC_1F_1 x 6*x* H-6909-5 have been germinated and grown successfully. These are planned to be evaluated morphologically and cytologically at later stages. The aneuploids with different chromosome numbers will provide useful material for *in situ* DNA hybridisation to identify chromosomal exchange between the parental genomes and potentially, association of specific characters with certain chromosomes.

The second congruity backcross (CBC_2) obtained after crossing the 4*x* BC_1F_1 plant with *T. repens* produced six seeds. The one plant so far grown from these seeds is expected to be tetraploid, as both of its parents produced euploid gametes with n=2*x*=16. This CBC_2 plant theoretically carries 3 genomes of *T. repens* and one genome of *T. nigrescens* in contrast to the tetraploid BC_1F_1 (CBC_1) with two genomes from each species. A backcross progeny with a 3:1 combination of parental genomes of these two species has not been reported before. The meiotic behaviour of these CBC_2 plants will provide additional information on the homology of chromosomes between these two species. All the BC_1F_1 progeny and their intercrossed progeny will be evaluated for clover cyst nematode resistance at later stages.

5.7 BACKCROSSES OF 4*x* AND 8*x* H-435 TO *T. repens* AND *T. ambiguum*

5.7.1 First Backcross (BC_1F_1)

Backcrosses of 4*x* H-435 to *T. repens* and 4*x* and 6*x* *T. ambiguum* were not successful. These results are partially consistent with the results of Williams and Verry (1981) and Anderson *et al.* (1991c) who successfully obtained BC_1F_1 progeny of 4*x* H-435 to *T. repens* but failed to produce backcrossed seeds with *T. ambiguum*. The failure to obtain backcross progeny of 4*x* H-435 to *T. repens* in the present study might be due to the use of only one genotype of *T. repens* (CC-1) in backcrosses. Evans (1962b) also observed that certain genotypes of one species showed greater compatibility than others in interspecific *Trifolium* crosses, suggesting that the growth and development of hybrid embryos may vary with the genotypes of the individual plants or strains that are crossed. Hovin (1962a) found that *T. repens* used as a tester was more cross compatible with Italian than Turkish *T. nigrescens*. This might be the reason that backcrosses of 4*x* H-435 to *T. repens* reported by Williams and Verry (1981) and Anderson *et al.* (1991c) were successful as they used different genotypes of white clover.

Self compatibility of 4*x* H-435 has been reported by Williams and Verry (1981) and of 8*x* H-435 by Anderson *et al.* (1991c). The production of 18 seeds from 6 selfed inflorescences of 8*x* H-435 confirmed its self-compatibility. The failure of 4*x* H-435 to produce F_2 progeny in the present investigation might have been due to very poor dehiscence of the anthers with a very low frequency of viable pollen.

First backcross progeny plants were successfully raised from reciprocal backcrosses of 8*x* H-435 to *T. repens*. However, backcrosses of 8*x* H-435 to 4*x* and 6*x* *T. ambiguum* were not successful. These results are once again partially consistent with those of Anderson *et al.* (1991c). These authors reported that 8*x* H-435 was both male and female fertile with *T. repens*, but was only female fertile with 4*x* *T. ambiguum*, and obtained 11 seeds from 528 backcrosses using 8*x* H-435 as the female parent. However, they did not evaluate these seeds to identify backcross progeny and so these 11 seeds might have resulted from self pollinations.

The success of backcrossing 8*x* H-435 to *T. repens* on the one hand and its failure with 4*x* and 6*x* *T. ambiguum* on the other hand again suggests the possibility that the relative dosages of genetic material of the two species may be involved in the regulation of the development of backcrossed embryos (Rabakoarihanta *et al.*, 1980; section 5.2.). Somatic chromosomes were counted for 8 BC_1F_1 plants having 8*x* H-435 as the female parent and *T. repens* as the male parent and 3 reciprocal BC_1F_1 plants out of a total 19 initially grown BC_1F_1 seeds. All were found to be hexaploid ($2n=6x=48$). This suggested that 8*x* H-435 was producing normal euploid ($n=4x=32$) gametes, thus giving a genomic combination of 4 *T. repens* and 2 *T. ambiguum* genomes in each 6*x* BC_1F_1.

5.7.2 Congruity (CBC) and Second (BC_2F_1) Backcrosses

The aim of generating 6*x* BC_1F_1 progeny between 8*x* H-435 and *T. repens* was to develop useful genetic material either for direct use as forage without the need for further backcrossing to either parental species, or inclusion in congruity backcrosses (Haghighi and Ascher, 1988) to hexaploid *T. ambiguum* types that are considered to be agronomically superior to tetraploid types (Kannenberg and Elliot, 1962; Spencer and Hely, 1982). However, again the congruity backcrosses of 6*x* BC_1F_1 to 6*x* *T. ambiguum* were not successful. Instead three seeds were harvested from one of the 6*x* BC_1F_1 plants pollinated with 4*x* *T. ambiguum* (cv. Treeline). On the other hand, second backcrosses involving 6*x* BC_1F_1 and *T. repens* were more successful (Table 4.7). The failure of 6*x* BC_1F_1 to produce seeds in congruity backcrosses with 6*x* *T. ambiguum* and its success in second backcrosses with *T. repens* can once again be explained on the basis of the assumption that the relative dosages of the genetic material from the two parental species might seem to be operating in the development of backcross embryos. It is evident from the first (BC_1F_1) and second (BC_2F_1) backcrosses that those crosses were successful where both the female and male gametes presumably had the two homoeologous genomes of *T. repens*. Any deviation from this resulted in the failure of the cross. This suggests that in a hybrid environment the specific dosage of genetic material of one species (in this case *T. repens*) might have more influence on the regulation of the development of the backcrossed embryos than the other species. This was evident when 6*x* BC_1F_1 were reciprocally crossed with 6*x* *T. ambiguum*. Although the cross was made at the same (6*x*-6*x*) ploidy level the presence of only one member of

each homoeologous pair from *T. repens* might be inadequate for normal development of the embryo in these backcrosses.

On the other hand in backcrosses of 6*x* BC_1F_1 to *T. repens*, the gametes of 6*x* BC_1F_1 had presumably two homoeologous genomes of *T. repens*, resulting in the success of the second backcross.

Three out of seven initially grown BC_2F_1 (6*x* BC_1F_1 x *T. repens*) plants were evaluated for ploidy level and were found to be pentaploid ($2n$=5x=40) with presumably 4 genomes of *T. repens* and one genome of *T. ambiguum*. The two successfully grown 6*x* BC_1F_1 x 4*x* *T. ambiguum* (CBC_2) seeds were also pentaploid ($2n$=5x=40) but presumably with a different genomic combination i.e. three genomes of *T. ambiguum* and two genomes of *T. repens*. The pentaploid BC_2F_1 plants with presumably four complete genomes of *T. repens* showed normal growth and development with an average of 59.3% pollen stainability (range 44.4-70.1%). In contrast, the two pentaploid CBC_2 plants exhibited developmental abnormalities. These abnormalities were expressed as altered leaf and inflorescence shape, low fertility and very short internode length. Both plants reached flowering but one of them failed to produce normal flower heads and the inflorescences did not grow beyond the bud stage.

Developmental abnormality or hybrid breakdown, also referred to as hybrid sterility or weakness, arises in interspecific F_1 hybrids or hybrid derivatives as a result of lack of proper co-ordinated function of the genetic material contributed by the parents (Haghighi and Ascher,1988). Haghighi and Ascher (1988) attributed the deviant growth and development of *Phaseolus* interspecific hybrids to incongruity. Incongruity has been defined as a pre- and/or post-zygotic reproductive barrier which results in the failure of intimate partner relationships because of a lack of genetic information in one partner about the critical factors of the other partner (Hogenboom, 1973, 1984; Haghighi and Ascher, 1988). Incongruity may therefore involve, in a hybrid, the mismatching of heritable information leading to the lack of coordinated development. The method of congruity backcrossing has been suggested by Haghighi and Ascher (1988) to overcome incongruity barriers. Congruity backcrossing can be defined as recurrent backcrossing of BC_1F_1 to each parent in alternate generations. Congruity

backcrossing in *Phaseolus* (Haghighi and Ascher, 1988) also produced individuals that exhibited symptoms of developmental incongruity (hybrid breakdown). Congruity backcrossing initially gives apparently slow improvement in fertility compared with recurrent backcrossing which can give rapid recovery in fertility but results in the loss of traits from non-recurrent parent. However by the fourth or fifth congruity backcross generation, recombinations seem to give rise to both recovered fertility and new and unique genetic combinations.

The abnormal development and low fertility of the two pentaploid CBC_2 plants in the present investigation are consistent with the concept of incongruity.

5.7. 3 BC_1F_1 x BC_1F_1 Intercrosses (BC_1F_2)

Anderson *et al.* (1991c) suggested the possibility of obtaining 6*x* BC_1F_1 by crossing 8*x* H-435 to 4*x* *T. ambiguum.* These hexaploid BC_1F_1 would be expected to carry 4 genomes of *T. ambiguum* and 2 genomes of *T. repens.* In fact, they were able to produce 11 seeds from 528 backcrosses using 8*x* H-435 as the female parent but, as already mentioned, they did not evaluate the plants for backcross origin. In the present investigation the backcrosses of 8*x* H-435 to 4*x* *T. ambiguum* failed and so another approach was adopted to generate similar hexaploids. This involved the crossing of already existing 6*x* BC_1F_1 (with presumably 4 genomes of *T. repens* and 2 genomes of *T. ambiguum*) with 6*x* *T. ambiguum* in congruity backcrosses. However, the cross failed once again despite the same 6*x*-6*x* ploidy level of the parents. Alternatively, the 6*x* BC_1F_1's were intercrossed among each other with the idea of selecting a more fertile 6*x* BC_1F_2 and testing its effectiveness in crosses with 6*x* *T. ambiguum.* At this stage one [(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-2]-1 out of six 6*x* BC_1F_2 has produced seeds when pollinated with 6*x* *T. ambiguum.*

Assuming that only a small amount of allosyndetic pairing among the genomes of 6*x* BC_1F_1 was occurring, the resulting 6*x* BC_1F_2 progeny from 6*x* BC_1F_1 x 6*x* BC_1F_1 intercrosses would presumably have an altered genetic (not necessarily genomic) combination as a result of recombination. Perhaps this altered genetic combination in one of the 6*x* BC_1F_2 [(CC-1 x 8*x* H-435)-2 X (CC-1 x 8*x* H-435)-2]-1 would have

resulted in its cross compatibility with 6*x* *T. ambiguum*. Three plants from 6*x* BC_1F_2 x 6*x* *T. ambiguum* have recently been grown in the glasshouse. These will be evaluated cytologically for ploidy level and meiotic chromosome configurations at later stages. These plants, in addition to the intercross (6*x* BC_1F_1 x 6*x* BC_1F_1), have provided useful genetic material for transferring characters of interest from both parental species and therefore, can be used as a "bridge" between *T. repens* and *T. ambiguum*.

Anderson *et al.* (1991c) had suggested that the use of crosses between *T. repens* and the octoploid hybrid (8*x* H-435) may have limited value because of depleting number of *T. ambiguum* chromosomes. The new approach described in the present investigation overcomes this difficulty.

The present investigation has resolved the difficulties originally perceived for the combining of superior white clover and caucasian clover traits without having to resort to further primary hybrids between these species.

The solution is to first hybridise superior white clover plants with 8*x* H-435 to generate 6*x* progenies selectable for superior white clover traits. Second, these selected 6*x* plants are recombined (intercrossed) to give 6*x* plants which will hybridise with 6*x* *T. ambiguum*. Third, superior 6*x* *T. ambiguum* plants are crossed with these selected 6*x* hybrids (white clover backcrosses) as a "fertile bridge" to effectively combine superior traits from both species. Further intercrosses among the 6*x* progenies will produce new combinations and genomic balances yet to be researched.

5.8 MEIOTIC CONFIGURATIONS IN FIRST BACKCROSS (8*x* H-435 x *T. repens*) PROGENY

The average frequency of 1.1 trivalents and 1.6 quadrivalents in four 6*x* BC_1F_1 plants in the present experiment is higher than the earlier findings of Anderson *et al.* (1991c) who reported an average of 0.5 trivalents and 0.07 quadrivalents in two 6*x* BC_1F_1 plants. They also found predominantly bivalent pairing i.e. an average of 22.5 bivalents in contrast to an average of 18.0 bivalents in the present study. Although presumably possessing the same genomic combination of the two parental species (4 genomes of *T. repens* and 2 genomes of *T. ambiguum*), the origin of 6*x* BC_1F_1's

generated in the present investigation is different from those reported by Anderson *et al.* (1991c) where the 6*x* BC_1F_1's were obtained through unreduced gametes contributed by the 4*x* H-435 female parent. Whether this difference in meiotic configurations was due to the difference in backcross origin of the two sets of 6*x* BC_1F_1's is not certain.

Chromosomes of *T. repens* and *T. ambiguum* were not distinguishable in meiotic cells of 6*x* BC_1F_1 plants. Therefore it was not possible to conclude whether the bivalent and multivalent pairing in these 6*x* BC_1F_1 represents intra or interspecific chromosome pairing. Meiotic configurations for the 4*x* H-435 were reported to be mostly bivalent with a low frequency of multivalent formation (Williams *et al.*, 1982; Anderson *et al.*, 1991c). It was impossible for these authors to conclude whether predominantly bivalent formation represented pairing of intraspecific homoeologous chromosomes (autosyndesis) or pairing between *T. repens* and *T. ambiguum* chromosomes (allosyndesis). However, the multivalent formation can only have been the result of both types of pairing.

Results obtained by Williams *et al.* (1982) for the meiotic configurations of the single tetraploid BC_1F_1 plant from backcrosses of 4*x* H-435 to *T. repens* (with presumably 3 genomes of *T. repens* and one genome of *T. ambiguum*) exhibited extreme meiotic irregularities with an average of 10 univalents per pollen mother cell. The multivalent (an average of 3.15 trivalents and 0.13 quadrivalents) formation in that 4*x* BC_1F_1 plant was suspected to be the result of homoeologous pairing of *T. repens* or *T. ambiguum* chromosomes with a homologous *T. repens* pair. In contrast, Anderson *et al.* (1991c) observed mostly bivalent (an average of 15.71 per PMC) pairing in two similar backcross plants. Reasons for these contrasting results are not known and Anderson *et al.* (1991c) have not attempted to provide a possible explanation for the differences in meiotic configurations of their two 4*x* BC_1F_1 plants and that of Williams *et al.* (1982). The observations of Anderson *et al.* (1991c), however, provided firm support for a large amount of allosyndetic pairing. In the 32 chromosome progeny of first backcrosses of 4*x* H-435 to *T. repens*, one homologous set of *T. repens* chromosomes was believed to be pairing as eight bivalents, and the remaining 8 *T. repens* chromosomes pairing as bivalents with *T. ambiguum* chromosomes.

Based on these assumptions it can be further assumed that in the presence of homologous genomes of white clover, homoeologous white clover chromosome pairing (autosyndesis) has been largely suppressed, thus allowing the third genome of white clover to pair mainly allosyndetically with caucasian clover chromosomes in the 32 chromosome BC_1F_1 plants. With regard to the 6*x* BC_1F_1 obtained in the present investigation, it can be assumed that four genomes of *T. repens* might be pairing mainly as homologues and the two genomes of *T. ambiguum* pairing as homoeologues. The multivalent formation might be the result of autosyndetic pairing of homoeologous *T. repens* chromosomes or allosyndetic pairing of *T. repens* and *T. ambiguum* chromosomes. This assumption is consistent with the earlier findings of Anderson *et al.* (1991c).

Based on the results obtained for meiotic configurations of 6*x* BC_1F_1 in the present investigation and the results of Anderson *et al.* (1991c) for 4*x* H-435, 4*x* BC_1F_1 and 6*x* BC_1F_1, it can be assumed that if the backcross progeny have an odd number of *T. repens* genomes, there will be a greater frequency of allosyndetic pairing, i.e. the odd genome of *T. repens* having no homologous set of chromosomes to pair with, might provide greater chances for allosyndetic pairing. This has almost certainly occurred in the 4*x* BC_1F_1 (Anderson *et al.*, 1991c) which had presumably 3 genomes of *T. repens* and one genome of *T. ambiguum.* The high frequency of bivalent pairing in that hybrid is an evidence of pairing between the *T. repens* and *T. ambiguum* chromosomes.

Apart from the 6*x* BC_1F_1, a pentaploid second backcross progeny (5*x* BC_2F_1) with presumably 4 genomes of *T. repens* and one genome of *T. ambiguum* was also produced in the present investigation. Although these 5*x* BC_2F_1 were not studied for meiotic configurations, these plants are likely to be potential candidates for further studying the chromosomal relations of these two species.

5.9 SELF-INCOMPATIBILITY IN 6*x* BC_1F_1 PROGENY

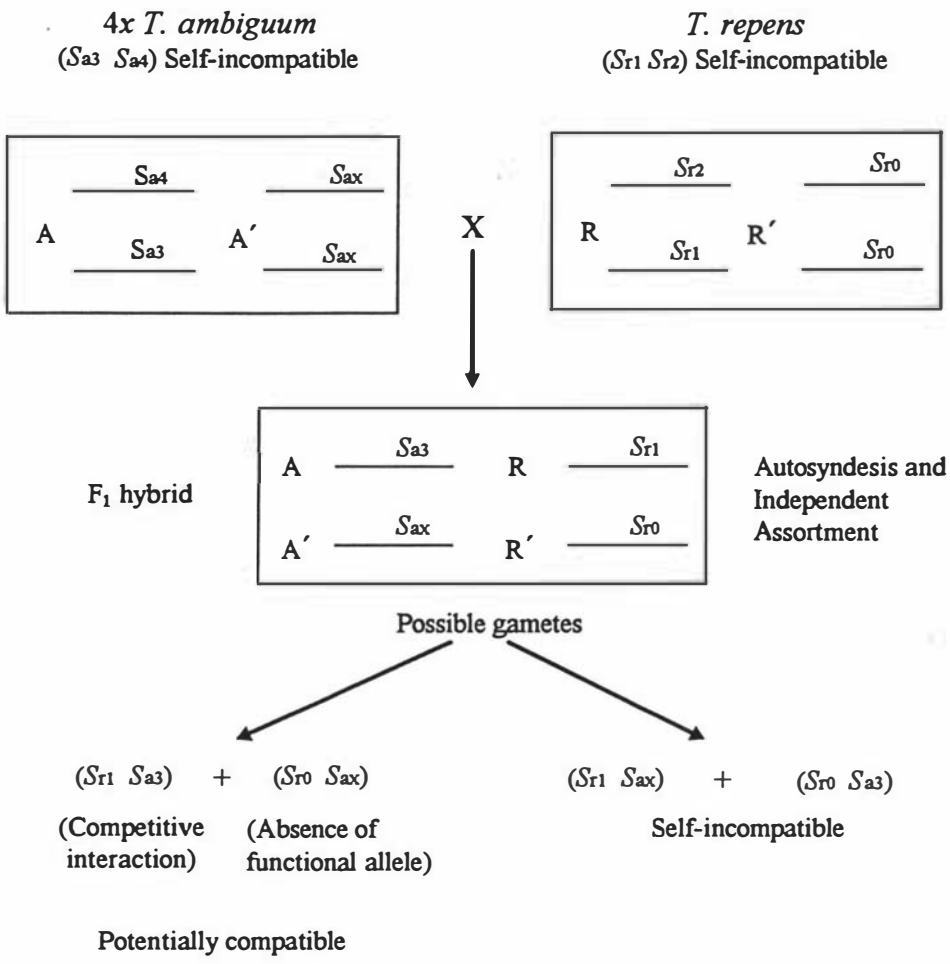
Colchicine doubled 8*x* H-435 was reported to be self-compatible (Anderson *et al.*, 1991c). In the present investigation the production of 18 seeds from selfing 6 inflorescences of 8*x* H-435 confirmed self-compatibility. Although no F_2 seeds were

produced by 4*x* H-435 in the present experiment, its self-compatibility was reported earlier by Williams and Verry (1981). The failure of 4*x* H-435 to produce F_2 progeny might have been due to its low fertility, as it produced a very low frequency of viable pollen.

The self-compatibility of 4*x* H-435 was explained by Williams *et al.*, (1982) using the following model (Fig. 5.2). Assuming autosyndetic pairing in the F_1 (4*x* H-435) hybrid, self-compatibility might result from either non-inclusion of a functional *S* allele in a pollen grain, or competitive interaction between two functional alleles from two different species within a single pollen grain (Atwood and Brewbaker, 1953, as quoted by Williams *et al.* 1982). Competitive allelic interaction in a pollen grain was also proposed to explain the self-compatibility of a hexaploid hybrid (WN2) obtained between colchicine doubled self-incompatible 4*x* *T. nigrescens* and a self-compatible 8*x* *T. repens* (Brewbaker and Keim, 1953). However, as discussed by Williams *et al.* (1982), Brewbaker and Keim (1953) assumed that competitive interaction was occurring between two *S* alleles from *T. repens* assuming allosyndetic pairing. Competition interaction between alleles from different species was apparently not considered.

When 4*x* H-435 was colchicine doubled (Anderson *et al.*, 1991c), the resulting 8*x* H-435 was found to be self-compatible. According to the proposed model of Williams *et al.*, (1982) for self-compatibility of 4*x* H-435, the 8*x* H-435 should have the genotype presented in Fig. 5.3 in relation to the *S* alleles from both parents.

As each chromosome in 8*x* H-435 has apparently a homologous partner, it can be assumed that pairing in this octoploid hybrid would be predominantly between homologues. Such pairing was evident from the results of Anderson *et al.* (1991c) who observed an average of more than 27 bivalents at metaphase I in 8*x* H-435. Homologous chromosome pairing should accordingly result in one type of pollen grain with respect to the self-incompatibility alleles (Fig. 5.3). According to the model proposed by Williams *et al.* (1982) these pollen grains should be self-compatible as competitive interaction is still presumably taking place between functional *S* alleles of the two species while the two non-functional alleles have no influence.



S_{r1}, S_{r2}	=	Functional *T. repens* self-incompatibility alleles.
S_{r0}	=	Non functional sites on *T. repens* chromosome homoeologous to the *S*-bearing chromosome.
R,R′	=	Homoeologous *T. repens* chromosomes (also homoeologous to A,A′)
S_{a3}, S_{a4}	=	Functional *T. ambiguum* self-incompatibility alleles.
S_{ax}	=	Non-functional site, or non competitive alleles, on *T. ambiguum* chromosome homoeologous to the *S*-bearing chromosome.
A,A′	=	Homoeologous *T. ambiguum* chromosomes (also homoeologous to R,R′).

Figure 5.2 Possible explanation for self-compatibility after autosyndesis of parental chromosome sets in an F_1 (4x H-435) hybrid between 4x *T. ambiguum* and *T. repens*. (after Williams *et al.*, 1982).

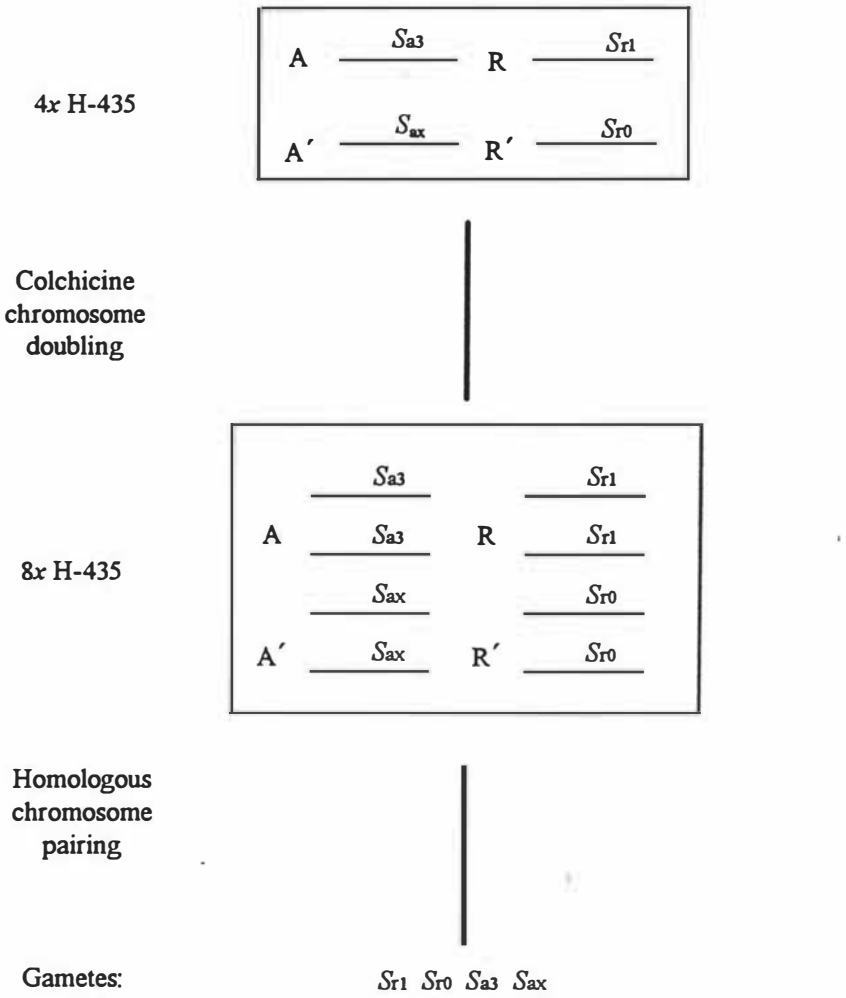
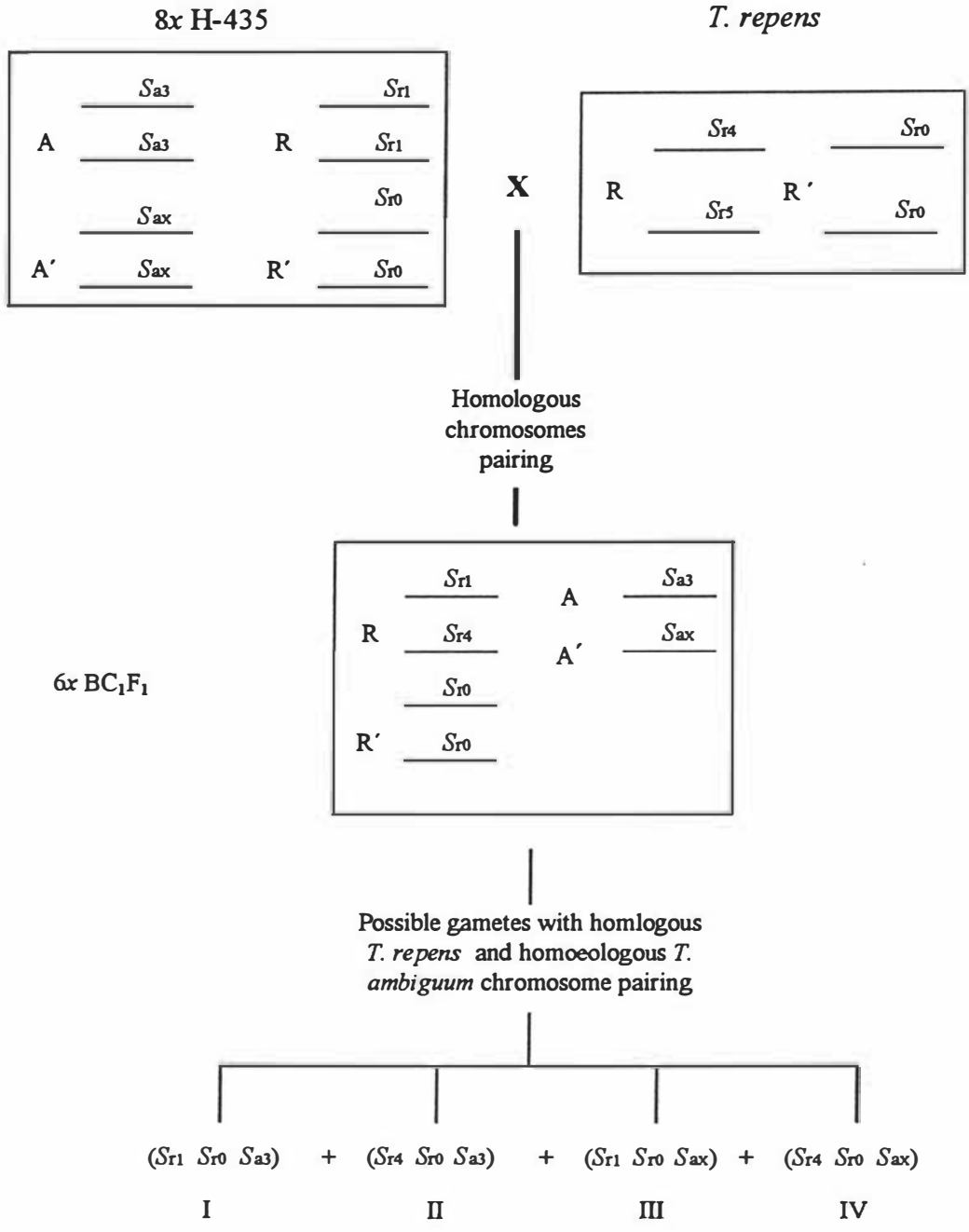


Figure 5.3 The genotypes of 4*x* H-435 and 8*x* H-435 in relation to self-incompatibility alleles from both parents. (Based on the model of Williams *et al.*, 1982).

Interesting results were obtained when 8*x* H-435 was reciprocally backcrossed with self-incompatible *T. repens* (Fig. 5.4). The resulting 6*x* BC_1F_1 progeny plants presumably have four genomes of *T. repens* and two genomes of *T. ambiguum*. Assuming that four genomes of *T. repens* pair as homologues and the two *T. ambiguum* genomes as homoeologues (Anderson *et al.*, 1991c, and from the present investigation) then every 6*x* BC_1F_1 plant should accordingly be self-compatible based on the model proposed by Williams *et al.* (1982) (Fig. 5.4). However none of the 19 initially grown 6*x* BC_1F_1 were self-compatible. Even if it is assumed that auto- and/or allosyndetic pairing is occurring in 8*x* H-435, self-compatible 6*x* BC_1F_1 would still have been expected. The absence of self-compatible plants from 6*x* BC_1F_1 progeny suggest that the model proposed by Williams *et al.* (1982) was not appropriate to explain the self-compatibility of 4*x* H-435. To explain the self-compatibility of 4*x* H-435 and 8*x* H-435 and the self-incompatibility of 6*x* BC_1F_1 plants, another possible explanation is proposed here. However, it is appropriate to list a number of assumptions before explaining the model.

1. Competitive interaction within a pollen grain occurs between alleles of the same species. Brewbaker and Keim (1953) also assumed that competitive allelic interaction was occurring among alleles of the same species (*T. repens*) in a hexaploid *T. nigrescens* (4*x*) x *T. repens* (8*x*) hybrid WN2. This assumption contrasts with that of Williams *et al.* (1982) who proposed that competitive interaction was occurring between alleles from different species.

2. (a) In a hybrid environment the "non-functional" site of the self-incompatibility alleles might become functional both in pollen and style and (b) interact competitively within the genomes of the same species and not with the alleles from different species. There is no direct precedent in the literature to suggest that a "non functional" self-incompatibility (*S*) locus can become functional in a hybrid. However, Pandey (1968) reported that the *S* gene is a complex locus controlling various aspects of reproductive physiology, and its effective functioning in the determination of compatibility behaviour depends on maintenace of the polygenic background in which it normally acts. He further argued that a hybrid or disturbed genetic background may impair the *S* gene action, and thus contribute to genetic sterility as well as to self-



Based on the model of Williams *et al.* (1982), I and II should be self-compatible. However none of the 6x BC_1F_1's were self-compatible.

Figure 5.4 Reciprocal backcrosses of 8x H-435 to self-incompatible *T. repens*.

compatibility. Liedl and Anderson (1993) reported that in *Lycopersicon* and *Arabidopsis* SC individuals have been shown to possess remnants of the SI system, which have been rendered inoperative. de Nattancourt (1977) described the various types of genetic changes e.g. the induction of polyploidy, mutations of the *S*-locus and the modifications in genetic background, all of which might lead to a state of self-incompatibility.

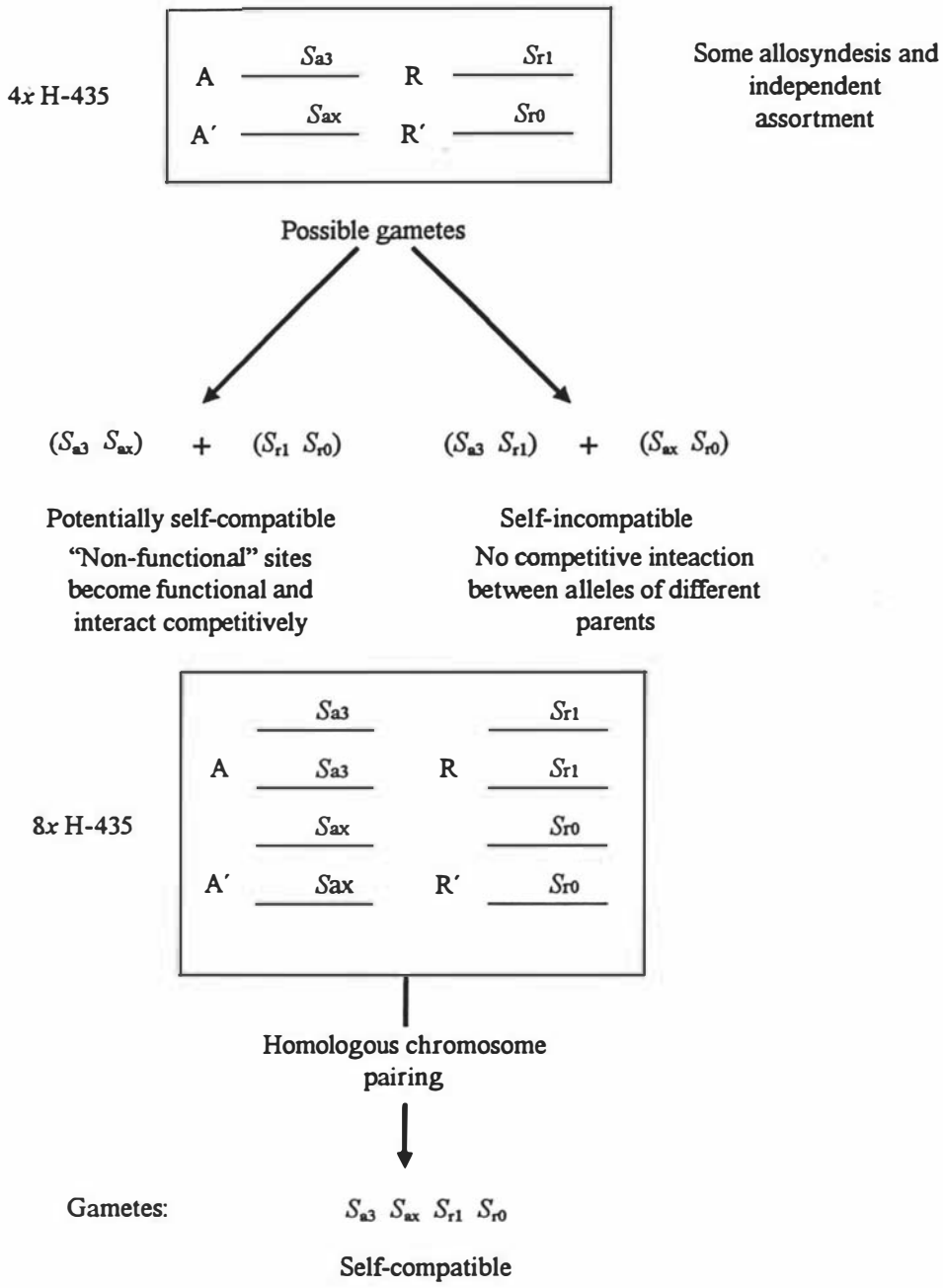
It is thus conceivable that *T. repens* and 4*x* *T. ambiguum* carry four copies of the *S*-locus, 2 of which are active (disomic inheritance) and two of which are non-functional (silent) but can be activated in some situations e.g. hybrid genetic background.

3. Autosyndesis might be a predominant phenomenon in 4*x* H-435, but some allosyndesis is required to bring self-compatibility to 4*x* H-435. In 8*x* H-435, pairing is assumed to be mostly between homologous chromosomes as each chromosome has a partner. However neither auto- nor allosyndesis would affect the self-incompatibility of 8*x* H-435.

Based on these assumptions, autosyndetic pairing in 4*x* H-435 (Fig. 5.2) would result only in self-incompatible pollen due to the absence of competitive interaction between the alleles of two different species. However, some allosyndetic pairing may allow the production of pollen grains that are self-compatible (Fig. 5.5).

Homologous chromosome pairing in 8*x* H-435 would presumably result in one type of gamete (Fig. 5.5). The "non-functional" sites may become active and, upon competitive interaction with alleles of the same species, might result in self-compatibility.

Meiotic configurations for 6*x* BC_1F_1 obtained from reciprocal backcrosses of 8*x* H-435 to *T. repens* were discussed in section 5.8. As reported by Anderson *et al.* (1991c) the four genomes derived from *T. repens* were believed to be pairing as homologues and the two genomes derived from *T. ambiguum* were pairing as homoeologues. Based on the proposed model as a possible explanation for self-compatibility in 4*x* H-435 and 8*x* H-435, all the 6*x* BC_1F_1 would presumably result in self-incompatible pollen (Fig. 5.6).



Competitive interaction between alleles of the same species. "Non-functional" sites become functional in a hybrid environment. The competitive interaction has to happen only in some pollen grains to get self-compatibility.

Figure 5.5 Model to explain self-compatibility in 4*x* H-435 and 8*x* H-435.

One out of six initially grown 6x BC_1F_2 (6x BC_1F_1 x 6x BC_1F_1 intercross) plants was self-compatible although derived from highly self-incompatible 6x BC_1F_1 parents. As mentioned earlier (section 5.8), the 6x BC_1F_1 progeny presumably showed homologous pairing between *T. repens* chromosomes and homoeologous pairing between *T. ambiguum* chromosomes, while multivalent formation represents both auto- and allosyndetic pairing. It is assumed that a limited extent of allosyndetic pairing in 6x BC_1F_1 plants would have resulted in recombination between the chromosomes of the two species, which in turn would have affected the genomic composition of 6x BC_1F_2 plants. This changed genomic composition in the intercrossed progeny could have caused one of the BC_1F_2 plants to produce some pollen grains with self-incompatibiltiy alleles of the same species. Competitive interaction among these alleles might be the likely cause of self-compatibility in this genotype. The assumptions listed to support the proposed model are at this stage speculative, and need further investigation.

Another explanation for the restoration of self-incompatibility in 6x BC_1F_1 plants which requires consideration is the segregation of modifier genes affecting the self-incompatibility locus. For example Townsend (1969) found in alsike clover (*T. hybridum*) the allele A_1 of the "*A*" locus which when present in heterozygous condition (A_1 A_2) and in certain genetic backgrounds, suppresses the action of a number of *S*-alleles. If it is assumed that self-compatibility in 4x and 8x H-435 was due to certain modifier genes affecting the *S*-locus, then it is possible that segregation of these modifiers or the appearance of another modifier allele from *T. repens* in backcrosses might have restored the self-incompatibility in 6x BC_1F_1 plants. As mentioned before, the *S* gene is a complex locus (Pandey, 1968) and it is possible that its expression might have been suppressed in the polygenic background of 4x and 8x H-435, but is capable of expression in the changed genetic environment of 6x BC_1F_1 plants. All these speculations, however, need further investigations.

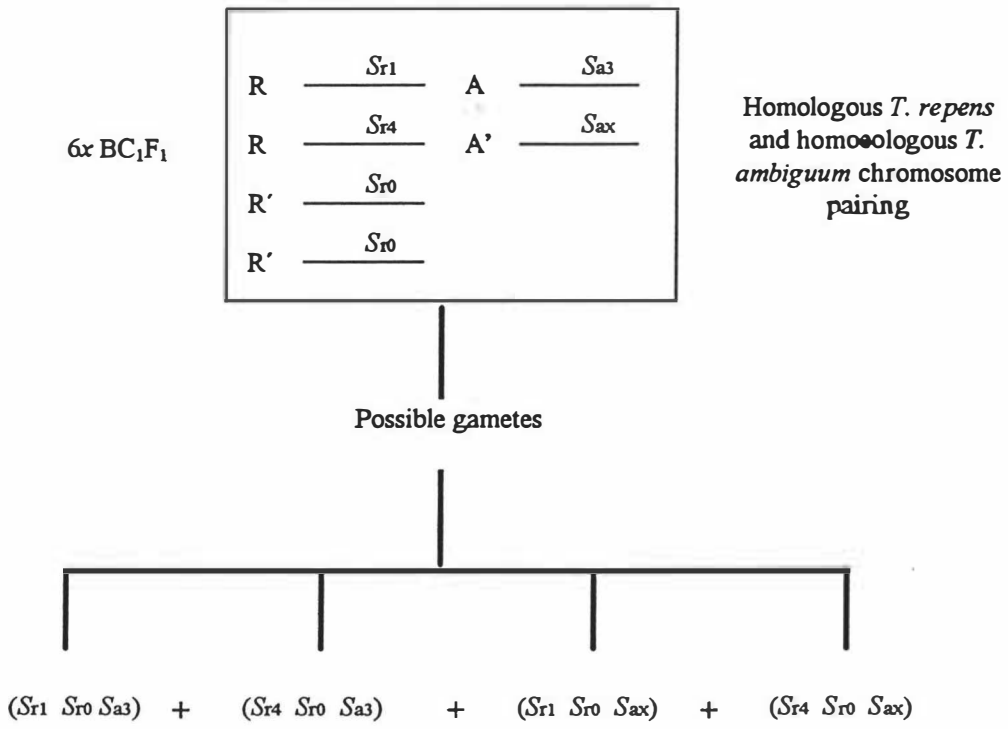


Figure 5.6 Possible explanation for self-incompatibility in 6*x* BC_1F_1 progeny based on the proposed model.

5.10 ENDOSPERM BALANCE NUMBER IN BACKCROSSES OF H-435 TO *T. repens* AND *T. ambiguum*

Evidence to support a 2:1 endosperm balance number (EBN) hypothesis in backcrosses of 6*x* H-6909-5 to *T. repens* and *T. nigrescens* has been already discussed in section 5.4.3. However backcrosses of 8*x* H-435 to *T. repens* do not necessarily support the 2:1 EBN hypothesis. Reciprocal backcrosses of 8*x* H-435 to *T. repens* were successful and approximately 9 seeds were obtained per 100 florets pollinated in both directions of the backcross (Table 4.7). An EBN=4 has been assigned to 4 genomes of *T. repens* (Parrott and Smith, 1986a, section 5.4.3.). 8*x* H-435 with four genomes of *T. repens* and four genomes of *T. ambiguum* should accordingly have an EBN greater than that of *T. repens*, due to the presence of four *T. ambiguum* genomes. Based on the 2:1 EBN hypothesis, successful crosses between two genotypes result when they share the same EBN. Therefore reciprocal backcrosses between 8*x* H-435 and *T. repens* would have not been so successful if a 2:1 EBN hypothesis was operating, unless *T. ambiguum* is assigned an EBN of zero.

However, some evidence obtained from the results of Anderson *et al.* (1991c) and the present investigation does suggest that a 2:1 EBN might be operating to determine the success and failure of backcrossed seed development. Anderson *et al.* (1991c) obtained 18 seeds from backcrosses of 4*x* H-435 to *T. repens*. Ten out of 18 (55%) of the BC_1F_1 were unexpectedly found to be hexaploid ($2n=6x=48$) and only 5 (27%) were tetrapolid ($2n=4x=32$). The remaining 18 % were near hexaploid aneuploids. Similarly when 4*x* H-435 was used as a female parent in crosses with 8*x* H-435 in the present experiment (Table 4.7), the two successfully grown plants from this cross were unexpectedly octoploids (the chromosome number was not fully confirmed but was very close to 64). On the other hand reciprocal backcrosses of 8*x* H-435 yielded an expected 6*x* BC_1F_1 progeny.

One of the important features of the 2:1 EBN requirement is that it can serve as a strong selective screen for the functioning of 2*n* gametes of the lower EBN species in inter EBN crosses (Johnston *et al.*, 1980; Parrott and Smith, 1986a). An unreduced (2*n*) gamete from a 2*x* plant is equivalent to the use of an *n* gamete from a 4*x* plant, as both such gametes would have the diploid (2*x*) complement of chromosomes. Therefore

the functioning of 2*n* gametes provides an alternative to chemical doubling of the chromosomes to overcome the EBN barriers (Parrott and Smith, 1986a). Based on this hypothesis, it can be assumed that 4*x* H-435 might have half the EBN of *T. repens* (i.e. an EBN=2).

The production of 55% hexaploid and 18% near hexaploid BC_1F_1 plants from the 4*x*-4*x* backcrosses reported by Anderson *et al.* (1991c) suggests that 2*n* gametes from the 4*x* H-435 female parent (presumably with an EBN=4) have functioned preferentially in this inter EBN cross. An EBN=2 assigned to 4*x* H-435 (with 2 genomes of *T. repens* and 2 genomes of *T. ambiguum*) is possible only if it is assumed that the two *T. repens* genomes might have suppressed the expression of the two *T. ambiguum* genomes in influencing the EBN of 4*x* H-435, or that *T. ambiguum* has an effective EBN=0. As mentioned earlier, chromosome doubling provides an alternative to functional 2*n* gametes to overcome the EBN barriers; therefore the 8*x* H-435 should have double the EBN (i.e. an EBN= 4) of the 4*x* H-435. This might be the reason for the success of reciprocal backcrosses of 8*x* H-435 to *T. repens*, as these crosses were presumably made at the same EBN level.

Intercrosses of 4*x* H-435 and 8*x* H-435 resulted in two successfully grown plants. The cross was apparently made at the 4*x* (2 EBN) x 8*x* (4EBN) level, but the resulting near octoploid level of these two plants suggested that 2*n* gametes from 4*x* H-435 have selectively functioned to bring the EBN of 4*x* H-435 to an equal level.

It seems difficult at this stage to assign an EBN of other than zero to *T. ambiguum* genomes. The great difficulty encountered in interspecific crosses of 4*x* and 6*x* *T. ambiguum* and *T. repens* (Williams and Verry, 1981; Yamada and Fukuoka, 1989) suggests that the species have a different EBN and it is conceivable that the explanation is that *T. ambiguum* has a 0 (zero) EBN.

Based on the assumption that *T. repens* genomes have suppressed the expression of *T. ambiguum* genomes in influencing the EBN of both 4*x* H-435 and 8*x* H-435 or that *T. ambiguum* has 0 EBN, the 6*x* BC_1F_1 (with presumably 4 genomes of *T. repens* and 2 genomes of *T. ambiguum*) would have an EBN=4. The second backcrossed progeny (5*x* BC_2F_1) was successfully obtained by backcrossing 6*x* BC_1F_1 to *T. repens*

(Table 4.7). Similarly 6*x* BC_1F_1 x 6*x* BC_1F_1 intercrosses were also successful (Table 4.7). In both cases, crosses were presumably made at the same EBN level and therefore give further support to the 2:1 EBN hypothesis in these crosses.

Further evidence for the 2:1 EBN hypothesis was obtained when 6*x* BC_1F_1 plants (EBN=4) were reciprocally crossed with 6*x* *T. ambiguum*. Although the cross was made at the same ploidy level (6*x*-6*x*), the complete failure of seed set may support the assumption of differences in EBN of the two genotypes. Interestingly when 6*x* BC_1F_1 were intercrossed, one out of six initially grown 6*x* BC_1F_2 plants produced 17 seeds after 318 pollinations from 6*x* *T. ambiguum*. As mentioned in section 5.7.3, the 6*x* BC_1F_2 plants might have a different genomic combination from 6*x* BC_1F_1, as a result of allosyndetic pairing and recombination. This might in turn, have resulted in the production of certain gametes that would have the same EBN as the gametes from 6*x* *T. ambiguum*. However this assumption needs further investigation.

It is emphasised at this stage that there has been no other case in the literature to suggest the suppression of the EBN factor i.e. this is a new idea, but is consistent with a genetic basis for EBN, and in this case, may involve dominant/recessive interactions. Without this assumption the EBN theory clearly does not apply to this material. On the other hand, the present results also fit the EBN model if an effective EBN of 0 (zero) is allocated to *T. ambiguum* genomes. One possibility is that *T. ambiguum* genomes have an EBN of 0 (zero) and no suppression has occurred at all.

5.11 MORPHOLOGICAL CHARACTERISTICS OF 6*x* BC_1F_1 PROGENY

The objective of measuring different morphological attributes of 6*x* BC_1F_1 genotypes in comparison to their parental species (*T. repens* and *T. ambiguum*) and 4*x* and 8*x* H-435 was to get an idea of the inheritance of different morphological characteristics of interest from the parental species. The main emphasis was to determine whether the 6*x* BC_1F_1 plants had combined the stoloniferous growth habit of *T. repens* with the rhizomatous growth habit of *T. ambiguum*.

The limitations of the experiment conducted for measuring these attributes were a short growth period of approximately three months, and limited space for growth as six vegetative cuttings were confined in a 42 x 30 x 5 cm plastic tray. The expression of most of the morphological characteristics may have been different if the plants had been allowed a longer growth period and more space. Such expression was observed in most of the 6*x* BC_1F_1 genotypes that had grown for more than one year in the glasshouse, as they developed much larger leaflets than *T. repens* and showed clear indication of rhizome formation.

Eight out of ten 6*x* BC_1F_1 plants showed the true stoloniferous growth habit of *T. repens* with very little expression of rhizomes (Table 4.10) The two 6*x* BC_1F_1 genotypes with apparently no true stolons produced significantly more rhizome-like growth from below the ground.

All the 6*x* BC_1F_1 plants exhibited great variation for leaflet length and width and stolon thickness. Larger leaflet length and stolon thickness were found to be positively correlated with herbage yield in white clover (Jahufer *et al.*, 1994). However as shown by Williams and Caradus (1979), this applies mainly to the first or second year growth. In later years, stolon number and density are more important. These variations among the 6*x* BC_1F_1's could be utilised as selection criteria for improving herbage yield in further studies.

Most of the 6*x* BC_1F_1's showed significantly higher number of nodules per plant than 4*x* *T. ambiguum* and 4*x* H-435, but only one was significantly higher than 8*x* H-435 in the number of nodules. However, it was not determined whether or not these nodules had effective *Rhizobium* strains. The presence of high numbers of nodules in 6*x* BC_1F_1 suggested that the character has the potential to be improved by backcrossing to *T. repens*.

In the present experiment a comparison of different morphological characteristics was made among 6*x* BC_1F_1 to both the parental species and 4*x* and 8*x* H-435. Later the 6*x* BC_1F_1 individuals were intercrossed and the resulting 6*x* BC_1F_2's were grown in the glasshouse. Most of these genotypes showed combined stoloniferous and rhizomatous growth with frequent nodal rooting and heavier nodulation than 6*x* BC_1F_1 plants (Fig.

4.18). This suggested that intermating of 6x BC_1F_1 plants had resulted in greater recombination between the parental chromosomes. Hanson (1959) determined that the break up of linkage blocks would be exceedingly slow with recurrent backcrossing, especially for blocks of short map distance, and suggested the use of at least one but preferably four sib-cross generations to disrupt linkage before backcrossing. Based on these proposals, it would be therefore desirable to intercross the most fertile individuals before the next backcrossing to the recurrent parent in order to get a greater frequency of recombinations.

CONCLUSIONS

The *in vitro* colchicine method reported by Anderson *et al.* (1991 b) was successfully applied to double the chromosome number of 3*x* H-6909-5. Fertility was restored in 6*x* H-6909-5 as a result of chromosome doubling. However the technique of *in vitro* colchicine application should be further refined to minimise the meristem mortality rate due to the bleaching effect of sodium hypochlorite and microbial contamination, to allow the recovery of a higher numbers of chromosome doubled plants.

T. nigrescens (Az 2225) was found to be a valuable source of germplasm to improve the resistance of *T. repens* to clover cyst nematode. Both the triploid and hexaploid H-6909-5 exhibited the same level of resistance to clover cyst nematode and their resistance did not differ from that of the resistant *T. nigrescens* plants. Although 6*x* H-6909-5 was markedly inferior to white clover agronomically, it provided useful genetic material for generating backcross progeny. These backcrosses need to be evaluated for agronomic characteristics in comparison to white clover, with special emphasis on clover cyst nematode resistance. Further investigation is needed to determine the genetic basis of resistance to clover cyst nematode in *T. nigrescens*, although results to date are consistent with a small number of dominant genes.

In the present investigations, two arrays of interspecific backcross progeny have been produced. In both arrays, hybridisation between *T. repens* and two other *Trifolium* species, *T. nigrescens* and *T. ambiguum*, which involved embryo rescue, produced initial hybrids of zero or near zero fertility. One achievement of the current research has been the development of these into highly fertile hybrids by subsequent manipulation of chromosome numbers, thus demonstrating that the initial sterility of the primary F_1 interspecific hybrids needs not be a barrier to successful interspecies breeding. A further major achievement has been the production of a range of hybrid plants combining agronomic characteristics of the parental species in varying genome balances, and at a range of ploidy levels. These plants represent a gene pool of potentially huge agricultural

significance as a possible answer to the world-wide search for truly perennial but vigorous clovers for sustainable pastoral systems.

The first array involved white clover (vigorous growth, high forage quality and nitrogen fixation, but poor drought tolerance and susceptibility to a range of pests and diseases) crossed with tetraploid caucasian clover (strongly perennial, rhizomatous, drought tolerant and virus resistant, but slow establishing, lacking cool season productivity and efficient nodulation). Beginning with an almost sterile tetraploid hybrid plant and a derived octoploid that was only a little more fertile, a range of fertile hybrids is now in existence at the 5*x*, 6*x* and 8*x* levels with varying balances of chromosome sets from the parental species. The creation of these backcross populations at different ploidy levels will enable the development of new clover types which are, for example, mostly like white clover but carrying some attributes (like the root system) from caucasian clover. Already some of the 6*x* backcross plants are showing combined stoloniferous and rhizomatous growth which may provide a vigorous but highly persistent legume for sustainable systems in difficult environments.

Although reported to be taxonomically related, *T. ambiguum* and *T. repens* are very difficult to cross. The very few hybrids so far reported between these two species were obtained only by embryo culture. This difficulty has been overcome in the present research by the creation of a "fertile bridge" between *T. ambiguum* and *T. repens*. In effect starting with a single 8*x* hybrid plant of low fertility, the way has now been opened for the breeding of new types of clovers. Characters of interest can now be transferred from *T. ambiguum* to *T. repens* by using the 6*x* BC_1F_2 plants as a "fertile bridge" without the use of sophisticated techniques like embryo culture with nurse endosperm or ovule culture.

The second hybrid population was derived from a completely sterile triploid hybrid between *T. repens* and an annual diploid species, *T. nigrescens*, carrying resistance to clover cyst nematode. The application of classical methodology and the doubling of chromosomes of triploid F_1 hybrid (3*x* H-6909-5) proved successful, and fertile hexaploids resulted. Subsequent backcrossing of the 6*x* H-6909-5 resulted in an array of hybrid plants from 3*x*, 4*x*, 5*x*, 6*x* and 7*x*, and despite starting from an initially

sterile hybrid plant, the way is now opened to develop a further set of unique new clover types of potential agricultural significance.

Aneuploids have been produced by inter-breeding the backcross progeny and from further backcrosses of 5*x* BC_1F_1 and 7*x* BC_1F_1 to *T. repens*. These confirmed aneuploids should provide useful genetic material for *in situ* DNA hybridisation to identify chromosomal exchange between *T. repens* and *T. nigrescens* and, potentially, association of specific characters with certain chromosomes.

The observations of meiotic configurations of 3*x* and 6*x* H-6909-5 and the first backcross progeny of 6*x* H-6909-5 to *T. repens* and *T. nigrescens* indicated considerable homology between the chromosomes of the two species. Although it was difficult to critically analyse meiotic configurations of the 6*x* BC_1F_1 progeny between 8*x* H-435 and *T. repens*, and thus determine the chromosomal relationships between *T. ambiguum* and *T. repens*, the observations of morphological characteristics of 6*x* BC_1F_1 and 6*x* BC_1F_2 indicated that genetic exchange between these two species is possible and that the two species must be closely related.

The failure and success of different crosses and backcrosses in the present research were assessed in relation to the "endosperm balance number" (EBN) hypothesis of Johnston *et al.* (1980). There is substantial evidence indicating that the 2:1 EBN concept might be applicable to different backcrosses, and be employed to determine the success or failure of a cross. However further investigations are needed on the genetic and physiological factors which control the EBN.

The current research reports for the first time the presence of functional 2*n* gametes in *T. repens*. This will allow the transferring of germplasm from tetraploid to at least hexaploid level in *T. repens* using 2*n* eggs. The frequency of 2*n* gametes in *T. repens* in the present investigation was very low, but the 2*n* gametes in white clover could be exploited by use of genotypes with different EBN as a selective screen for 2*n* gametes in intra- or interspecific crosses.

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APPENDIX 3.1

PLANT GROWING CONDITIONS

Seedlings and vegetative cuttings for clover cyst nematode resistance/ susceptibility screening were transplanted to 6.5 cm diameter plastic pots containing a 1:1 Manawatu silt loam:coarse river sand. The pH of the mixture was very close to 6.5. The mixture was heat-sterilised at about 85°C in a soil steriliser for one cycle of about 20 hours. Plants were placed in a glasshouse maintained at a temperature of 20±3 °C.

Plants in other experiments were grown in a potting mix containing 6:4 by volume Peat moss:washed coarse sand with the following fertilizer mix:

0.6 kg/m^3 Osmocote 3-4 months time release fertilizer (15:5.2:12.5 N:P:K);
2.4 kg/m^3 Osmocote 8-9 months time release fertiliser (18:4.8:8.3 N:P:K);
1.0 kg/m^3 Superphosphate
0.3 kg/m^3 Trace element mixture (12%Fe, 2.5% Mn, 1.0% Zn, 0.5% Cu, 0.1% Bo, 0.005% Mo, 15% S).

The pH of the potting mix was adjusted to 6.5 by the addition of lime.

Mean daily glasshouse temperature during summer was 22°C with a range of 18-26°C. Mean daily glasshouse temperature during winter was 19°C with a range of 16-22°C. All plants in the glasshouse were grown under natural day length.