Analysis of seed production traits in interspecific hybrids between *Trifolium repens* (white clover) and *Trifolium uniflorum*

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I dedicate this effort to my homeland

PAKISTAN

Analysis of seed production traits in interspecific hybrids between *Trifolium repens* (white clover) and *Trifolium uniflorum*

Trifolium repens L. (white clover) is an important component of New Zealand's pastoral and herbage seed industries. It has delicate shallow roots vulnerable to drought and pest attack. *T. uniflorum* is a wild species, adapted to dry environments, with deep woody roots but poor vegetative growth and only 1-3 florets per head. Hybridization was done to incorporate the drought tolerance and root characteristics of *T. uniflorum* into white clover. The primary hybrids (F_1 and BC_1F_1) showed poor seed production. The project aimed to analyse this problem and improve seed production by further hybridization and selection.

The hybrids expressed high variation for almost all seed production traits. Heads per plant, florets per head and seeds per floret were important determinants with moderate to high heritabilities. The derived traits seeds per head, florets per plant and seeds per plant expressed low-moderate to moderate heritabilities. Flowering pattern showed high heritability. No negative correlations between seed production and root traits were found. Whereas nodes per stolon favoured head production, stolon production had a negative effect on nodes per stolon. No negative associations between head production, persistence and foliage production of the hybrids were observed. No cytoplasmic influences were evident.

The hybrids showed, higher self fertility and a wider gap between potential and actual seed yield than white clover. The BC_1F_1 generation produced low numbers of heads per plant and seeds per floret. Intercrossing coupled with recurrent selection improved variation, fertility and realization of potential seed yield. The second backcrossed and intercrossed generations had high seeds per floret and plant. Backcrossing of selected hybrids effectively recovered the white clover phenotype and created new variation. The BC_2F_1 and BC_3F_1 generations expressed the better head production ability of *T. uniflorum* combined with the bigger head size of white clover.

Seed production capability of the hybrids was successfully restored. More genetic variation was created in the hybrids for future breeding and selection endeavours.

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Abbreviations and terminologies

The abbreviations and terminologies used are defined and explained below.

F_1: Filial 1 generation, produced by hybridization between white clover and *T. uniflorum*.

BC_1F_1: First backcrossed generation, produced by crossing between white clover and F_1.

BC_2F_1: Second backcrossed generation, produced by crossing between white clover and BC_1F_1.

BC_3F_1: Third backcrossed generation, produced by crossing between white clover and BC_2F_1.

BC_1F_2: Second intercrossed generation, produced by crossing among BC_1F_1 hybrids.

BC_1F_3: Third intercrossed generation, produced by crossing among BC_1F_2 hybrids.

BC_2F_2-BI: Second backcrossed and intercrossed generation. The BC_1F_1 hybrids were backcrossed with white clover to produce BC_2F_1 progenies. In the subsequent year, crosses were made among these BC_2F_1 hybrids to produce BC_2F_2 hybrids.

BC_2F_2-IB: Second intercrossed and backcrossed generations. Crosses were made among BC_1F_1 hybrids to produce BC_1F_2 progenies. In the subsequent year, these BC_1F_2 hybrids were backcrossed with white clover to produce BC_2F_2.

Interspecific hybridization: A type of wide hybridization in which plants representing two different species are crossed to produce F_1 hybrids.

Backcrossing: Crossing of a hybrid with one of its parents (recurrent parent). In this study, the recurrent parent was always white clover. The backcrossed nature of the hybrids is represented by the abbreviation “BC”. For example, the F_1 hybrids between white clover and *T. uniflorum* were backcrossed with white clover to produce BC_1F_1 hybrids.

Intercrossing: A synonym of cross breeding used for crossing between different breeds, varieties, or species. In the current study, this term was used for crossing of the hybrids with each other. The letter “F” in the name of a generation is used to show intercrossing status of the hybrid. For example, the BC_1F_1 hybrids were intercrossed among themselves to produce BC_1F_2 generation. The pollination of a plant with its own pollens was described as selfing.

Recurrent selection: The process of selection for particular traits repeated after each cycle of hybridization, in order to bring improvement and stability in the traits. The target in the current study was improvement in seed production traits.

Family: In this study, the term “family” was used to denote a group of plants having at least one identifiable common parent. For example, all plants belonging to the ‘family A’ were the progeny of plants having white clover cultivar ‘Aran’ as their recurrent parent.

System of naming the families: Families of generation BC_1F_1, BC_2F_1 and BC_1F_2 are named after the white clover cultivar used as the recurrent parent in hybridization with the F_1 in the development of these generations. For example, in the BC_1F_1 generation, plants A_1 and A_2 are members of same family (family A) as they had cultivar "Aran" involved as recurrent parent in their production. The BC_1F_2 plants produced by intercrossing of A_1 and A_2 were named as family A. Families of BC_3F_1 and BC_1F_3 had unique parental compositions and represented by their unique family codes.

Generation: A group of families at the same stage of a breeding strategy belonged to the same generation. For example, families A and B of generation BC_1F_1 were products of the first backcrossing of F_1 hybrids with white clover cultivars Aran and Barblanca, respectively.

Direction of a cross: Every individual cross was set out as "female parent x male parent". For example, "white clover x *T. uniflorum*" indicates that white clover was used as the female parent and *T. uniflorum* was used as the male parent.

Chapter 1

INTRODUCTION

1.1 BACKGROUND

1.1.1 General background

Trifolium repens (white clover) is an important component of temperate pastures worldwide. For New Zealand, it has dual importance. Firstly, it supports the country's livestock based economy (Woodfield and Caradus, 1996). Secondly, New Zealand is one of the world's major exporters of white clover seed (Mather et al., 1996). Continuous research in cultivar improvement can sustain the competitive edge and increase national and international usage of this important pasture species (Rolston et al., 1990). White clover is adapted to temperate climates, but has a delicate shallow root system making it susceptible to drought (Caradus and Woodfield, 1998). Improvement in the root traits and drought tolerance of white clover are not only expected to make the livestock based economy less vulnerable to dry climatic spells, it can also expand cultivation of this highly valuable pasture component to marginal areas.

The white clover gene pool is deficient in drought tolerance. However, its wild relatives can be a potential source to improve this deficiency (Williams et al., 2007). *T. uniflorum* is a perennial wild species, adapted to a dry climate that has traits absent from white clover, including a deep woody tap root system, pest resistance and drought tolerance (Plate 1.1). Interspecific hybridization between white clover and *T. uniflorum* has the potential to transfer these desired traits from *T. uniflorum* into white clover (Williams, 1987a). This hybridization is feasible as both species have the same chromosome number and produce fertile hybrids with the aid of embryo rescue techniques. There are evidences of chromosomal pairing and crossing over, giving chances of genetic exchange between these two species and creation of new and useful genetic variations (Williams and Nichols, 2011).

Interspecific hybridization involves mixing of two differently evolved genome complexes; hence it has the potential to create a wide range of genetic variation. The co-evolved allelic combinations in each species are expected to be disrupted during recombination of the two genotypes (Stephens, 1949). Specific breeding strategies are

White clover **BC_1F_1 hybrid** ***T. uniflorum***

a

b

c

Plate 1.1. Comparison of shoots (a), roots (b) and inflorescences (c) of white clover, BC_1F_1 hybrid and *T. uniflorum*.

required to break undesired linkages and manage newly created variation in a desired direction. Backcrossing improves the genomic content of the desired genotype by using it as recurrent parent in repeated crossing cycles. Intercrossing breaks the undesired linkage within parental genomes and creates new genomic combinations. The use of selected desired parents in each breeding cycle speeds up the emergence of required phenotypes (Williams and Hussain, 2008).

1.1.2 Specific background

Interspecific crosses were made during 2005-06 between white clover and *T. uniflorum* at AgResearch Grasslands, Palmerston North. Hybrids were produced with the aid of the embryo rescue technique. The aim was to incorporate the better root, drought and pest tolerance characteristics of *T. uniflorum* into the genome of white clover. Five different white clover cultivars were crossed with three accessions of *T. uniflorum* to create F_1 interspecific hybrids. The backcrossing process was adopted to eliminate the undesired traits, including the poor seed production inherited from *T. uniflorum*. Additional white clover cultivars were used as recurrent parents to create BC_1F_1 hybrids. Based on different white clover recurrent parents, nine families of these BC_1F_1 hybrids were planted at Lincoln during September 2007. These hybrids showed significant variation for desired agronomic traits. However, they were extremely poor in seed production which was a potential hurdle for commercial success and wider usage of this improved product. Plants showing better agronomic characteristics were selected in 2008 and used as the base population in this study at AgResearch Grasslands, Palmerston North, New Zealand.

1.2 IMPORTANCE OF THE STUDY

This project has academic as well as applied aspects. Wide hybridization is a promising research area for a wide genus like *Trifolium* (Williams et al., 2007). This study is about an interspecific hybridization with a wild donor species, i.e. *T. uniflorum*, used to improve the highly domesticated species white clover. Interspecific incompatibility was faced only in the production of F_1 hybrids and a diverse range of fertile advanced hybrid generations were developed. The existence of high variation at each generation level, despite selection pressure, showed retention of traits contributed by the donor parental species. The aim of the study was to improve the composite quantitative trait of seed production. The perennial and plastic nature of the plant, the intermittent flowering pattern and usage of an alternative way of propagation by stolons, makes this characteristic very complex in white clover (Thomas, 1987; Marshall et al., 1995). The contrasting seed production trends of the parental species made handling this trait in their hybrids more interesting as well as challenging. This study was expected to contribute to the understanding of issues ranging from genomic compatibility of the species to suitable breeding designs for a complex trait. There are very few examples where interspecific hybrids between two very distant species have been studied with such a large population, at diverse generation levels and for a complex trait.

From an applied point of view, drought, low soil fertility of marginal areas and variable seed production are important issues in white clover worldwide. The interspecific hybrids between white clover and *T. uniflorum* have shown improved tolerance to drought and low soil infertility relative to white clover (Nichols, 2012). However, low seed production of these hybrids could restrict their wide distribution and commercial success due to unavailability of sufficient seed at reasonable cost (Jahufer and Gawler, 2000; Marshall et al., 2008). Moreover, white clover seed is a prominent export commodity of New Zealand. An improved plant variety, especially with drought and soil infertility tolerance, can broaden the market range as well as sustaining the competitive edge. With inefficient seed production, these highly valuable hybrids would not have a fair chance of dissemination and utilization.

This study was an effort to understand the dynamics of seed production in an interspecific hybrid background and improve the seed production of these hybrids by selection and breeding.

1.3 HYPOTHESIS AND OBJECTIVES

The underlying assumption for this study was that the hybrids have inherited poor seed production from the *T. uniflorum* parent, and the hypothesis to address this issue was that the seed production can be improved by recurrent selection after backcrossing of the hybrids with white clover and intercrossing among the hybrids to break any association between desired agronomic traits and poor seed production. A wide range of hybrid generations of varying genomic composition was therefore required to study the dynamics of seed production of these interspecific hybrids.

The overall aim of this study was to recover the seed production ability of these hybrids to make them commercially viable and usable in general as well as in marginal areas with regular dry periods.

The objectives of this study were to analyze:

1. Sources and levels of variation expressed by the hybrids.
2. Fertility and seed production of the hybrids.
3. Inheritance of seed production traits under interspecific hybrid background.
4. Effects of inbreeding on seed production traits of the hybrids.
5. Effects of different breeding strategies on the improvement of seed production traits of the hybrids.
6. Associations among traits with special focus on seed production components.

1.4 METHODOLOGY OF THE STUDY

Two breeding strategies, backcrossing and intercrossing, were adopted to analyse the seed production potential of the hybrids. Recurrent selection pressure was applied to speed up the recovery process and to keep the population size manageable.

The backcrossing process was used to maintain the elite agronomic traits in the hybrids by increasing the genomic proportion of the agronomically better parent white clover, in them. Backcrossing of BC_1F_1 hybrid with white clover as recurrent parent theoretically reduced the genomic proportion of *T. uniflorum* (donor parent) to 12.5% in BC_2F_1 progenies (Figure 1.1). Backcrossing of BC_2F_1 hybrids further reduced this proportion to 6.25% in BC_3F_1 progenies. Intercrossing among hybrids was aimed to reshuffle the genomes of the two parental species to create new desired combinations. Intercrossing among BC_1F_1 hybrids produced BC_1F_2 and BC_1F_3 generations from the first and second cycles of intercrossing, respectively. All of these hybrids retained the same theoretical proportion of *T. uniflorum* i.e., 25%. A blend of two breeding strategies, (a) backcrossing of BC_1F_1 hybrids and then intercrossing of their progenies and (b) intercrossing among BC_1F_1 hybrids and then backcrossing of their progenies, was used to create two types of BC_2F_2 generations, i.e. BC_2F_2-BI and BC_2F_2-IB, respectively. Theoretically both of these generations would have the same parental genomic proportions. The difference is that BC_2F_2-BI has first improved genomic proportion of white clover and then reshuffled the parental species genomes in the next breeding cycle, whereas, the BC_2F_2-IB has first reshuffled the parental genomes and then improved the proportion of white clover in the second breeding cycle.

Theoretically backcrossing was expected to cause a rapid shift of the hybrid population towards the white clover parent (Figures 1.1, 1.2). The intercrossing process was anticipated to increase the variation in the hybrid population. The recurrent selection at each breeding cycle was aimed to speed up the recovery of desired traits.

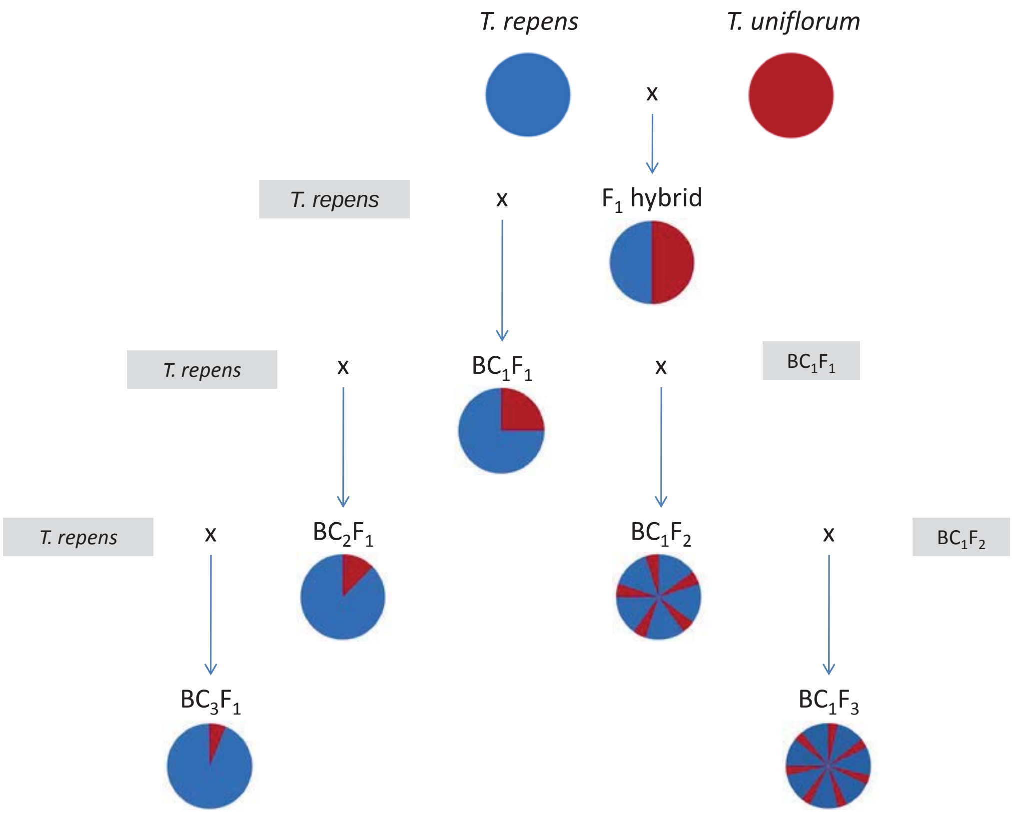


Figure 1.1. General pattern of genomic inheritance. Colours represent the genomes of each species.

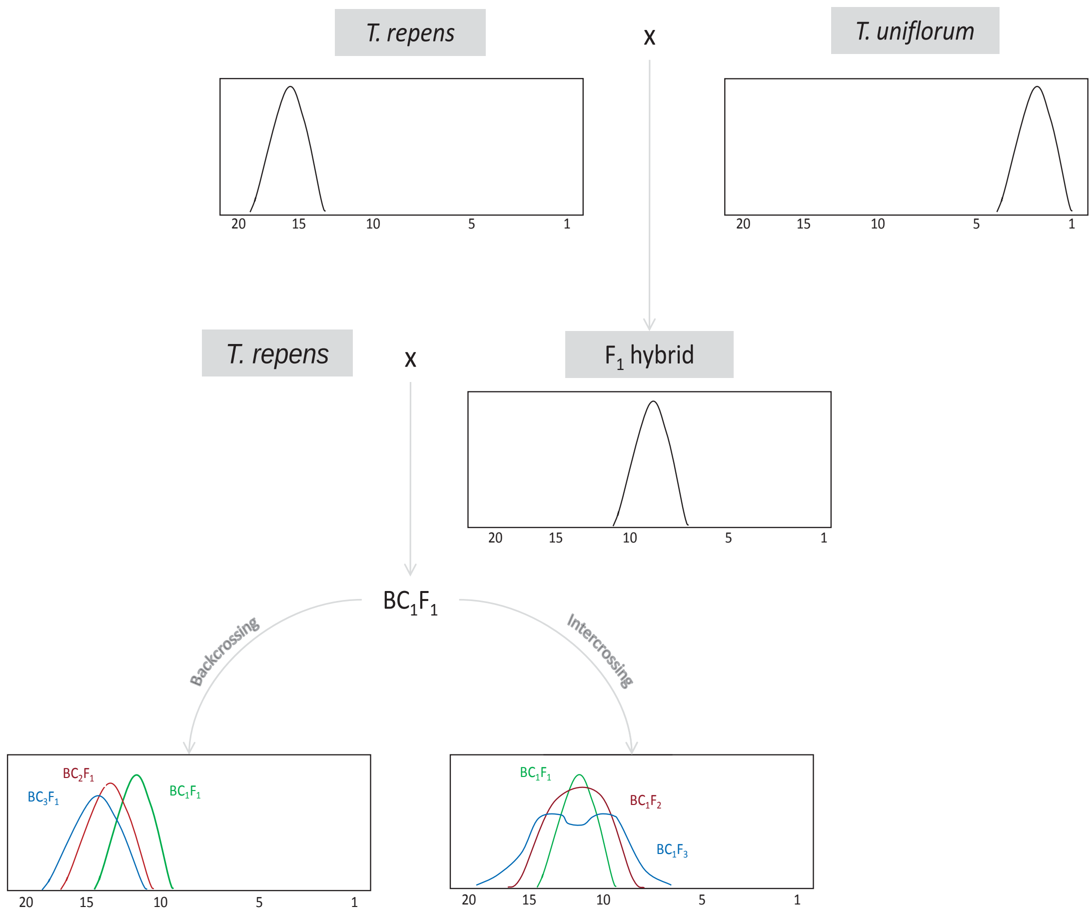


Figure 1.2. General schematic shapes of population distributions for large independently mating populations in the absence of selection.

1.5 PHASES OF THE STUDY

A schematic, workflow chart showing generations involved in different phases of the study is given in Figure 1.3. Diagrammatic representation of important developmental stages of the project is shown in Appendix 1. The project started in November 2008 with planting the cuttings of nine BC_1F_1 hybrid families under glasshouse conditions in experiment-1. This was a base-population study to explore the variation present in the BC_1F_1 generation for successive analyses. Superior genotypes were selected and second backcross and intercross generations were created at the end of this experiment. During the subsequent season, different aspects of seed production were studied in experiment-2 under field conditions (in pots) with seventy five families belonging to two generations i.e., BC_2F_1 and BC_1F_2. Advanced hybrid generations were created by both backcrossing and intercrossing of selected parents at the end of this experiment. In the third season there were 81 families representing eight different generations with broad genetic backgrounds. The material in experiment-3 provided an opportunity to study the effects of different breeding strategies with a wide range of hybrid generations.

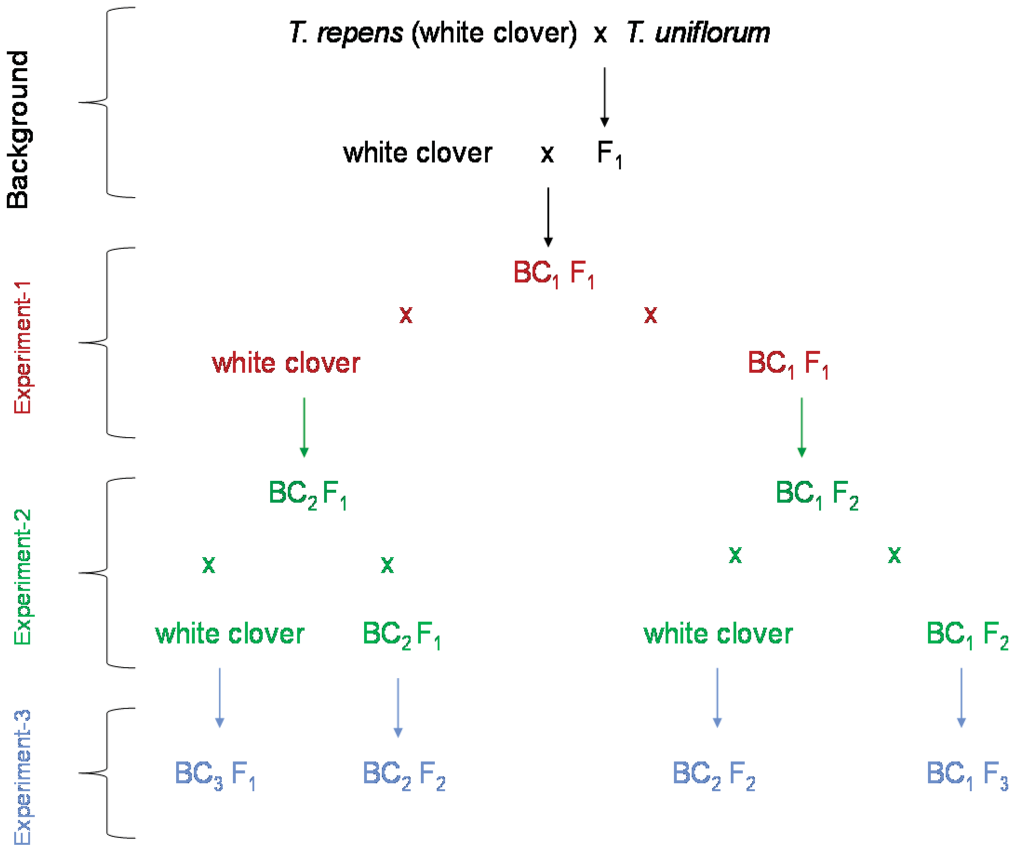


Figure 1.3. Crossing scheme used in this study.

The whole project was a consistent progress towards understanding and improving seed production traits of the interspecific hybrids. In each experiment, new hypotheses were added, and those made during earlier experiments were retained for study with wider diversity. The results mostly remained consistent across the experiments, which made it possible to later bring the results together and discuss them comprehensively under broad headings. The outline of the thesis is shown in Figure 1.4.

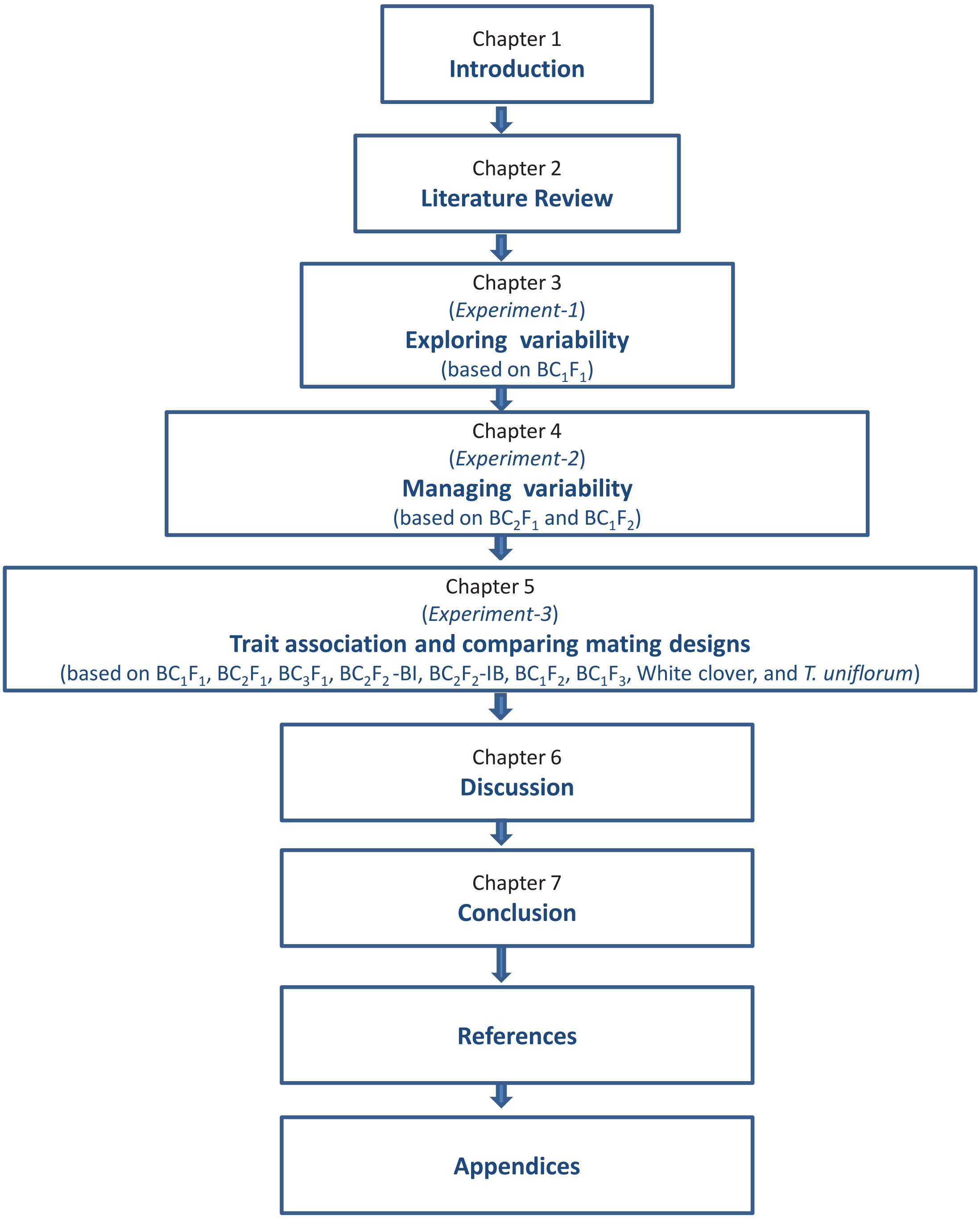


Figure 1.4. Outline of the thesis structure.

Chapter 2

LITERATURE REVIEW

2.1 THE NEW ZEALAND SEED INDUSTRY

According to the Ministry of Agriculture and Forestry (2008), seed production started in New Zealand during the 1800s with the introduction and multiplication of English ryegrass. Subsequently, through the development of local ecotypes and breeding programs it has become a well reputed export industry. Data from Statistics New Zealand (2012) show that vegetable seed business has gradually emerged as a major source of export earning followed by seeds of rye grass and clover (Figure 2.1). Export trends are improving for vegetable and rye grass seeds but are stagnant or declining for clover seeds.

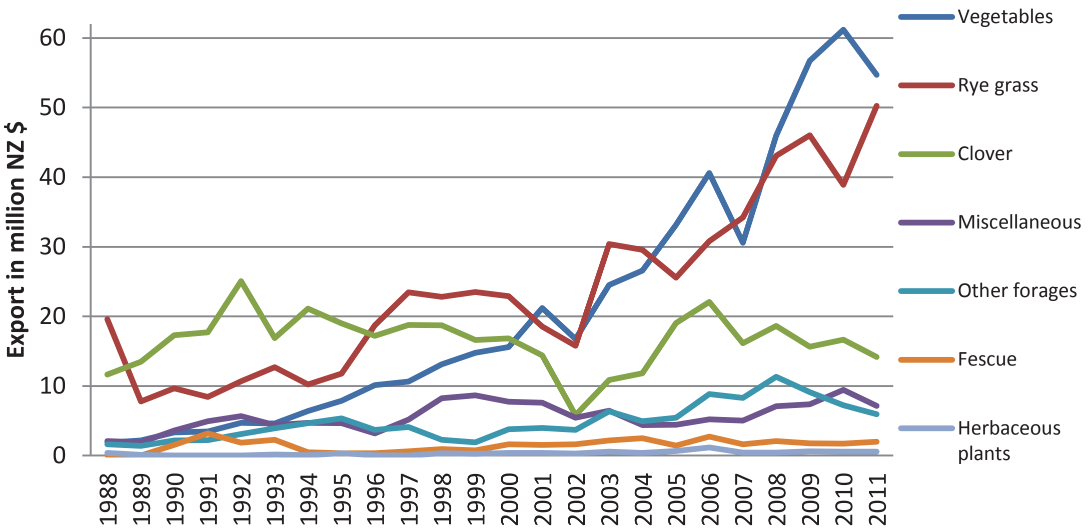


Figure 2.1. Seed export earnings of New Zealand from 1988 to 2011 (Statistics NZ, 2012). The category "miscellaneous" represents export of beet seeds and seeds of other plant species not elsewhere classified. The category "other forages" carries seed export data of plants having small volumes like that of lucerne and timothy grass or of plants not mentioned individually in these seed export statistics. This data does not include leguminous vegetables, cereals and oil seeds.

The herbage seed sector is a prominent component of New Zealand's overall seed production. Rowarth et al. (1998) reviewed the evolution and importance of the New Zealand herbage seed industry. Herbage seeds are mainly produced along with grains, vegetables and livestock on arable farms in the Canterbury region (Figure 2.2). This sector is not only a source of export earnings but also provides green pastures supporting sports, leisure, agriculture and tourism. Herbage seed production has gradually evolved into an established industry with developed infrastructure and specialized growers supported by high profile research and a favourable climate. New Zealand herbage industry has to face multifaceted challenges like contamination of seed lots by weed seeds, difficulty in maintaining varietal purity due to a rise in numbers of cultivars, climatic uncertainty and price competition due to globalization. The key for future survival of this industry is production of high quality seed at low cost.

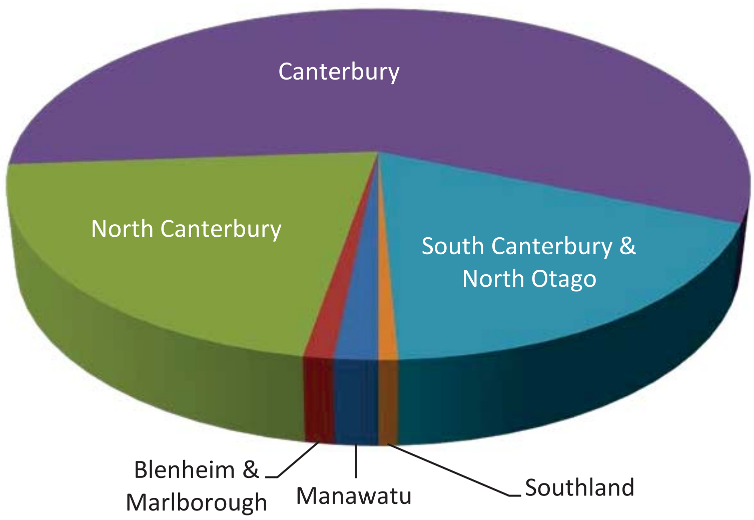


Figure 2.2. Percentage shares of different areas in New Zealand herbage seed production (adopted from Rowarth et al., 1998).

Pyke et al. (2004) analysed the performance of and challenges faced by the New Zealand herbage seed industry. The seed sector relies on temperate grasses and legumes having perennial rye grass (*Lolium perenne*), Italian ryegrass (*Lolium multiflorum*), tall fescue (*Festuca arundinacea*) and white clover (*Trifolium repens*) as the predominant species. Despite a decrease in area, herbage seed production has remained sustainable due to increase in per hectare seed yields by improved management practices, application of plant growth regulators and better genetic resources. The introduction of Plant Variety Rights (PVR) encourages production of proprietary cultivars. The availabilities of a favourable climate, expertise and stringent certification standards have facilitated multiplication of overseas cultivars in New Zealand, especially of white clover. Innovations like the introduction of novel endophytes in ryegrass has given substantial support to the herbage seed industry. The industry faces challenges like competition for land use with low risk and highly profitable sectors, such as livestock production and vegetable seed production. Management of water resources, especially in Canterbury, the main herbage seed production area, and competition with international competitors are also challenges. The New Zealand herbage seed industry can sustain its position by improving crop management practices, increasing seed yield potential of the crops and by adding identifiable value to the crops.

2.1.1 White clover seed

New Zealand is the world's major exporter of white clover seed. This export has been to more than 40 countries with Australia, USA and European countries being the major markets. According to the Statistics New Zealand (2012) clover is an important contributor to seed export earnings (Figure 2.3). A dominant fraction (98%) of clover export earnings was contributed by white clover. New Zealand exported 2524.68 metric tons (Mt) of white clover seed worth NZ$13.80 m during 2011. Most (96%) of the white clover seed was certified (Figure 2.4). The variety Grasslands Huia has remained a prominent contributor to white clover seed exports. Mather et al. (1996) reported that the world consumes 9000–10,000 Mt of white clover seed annually. Western Europe and USA are the main consuming regions. New Zealand has an edge in research, production and marketing of white clover seed. This dominance could be maintained by breeding new cultivars capable of meeting future demands and ensuring consistent and reliable seed supply. Along with other measures, research is required to improve seed yield at low cost (Rolston et al., 1990).

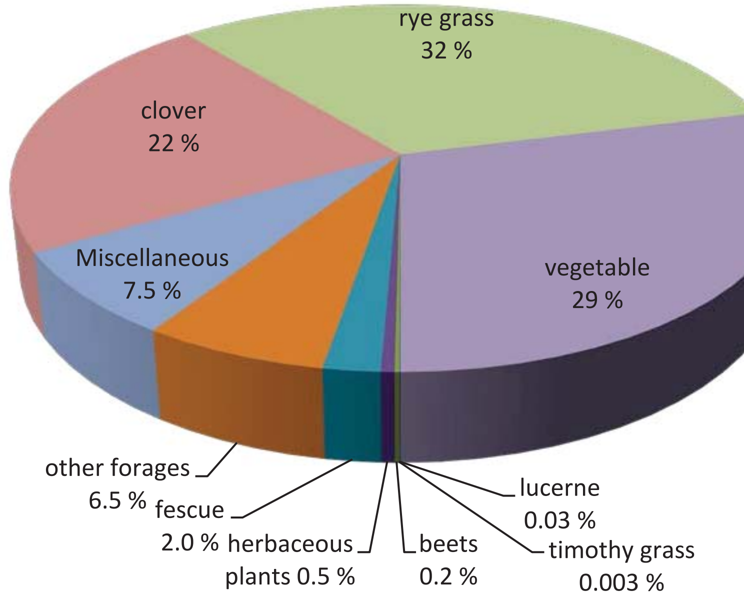


Figure 2.3. Share of different plant species in New Zealand seed export earnings from 1988 to 2011.

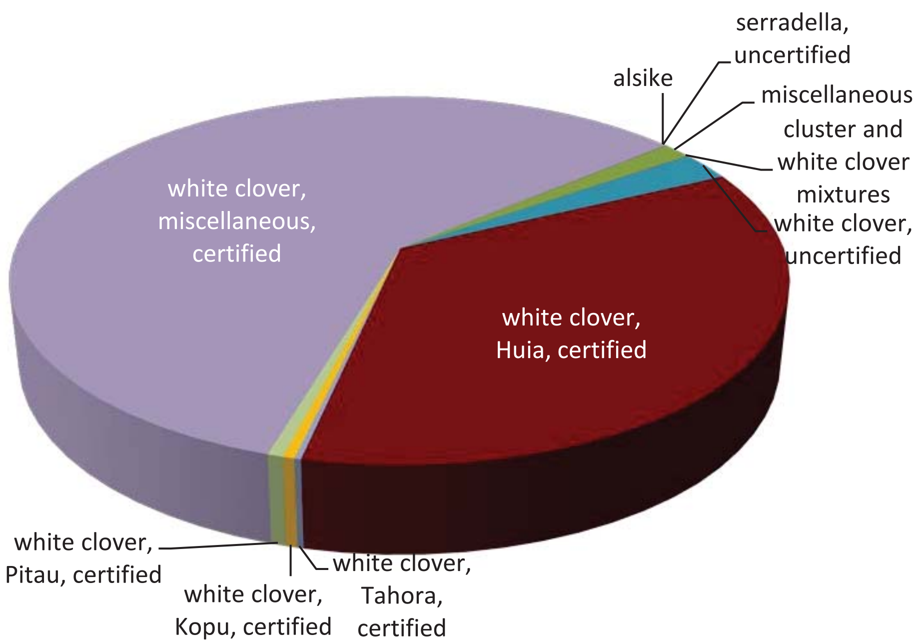


Figure 2.4. Shares of different clover types in New Zealand seed export earnings from 1988 to 2011.

Rolston and Clifford (1989) showed that from the mid-1940s to 1989 the area under seed production of white clover had reduced from 18,000 hectares to 15,000 hectares. However, the national average seed yield increased from 80 kg/hectare to 230 kg/ hectare due to effective in research seed production. Reduction in white cover seed production area has been successfully compensated by research causing an increase in yield per unit area. However, it appeared that New Zealand’s white clover seed production was declining at the rate of 168 tons per year. The viability of the New Zealand herbage seed industry is crucial for the delivery to farmers of improved traits, either of a direct (in genetics of plant) or indirect (endophyte, rhizobia) nature.

Woodfield and Caradus (1994) evaluated a collection of 110 white clover ecotypes and cultivars originating from 24 countries, representing more than six decades of plant improvement. They found a 6% genetic gain per decade for clover herbage yield and clover content. However, no significant difference was found for the number of flower heads per square meter. This shows that improvement in white clover had occurred without reducing flower head production. This also indicates that there has probably been less focus on improvement of seed production traits.

High seed production potential is a very important factor in commercial success and survival of a variety (Marshall et al., 1995; Abberton and Marshall, 2005). It ensures appropriate seed supply and production costs. Inadequate seed yield of *T. ambiguum* is one of the major hindrances to the commercial success of this persistent, high quality forage, and drought and pest resistant clover species (Williams and Nichols, 2011). Low seed production of many white clover cultivars like Tillman (USA), Siral (Australia) and many European cultivars has resulted in their commercial failure (Woodfield et al., 2004). Ayres et al. (2002) described the need for development of ‘NuSiral’ from ‘Siral’ white clover. ‘Siral’ was developed from an introduction from Algeria as a variety with broad adaptation, strong winter growth in mild climates and efficient recovery of vegetative growth after summer-autumn moisture stress. However, it lacked a distinct and intense flowering phase and had a tendency to retain the leafy phase during the reproductive period. This resulted in poor seed production, which made it commercially unviable. It required three additional cycles of selection on a phenotypic basis, primarily for increased seed yield and disease resistance to create the commercially viable ‘Grasslands Nusiral’ cultivar. Seed production attributes should be focused on during the early stages of cultivar development for commercial success (Jahufer and Gawler, 2000).

White clover seed production is an output of complex interactions among a wide range of environmental and genetic components (Thomas, 1987). There is a substantial gap between potential and actual seed yield (Pasumarty et al., 1993a). Moreover, adaptation and usage of white clover can be increased by improving its gene pool through wide hybridization with its wild relatives (Williams and Nichols, 2011). New Zealand's edge in white clover seed production can be maintained or enhanced by improving seed yield per unit area and producing improved cultivars.

2.2 WHITE CLOVER SEED PRODUCTION

Zohary and Heller (1984) described most species of *Trifolium*, including white clover, *T. uniflorum*, as allogamous (cross pollinated) having insects as the main pollinators. White clovers can propagate sexually by seeds and vegetatively by stolons (Williams, 1987b). Thomas (1980, 1987) explained reproductive development of white clover. The production of floral primordia initiates at terminal apical meristems of elongating lateral stolons. Only lateral and axillary positions of the stolon apex produce floral primordia. Each new inflorescence emerges a few days after the appearance of its subtending leaf. Low temperatures of autumn, winter and/or spring are the most influential factors for floral initiation for most cultivars studied in New Zealand. Long days with high temperature also increase inflorescence initiation for many white clover cultivars. Observations based on 'Grasslands Huia' under Palmerston North conditions, showed that inflorescences emerged out of the stipular sheath after seven or eight leaf primordia had formed at the same stem apex. Adaptation to different latitudes also influences flowering. Cultivars from low latitudes e.g., Tamar, Spanish and Louisiana ecotypes, categorized as 'Mediterranean', initiated flowering in response to cool conditions and terminated it with the start of warm days. By contrast, cultivars from high latitudes e.g., Kent wild white, Ladino and Russian lines, categorized as 'summer growing, from high Latitude; did not initiate flowering in response to cool conditions until June or July. This initiation trend extended until December and peaked during November and December, with no further progress in January. These genotypes are called short-long-days plants, for responding positively to both short and long days for floral initiation. Favourable environmental conditions for flowering promote reproductive bud formation. The ratio of flowering to non-flowering nodes is dependent on genotype as well as environment. The developmental sequence of floral parts is sepals, stamens, petals and lastly the carpel. An inflorescence, normally consisting of 20-40 white or pinkish white florets arranged in a raceme, is born on a peduncle originating from the axil of a leaf. The peduncle is usually longer then the adjacent leaf petiole. Each floret is a typical papilionaceous flower. The calyx consists of five sepals. It encircles the corolla of five petals identified as a larger posterior petal, the vexillum (standard petal) enclosing two lateral wing petals (alae) which further surround two anterior keel petals. The white clover flower is hermaphrodite with 10 stamens and a single carpel. Nectary is located at the base of the staminal tube. Anthers dehisce before full elongation of the petals. Flowers are self-incompatible or pseudo-self-compatible and they are cross pollinated mainly by bees. Seeds become mature after 4-6 weeks from pollination. Mature seed is small in size i.e., 1 mm wide and 0.5 to 0.7 mm thick, cordate (heart) shape and yellow to brown in color. A significant proportion of seed has an impermeable seed coat (hard seed) and can survive in the soil for many years.

Seed production of white clover is highly variable and often low (Marshall, 1995). However, considerable natural variation exists in white clover for this trait. Jahufer and Gawler (2000) evaluated 36 wild accessions, collected from Morocco and Tunisia, along with three varieties and 14 commercial cultivars of white clover for seed yield components in Australia. The populations showed significant variation for all seed components. They found strong phenotypic and genotypic correlations between the total number of ripe inflorescences and potential seed yield. Florets per ripe inflorescence were also moderately associated with potential seed yield. Finne et al. (2000) studied vegetative and seed yield traits of eleven Norwegian white clover cultivars. The genotypic coefficient of variation for seed yield was considerably higher than those of the studied vegetative traits. This suggested that reproductive characters are genetically more variable than vegetative attributes. Except ‘Milkanova’, all cultivars showed significant within population genotypic variation for all traits. However, even this cultivar possessed significant ($P < 0.001$) genotypic variation within a population for seed yield along with winter survival and spring growth. The test of heterogeneity of genotypic variance showed that seed yield was the most variable trait within the populations. Williams et al. (1998) studied a wide range of commercial varieties of different leaf sizes under field conditions of UK. They observed significant variation for various seed yield components.

New Zealand has an edge in knowledge, skill and the favourable environmental conditions in the Canterbury area for white clover seed production. According to Woodfield et al. (2004), in New Zealand white clover inflorescence density regularly exceeds 800 inflorescences per square meter. The 1000 seeds weight of 0.7 gram (g) and seed yield of 0.25g per inflorescence is achievable. A conservative estimate for potential seed yield is 2000 kg/ha, whereas, the best commercial cultivar averages around 1000 kg/ha. The researchers selected high seed producing plants from a range of pre-released cultivars at Lincoln, New Zealand. These cultivars were polycrossed to produce F_2. This F_2 population was screened to isolate 4 groups of plants i.e., High inflorescence density (HID), Low inflorescence density (LID), High seed yield per inflorescence (HYI) and Low seed yield per inflorescence (LYI). The groups were selected such that the HID and LID had a four-fold difference of seed yield and inflorescence density but were not different from each other for seed yield per inflorescence. Similarly, the HYI and LYI had four-fold differences between them for seed yield per inflorescence but had the same inflorescence density. These four groups were crossed with each other in various combinations. It was observed that an increase of 33% in inflorescence density resulted in 34% increase in seed yield from LID to HID. There was no change in yield per inflorescence associated with this improvement. Similarly, an improvement of 24% in yield per inflorescence caused a 17% increase in seed yield from LYI to HYI without affecting inflorescence density. This showed that traits of inflorescence density and seed yield per inflorescence are under different genetic control and can be manipulated independently. Leaf size was negatively associated with increase in inflorescence density but was not affected by seed yield per inflorescence.

2.2.1 Pollination

Thomas (1987) described a close relationship among bee foraging behavior, floral nectar production, pollination and environmental conditions. Generally warm and dry conditions increase the pollinator’s activity. For example, at 27°C bees are more attracted by nectar. As the self-incompatibility barrier breaks down at higher temperatures, particularly over 35°C, the improved activity of bees at higher temperatures facilitates more selfing than normal. Rodet et al. (1998) observed that the first visit of a honey bee provided a sufficient pollen load (280-300 pollen grains) to saturate the stigma and ensure 60-70% fertilization. The number of pollen tubes in the styles and at the ovary entrances correlated with the pollen load on the stigma. Generally this correlation remained effective up to 150 pollen grains per stigma. Several visits by the pollinator facilitated uniform distribution of pollen on the stigma, improved the proportion of compatible pollen in the pollen load and maximized pollen tube access to ovules. The researchers proposed that frequent visits of bees added pollen to poorly loaded stigmas and removed pollen from over saturated stigmas. They found no effects of self-pollen on the performance of outcross pollen during pollination, pollen tube growth and fertilization. Self-pollen germinated and pollen tubes grew in the upper part of style but their success rates for entrance into the ovary and penetration of ovules were negligible (0.28%).

White clover has gametophytic self-incompatibility system which is controlled by multiple oppositional S alleles. The incompatibility is of pseudo nature and gets broken at high temperatures. This species shows two interference zones i.e., on the stigma and about three quarter way down to stigma, inhibiting fertilization by incompatible pollens (Williams, 1987b). Chen and Gibson (1973) studied the effects of temperature on pollen germination, pollen tube growth and fertilization. They found that temperature stimulates the germination of both, compatible and incompatible pollens in the same way. The compatible pollen took 8, 16 and 24 hours after pollination to fertilize the most distant ovules in the ovary at 35°C, 25°C and 15°C, respectively. Temperature affected early growth in the stigmatoid tissues more than late growth in the hollow canal of the style. Cowan et al. (2000) studied the contributions of inadequate pollination, the self-incompatibility system and competition for nutrients as causes of low seed setting of white clover in the UK environment. They found that incompatible pollen did not inhibit compatible pollen from fertilizing ovules and producing seeds. At 20°C the stigma remained receptive until 32 hours after initial pollination with compatible pollen. After pollination with incompatible pollen, the stigma receptivity was viable for 40 hours, or probably more, as the researchers did not study it beyond 40 hours. Pollination from more than one compatible source yielded more seeds than a single source. They provided a sufficient load of pollen (two loads of compatible pollen) by hand pollination, thus eliminating the effects of variability of insect pollination and inadequate pollen supply. Only 20 florets, selected from 15 inflorescences, were pollinated. This eliminated the chances of inadequate availability of resources to florets to support pollination and seed development. Provision of both adequate pollen load and nutrient resource could not produce seeds up to the full potential.

2.2.2 Ovule abortion

Understanding the causes of low seed production per floret is very important for planning breeding strategies and management practices for seed production. Thomas (1996) explored the fact that in white clover all available ovules do not form seed and there is scope for 20-50% increase in seed yield per inflorescence. The average number of ovules per floret was 5.1 with lower florets producing more ovules than upper florets. The fertility of these ovules was very sensitive to light intensity. However, even the brightest conditions could not improve ovule fertility beyond 70-80%. Thus ovule sterility is a major contributor to the difference between potential and actually harvested seed yield.

Pasumarty and Thomas (1990) discussed the effect of light intensity on ovule fertility, seed abortion and seed yield of Grasslands Huia and Pitau white clovers. For Huia they observed that dense canopies reduced the production of normal embryo sacs to 54%. The seed yield was reduced from 67% to 49% for open and dense canopies, respectively. Eventually, only 71% of ovules per head were fertile and capable of setting seed in good growing conditions. Similarly for Grasslands Pitau, the seed setting was 58%, 47% and 50% of ovules per head for open canopy, dense canopy and pre fertilization shading, respectively. The 1000 seed weight and number of ovules per floret remained unaffected by canopy and shading effects. This showed that low light intensity reduced seeds per head by ovule sterility, and abortion of seeds and florets.

Pasumarty et al. (1993b) studied seed development, ovule degeneration and seed abortion under controlled conditions to explore the causes of poor seed set in three Japanese cultivars of white clover. Cytological studies showed that apparently normal looking ovules may have abnormalities such as under-developed embryo sacs, which make them sterile. Even in favourable environmental conditions, only about 79% of ovules were capable of setting seed. It could not be ascertained whether nutrient shortage or meiotic irregularities resulted in ovule sterility. Major ovule or seed abortion

(41.8%) took place within 5 days after pollination (DAP). This reduced the available reproductive sites from more than four per floret to approximately 2.5, which is almost the same as the average number of successful seeds produced per floret (2.3 – 2.7). The distal florets in a head produced slightly less seeds than the lower florets. Within a floret, ovule sterility and seed abortion was random. Moreover, they found inter-varietal differences for seed retention capability. This indicated that genetic variation exists, and that an increase in ovule fertility and embryo retention are important objectives to improve seed yield. The researchers compared the results obtained in this study under optimum conditions with their results obtained under field conditions (Pasumarty et al., 1993a) and suggested that cool, rainy and overcast weather conditions reduced photosynthesis, and ultimately, the food supply to developing embryos. This created competition among developing seeds in a floret and/or among florets in a floral head and caused embryo abortion. In both of the studies of Pasumarty et al. (1993a; b) chances of pollination defects were removed by providing adequate pollen loads. Sufficient pollen tubes to fertilize ovules were also observed in these studies.

Dhar et al. (2006) compared obligatory self (*T. dubium*), outcross (*T. pratense* and white clover) and mixed (*T. fragiferum*) pollinated genotypes in a study of ovule abortion. Enclosure of closely placed anthers and stigma in the keel, blended with low insect visits, facilitated selfing of *T. dubium*. In *T. dubium* and *T. fragiferum* ovule abortion was non-random with more frequent abortion taking place at the peduncular and stylar ends, respectively. Although ovule abortion was random in white clover and *T. pratense*, ovules near the middle of the ovary were involved more frequently in seed setting of white clover. The mixed mating system species *T. fragiferum* provided the highest (63%) and the obligatory outcrossed species *T. pratense* produced the least (15%) seed set percentage on open pollination. High within species variation in reproductive success showed that the mating system had no effect on seed setting. However, species with a mixed mating system enjoy the availability of a selfing option in cases of failure of outcrossing. Their pollinator not only conducts outcrossing but also improves selfing. Dependence on a pollinator and the existence of an incompatibility system made white clover the lowest seed producer among the studied species. Bogaert (1977) studied 18 clones of white clover in Belgium for two years to explore possibility of selection for head size and seed setting ability per floret as means to improve seed yield. Both of these traits were found highly heritable. Size of the inflorescence had no significant direct effect on fertilization. Plant genetics and pollinating agents are crucial in fertilization success.

2.2.3 Inflorescence

The number of inflorescences produced by a plant is the most influential factor in seed production of white clover (Hollington et al., 1989; Jahufer and Gawler, 2000; Montardo et al., 2003). This characteristic is dependent on the availability of reproductive nodes, intensity of stimulus for floral initiation, the vigor of the response to that stimulus and leaf emergence rate. Low temperature and long days stimulate flower initiation. Genotypes of high latitude origin respond as long day plants. They start floral initiation as the photoperiod increases in spring. Genotypes adapted to low latitude stop flower initiation with increase in day length and temperature. They continue floral initiation from autumn through winter and into spring (Thomas, 1987). The indeterminate nature and intermittent pattern of flowering make it impossible to harvest all inflorescences produced by a plant. This led Marshall et al. (1998) to make interspecific hybrids between *T. nigrescens* and white clover to transfer the traits of high intensity and less intermittent inflorescence production from the former to the latter. The closing date for grazing of the field and environmental conditions during the floral development and harvesting also play important roles in obtaining harvestable mature intact inflorescences. Only ripe inflorescences at the time of harvesting can contribute to seed yield. Jahufer and Gawler (2000) found strong phenotypic and genotypic correlations between total numbers of ripe inflorescences and seed yield.

Number of florets per inflorescence (head size) is another important contributor to seed production. A bud present on a stolon can form an inflorescence or a new stolon. This causes inflorescence production to be at the cost of stolon production and vice versa. This also highlights the importance of head size. Fewer but bigger heads can spare more nodes for stolon production without compromising seed yield. Head size is affected by genotype as well as environment. Long days and low temperatures improve head size (Thomas, 1987). A very weak correlation was observed between total ripe inflorescences and florets per ripe inflorescence in the study of Jahufer and Gawler (2000). Annicchiarico et al. (1999) estimated a genetic correlation coefficient of 0.17 between head size and numbers. In another study, Kahn et al. (1995) estimated a genetic correlation of 0.29 between head size and number and a phenotypic correlation of 0.39 between these traits.

2.2.4 Peduncle

The peduncle plays an important role in pollination and harvestability of the inflorescence. Marshall (1995) studied the effects of peduncles on potential seed yield. He studied the white clover cultivar ‘Aberdale’, a small leaved variety bred for stronger peduncles without compromising dry matter yield and persistence. It produced thicker and heavier peduncles than the other compared varieties. Peduncle length was longer relative to the subtending petiole. These peduncle qualities maintained the integrity of the inflorescences and contributed towards higher overall seed yield. ‘Aberdale’ also had other superior heritable seed components, including higher numbers of inflorescences and seeds per floret. These traits may have independent bases and contributions or it might be speculated that they are linked because of the availability of early maturing inflorescences for harvesting due to the strong peduncles. However, the researchers did not show any relationship between maturity age of inflorescences and number of inflorescences, florets per inflorescence or seeds per floret. They also provided no evidence that strong peduncles increased attraction for pollinators. This study showed the potential for increasing seed yield by focusing on peduncle strength without having a negative impact on vegetative growth of white clover, which is after all a pasture crop. Williams et al. (1998) also discussed the importance of the peduncle in seed production with reference to ‘Aberdale’ in comparison with other varieties. Generally the small leaved cultivars produced higher numbers of inflorescences per square meter and this variety also had the highest number of seeds per floret and the heaviest seeds per inflorescence. All these positive seed attributes of ‘Aberdale’ became harvestable due to its peduncle strength. The optimum range of the flowering peak can also contribute to successful harvesting. The contributions of peduncle strength and optimum range of flowering peak appear to be important for high actual seed yield. A considerable difference ranging from 36% to 50% depending on cultivar was observed between potential and actual seed yield during a favourable season (1995). Potential seed yield could not be actually harvested in Britain because harvest is taken once a year and its success depends on an optimum combination of favourable weather and ripeness of inflorescences at that time. This study demonstrated the importance of applied research.

Hill et al. (1989) studied heritabilities of peduncle length, floral buds per stolon, floret numbers and floral density per square meter for diallelic crosses of 7 white clover varieties. Their analyses were based on the assumption of homozygosity for the traits under investigation; hence this requires careful interpretation of their data and results. For peduncle length they observed low heritability, particularly the additive genetic component. Non-additive variation for this character was due to non-allelic interactions (epistasis). Similarly heritability of seeds per floret was found to be very poor. However, there were strong additive and dominance effects for floral buds per stolon and floral density. The researchers proposed to focus on these two traits to improve the overall seed yield. They also suggested that efforts be made to improve the components of seed yield independently as there were weak correlations among them. No maternal effects were detected in the inheritance of any of the observed traits.

2.2.5 Environment

White clover seed production is greatly influenced by environment. Marshall et al. (1989) and, Langer and Rohde, (2005) found the environment to be an important contributor to the low seed yields in UK and Denmark, respectively. Thomas (1961) analysed the role of the environment in seed production of white clover. Number of fertile ovules per plant appeared to be a fundamental factor for seed yield. Numbers of inflorescences produced, along with florets per inflorescence and ovules per floret influenced the total number of ovules formed on the plant. Number of inflorescences per plant was influenced by floral initiation and rate of vegetative and reproductive growth. Long hot days maximized floral initiation and ratio of peduncle to petiole length. Long cold days improved inflorescence size by increasing florets per inflorescence and floret size. It also increased ovules per floret. High temperatures supported pollen fertility. After initiation, the emergence of floral buds was tightly linked with the rate of leaf appearance on the same runner.

In Australia, the morphological attributes and herbage yield studied by Jahufer et al. (2002) for Ladino white clover expressed complex genotype by environment (GE) interactions. This GE interaction was greater than the genotypic contribution, highlighting the importance of GE interaction studies. Temperature and light were two important environmental factors involved in reproductive development of white clover (Thomas, 1987). Generally white clover is considered as a long day plant. Temperature and day length influence traits like inflorescence emergence, size, florets per inflorescence etc. For example, low light intensities and warm short photoperiods cause inflorescence abortion.

Williams et al. (1998) evaluated seed components and, actual and potential seed yields of small, medium and larger leaved white clover varieties under UK field conditions for two years. Higher temperature and less rainfall during the harvesting months of 1995 produced better seed yield relative to 1994. There were significant effects of year, leaf size and their interactions on inflorescence appearance, number and ripeness. Large leaved varieties were generally better than small and medium leaved varieties for the traits including number of florets per inflorescence, number of seeds per floret, seeds per inflorescence and seed weight. Pederson and Brink (2000) compared local small leaved naturalized populations with large leaved Ladino type cultivars of white clover for seed production in pastures in South East USA. They observed considerably higher production of seeds, inflorescences and seed bearing inflorescences and ratio of seed bearing inflorescences to total inflorescences for naturalized populations as compared to the cultivars. Only cultivar ‘Louisiana S-1’ showed results close to the naturalized populations. Naturalized populations were earlier flowering than the Ladino cultivars. High correlations existed among numbers of flower, number of seed bearing florets and seed yields in both years of observation. Relationships between reproductive and vegetative traits were not investigated in that study. The researchers highlighted the superiority of the small leaved naturalized populations for higher re-seeding potential under intense grazing, even under unfavourable conditions.

2.2.6 Seed crop management

Management practices are also very important in seed yield. Clifford (1980) highlighted the main contributions of management practices in white clover seed production. The appropriate sowing rate for a clover crop grown in mixture with ryegrass or under-sown in a cereal crop for seed harvest in the next year was 3 kg/hectare. In the case of a white clover crop for seed production in the first year, a sowing rate of 6 kg/hectare gave the best seed yield. The author suggested that in deciding sowing rate, the developmental rate of the cultivar should also be considered. For example, Pitau, being slow growing, required a higher sowing rate than Huia. Use of 30 cm and 45 cm rather than 15 cm row spacing improved seed yield in the second year. This increase was very significant if, after the first harvest, plant growth in the inter-row space was restricted by inter-row cultivation until field closing for flowering. Heavy grazing removed primary stolons and stimulated production of secondary and tertiary stolons, which were less vigorous. At the time of closing of a field for flowering, heavy defoliation could be reduced with cattle grazing or topping with a mower. The study highlighted that the type of herbicide and its application timing should not be allowed to hinder flowering. Low seed yields obtained because of early closing were found to be caused by losses of inflorescences due to deterioration under the dense foliage canopy. Late closing was proposed to reduce the loss of inflorescences due to short peduncles. Harvesting no later than one month after main flowering was found to minimize seed head lost. The peak flowering was observed in late December (close to longest day) and it was suggested that harvesting should not be later than late January to early February.

Seed yield and weight are affected by factors like genotype, climate, closing date for grazing, etc. Clifford (1979) studied effect of closing date on potential seed yield of Huia and Pitau cultivars under Canterbury, New Zealand, conditions. Four closing dates i.e., mid-September, mid-October, mid-November and mid-December, were tested for their effect on seed yield. Mid November closing produced the highest seed yield due to a higher daily rate of inflorescence appearance. Closing in mid-December drastically reduced seed yield due to a decrease in inflorescence production caused by a slowing of lengthening days and risinf temperatures. Inflorescence production was promoted more by rapidly increasing daylength and mild days than declining long days. However, florets per plant and individual seed weight were reduced as closing date was delayed after mid-September. Seasonal and genotypic effects on seed yield components were also apparent from the data. Clifford et al. (1985) found that a mid-November closing date provided the shortest flowering span along with high harvestable seed yield for New Zealand bred cultivars. A short flowering span with high seed yield could reduce the contamination caused by buried seeds from the previous year’s crop. The best seed harvesting time was found to be six weeks after peak flowering in crops with one significant flowering peak. In the case of multiple significant flowering peaks, harvesting four weeks after the last flowering peak gave maximum seed yield.

Hollington et al. (1989) experimented on S184 (small leaved), Menna (medium leaved) and Olwen (large leaved) varieties of white clover to investigate the effect of spring management on seed yield in UK conditions. The period of availability of maximum ripe inflorescences appeared to be short, demanding careful monitoring for obtaining maximum seed yield. Florets per inflorescence, seed setting percentage and 1000 seed weight remained constant during the harvest period. Plants subjected to cutting (with hand shears) showed more potential seed yield than grazed plants. There was also significant interaction between varieties and defoliation method. For example, Olwen produced the highest seed yield under the cutting treatment only. As a whole, Olwen appeared to be more suitable for conservation systems. S184 seemed to be suitable for grazing systems. Menna showed flexibility in adapting to different management systems for seed production. Defoliation, for example by cutting or grazing, stimulates inflorescence development by increasing availability of light and reducing competition imposed by other forages (Thomas, 1987). Also in the UK, Marshall et al. (1993) studied effects of defoliation at pre- and post-bud emergence stages on seed yield components and potential seed yield. Defoliation before bud emergence was ineffective. By contrast, defoliation three weeks after bud emergence improved inflorescence numbers, 1000 seed weight and potential harvestable seed yield and reduced florets per inflorescence for brown inflorescences. This showed that defoliation after bud appearance could improve the seed yield. The effect of defoliation after bud emergence was independent of cultivar. However, Marshall and Hides (1990) observed more negative effects of defoliation on large leaved cultivars as compared to small leaved cultivars in the context of seed production potential. This might have been due to stolon damage caused by sheep grazing. Seasonal variation contributed significantly to cultivar performance and defoliation effects.

Clifford (1985) studied the relationship between leaf area and seed production of white clover. Initial growth in leaf area accompanied increases in inflorescences and florets per inflorescence. This relationship remained consistent until leaf area became 5.6 cm^2 per trifoliate leaf. At this leaf size, maximum number of inflorescences and florets per inflorescence were observed, resulting in peak seed yields. With an increase in leaf size beyond 5.6 cm^2, florets per inflorescences kept on increasing but production of inflorescences started declining. Improved numbers of florets per inflorescence could not compensate for the loss of inflorescences and seed yield decreased. Proper seeding rate and removal of companion species provided space for better plant growth and expression. Grazing or any defoliation practice removed primary stolon tips and stimulated production of secondary stolon formation along their length. Thus grazing, before closing a field for flowering, favoured inflorescence production. Delay in closing date reduced leaf size and resulted in an increase in floret per inflorescence and reduced seed yield. In area with sufficient water availability, a suitable closing date could be mid-November. In dry areas it could be earlier than mid-November to avoid any severe stress. Water stress above wilting point or, more suitably, about 50% plant available water, favoured inflorescence production by reducing leaf size during flowering time. Less water also reduced phosphate uptake, which also favoured inflorescence production. Suitable water provision kept leaf size at optimum level and supported growth of fertilized embryos, resulting in high inflorescence production and seed yield.

Evans et al. (1986) compared seed production potential of the national list and potential varieties in the UK over two years. Bad weather conditions (wet and cold) and increased clover sward density during the second year reduced florets per inflorescence, seed setting, seed weight and bee activity. Larger leaved varieties produced fewer inflorescences than small leaved varieties. However, larger leaved varieties overcame this deficiency by improving seed yield per inflorescence supported by increased seed weight, seeds per floret and florets per inflorescence, which all appeared to be positively correlated with leaf size. The Danish variety Milkanova was reported to produce 400 kg/hectare in Denmark while the average yield of British varieties S100 and S184 was 78 and 68 kg/hectare in UK. Milkanova produced similar low seed yields to S100 and S184 in the environment of Aberystwyth, UK. This showed the importance of environmental effects on seed production.

Severe water stress is reported to have negative effect on seed yield. Iannucci and Martiniello (1998) found that water stress reduced the proportion of dry matter allocated to reproductive growth of the studied legumes. Specifically, water stress severely reduced dry bio-mass, stolon weight, seeds per head and stolon density while there were limited adverse effects of water stress on inflorescence per stolon and seed weight. Bissuel-belaygue et al. (2002) made their observations under greenhouse and growth chamber conditions at Montpellier, France and Aberystwyth, Wales. They found that severe water stress reduced pollen viability, nectar formation, pollination, fertilization, seed setting and seed filling in white clover. Embryo abortion appeared to be the biggest reason for low seed set. However, a moderate water deficit produced better seed weights and numbers and, higher and more consistent numbers of florets containing nectar as compared to plants grown under normal and severe water stress conditions. Crosses between moderately stressed plants produced more seeds per inflorescence than crosses amongst well watered or severely stressed plants. The researchers concluded that reduction of soil water potential in the rooting zone, sufficient to inhibit stolon branching and induce a reduction of leaf growth without causing wilting in the mature leaves and without reducing net photosynthesis or dry matter accumulation in growing stolons, was beneficial to seed yield. In Oregon USA, Oliva et al. (1994) found that drying down to 68% depletion of soil water content by delaying irrigation led to restricted vegetative growth and improved flowering and seed production, giving high seed yield. Flower density appeared to be the most distinctly effective contributor to seed yield. However, the same treatment did not increase seed production in the second year, probably because of an increase in stolon density during late autumn and early spring between the two years. These stolons grew between the planted rows and reduced production of inflorescences.

Widdup et al. (2004) successfully made selection for seed production during later stages of cultivar development. The morphology of cultivars, as determined by leaf area, was maintained, uniformity was improved and flowering peaks became more concentrated. They compared three generations of six cultivars for two growing seasons. The pre-release generation was selected for improved agronomic traits. The nucleus generation was the product of two years selection pressure for seed yield on the pre-release generation. The basic generation was the expression of this selection pressure after four years in commercial seed production. The selection resulted in a 21% increase in seed yield between the pre-release and nucleus generations. Selection accumulated favourable alleles to give a further 10% seed yield increase in the basic generation. Small leaved varieties, like Prestige, improved seed yield by increasing inflorescence density. On the other hand, the large leaved cultivar Kopu-II improved seed production by having moderate to high inflorescence density, complemented by high seed yield per inflorescence. This strategy made Kopu-II the highest potential seed producer amongst the compared cultivars. The researchers suggested that due to the high heritabilities of the seed production traits (flower density and seed yield per flower), selection for high seed production is possible at the late stages of variety development without disturbing the agronomic performance.

Woodfield et al. (1996) argued that improvement in root traits will enhance production, nitrogen fixation and quality of white clover under water stress conditions. In moist environments, the tap root is less important than nodal roots as moisture is not an issue and nutrient uptake is more important. But in dry environments or where moisture stress occurs in dry soils, nodal roots are inhibited, and tap roots become important as moisture uptake becomes a vital issue for plant survival. The researchers studied the effect of selection for tap root diameter on leaf size and stolon density of white clover cultivars. Most of these cultivars (Aran, Dusi, SC-1, Titan and Huia) were large leaved and already had large taproot diameters. Three cycles of selection on 10-14 week-old (seedling) plants resulted in a 2.4% genetic gain per cycle for taproot diameter. This improvement in tap root diameter did not significantly affect leaf size and stolon numbers. Notably, gain in taproot diameter remained independent of leaf size, showing potential for independent improvement of these traits.

2.2.7 Growth regulators

In Palmerston North, New Zealand, Thomas (1987) observed that application of GA_3 hormone delayed flower initiation for 2 to 3 weeks and doubled the numbers of inflorescence produced per stolon in some high latitude white clover cultivars. Hampton (1991) studied the effects of Paclobutrazol application at floral initiation (9 October) and at first floral bud appearance (2 November) on inflorescence production and seed yield. In general, Paclobutrazol enhanced inflorescence production and seed yield. The effect was more evident when this growth retardant was applied at first floral bud emergence. It reduced leaf size and petiole length. It reduced peduncle length after the November application but not after the October application. The inflorescences were elevated 2-5 cm above the leaf canopy, providing more light intensity to enhance ovule fertility and seed setting. A significant correlation between leaf size and reduction of leaf size due to Paclobutrazol application was observed. Responses of seed yield components (seed numbers per inflorescence, seed yield per inflorescence, ripe inflorescence per square meter and 1000 seed weight) were dependent on variety and time of chemical application. In another study at the same location, Budhianto et al. (1995) found that, after closing a field in mid-November for seed harvest, the maximum potential and actual seed yields of cultivar Pitau could be achieved at a harvest 25 days after the flowering peak. Seed yield did not fall significantly until 35 days after peak flowering. However, delays in harvest until 40 and 45 days after peak flowering considerably reduced seed yield. Use of Paclobutrazol significantly increased inflorescence numbers at peak flowering and did not change normal optimum harvesting time.

Zubal and Drobny (1995) studied effect of 3 growth regulators (Relan PGR, Atonik, Nevirol 60 WP) on seed yield and thousand seed weight of white clover variety Dubrava. They conducted trials at Borovce (warmer and drier area) and Bystricka (colder and wetter) area of Slovenia for 3 years. Only the growth regulator Nevirol 60WP could improve the seed yield significantly at Bystricka. These growth regulators also could not affect thousand seeds weight. The effect of different growth regulators was inconsistent with respect to years and location. Hampton (1996) reviewed reports on Paclobutrazol performance in seed production of white clover. Inconsistent findings were apparent from different studies. A range of factors from environment to genotype were suspected to contribute in this variability of findings. Moreover, the high cost of the chemical and its residual effects were also considered to be potential hindrances to its commercial use.

2.2.8 Relationship between vegetative and reproductive growth

In white clover, an axil at a node produces one bud which develops into either a stolon or an inflorescence. Each inflorescence occurs at the expense of a stolon and vice versa. This creates an inbuilt inverse relationship between vegetative and reproductive growth. Inhibitory effects of inflorescences on stolon production and growth, and competition for nutrients also contribute to a negative correlation between vegetative and reproductive development (Thomas, 1987). Woodfield and Caradus (1996) emphasized importance of persistence of white clover plant to improve the competitive edge of New Zealand farming. They suggested breeding for higher stolon growing points at a particular leaf size can improve plant persistence and yield. Gibson (1957) found a strong negative relationship between inflorescence production and persistence of Ladino clover. He used profuse, medium and non-flowering genotypes under both normal and long day length conditions. Long light periods were used to induce flowering to the genotypes that produced few flowers in Alabama, USA, conditions. Flowering, induced by plant genetics or prolonged light, negatively affected persistence. Interestingly, extended light periods made profuse flowering genotypes to change their normal tendency of high flowering and low stolon production. They produced more stolons than non- flowering genotypes under those conditions. The researchers hypothesized that long days were sensed by plants as an occurrence of unusual hot weather and plant's response of improved stolon production to ensure their survival was turned. Crowder and Craigmiles (1960) studied the effects of soil temperature, soil moisture and flowering on the persistence and forage production of Ladino (large type) and Louisiana (intermediate type) white clover varieties under Georgia, USA, conditions. They found that a decrease in stolon population did not reduce forage production during the spring season. Repeated defoliation, flowering, and to some extent, deterioration due to diseases, weakened the plants. The weakened plants, when exposed to high soil temperatures and moisture stress, lost large proportions of their stolons due to desiccation. Both studies (Gibson, 1957; Crowder and Craigmiles, 1960) showed that high flowering reduced stolon production during summer, making them vulnerable to drought and lowering their persistence.

In Italy, Piano and Annicchiarico (1995) observed agronomic traits of 16 landraces and 12 natural populations of Ladino white clover. They found differences between land races and natural populations for leaf size, stolon density and long and short term dry matter (DM) yield. The natural populations showed stolon densities typical of intermediate-leaved white clovers, while their leaf lamina size and petiole length were similar to Ladino white clovers. This combination of traits could lead into a variety having high DM yield, tolerance to competitive stress, better persistence and tolerance to grazing, if seed production of these natural populations could be improved. In Ladino clover, improvement in seed production and less emphasis on long term survival during the domestication process has decreased stolon density and its associated agronomic characteristics. The phenotypic observations showed a strong positive correlation ($r = 0.74$) between persistence and stolon density in the third year of production. However, there were negative correlations between persistence and seed yield components. In particular, seed head production showed a strong negative correlation ($r = -0.7$) with persistence. Stolon density appeared to be negatively associated with production of seed heads. The researchers showed that very few ecotypes, for example 'landrace 19', had the combination of persistence and high seed yield. These two traits appeared to be difficult to combine. However, high seed yield could be combined with high DM yield, at least in earlier years.

Cain et al. (1995) did correlation studies for biomass and reproductive growth components of white clover. They studied 30 possible trait pair combinations for number of inflorescences, number of flowers per inflorescence, number of fruits (pods) per inflorescence and number of seeds per pod in both late and early maturing pods. There were no significant negative correlations on a genetic, phenotypic or micro-environmental basis amongst the studied traits. There was a strong positive association between numbers of pods and florets per inflorescence, showing potential for improvement in seed yield. Number of florets per inflorescence was moderately correlated with biomass production on both genetic and phenotypic bases. Except for early seed weight, biomass appeared to have positive phenotypic and genetic correlations with all observed reproductive traits. There was weak evidence for trade-offs among reproductive components and between reproductive traits and biomass. However the environmental conditions of this experiment were not optimal and, according to the researchers, resources and (or) pollen might have been insufficient to express the maximum potential of the plants. Annicchiarico et al. (1999), while working on ladino ecotypes and their sibs, also found negative genetic correlations of persistence (as indicated by stolon density) with seed yield (r_g= - 0.70) and DM yield (r_g = - 0.6). Their results also showed a slight negative genetic correlation (r_g = - 0.45) between seed yield and DM yield. The narrow sense heritabilities for seed yield and its components including peduncle thickness were moderate to high. Number of florets per head and seeds per floret showed high narrow sense heritabilities. There were positive genetic correlations between sizes of different vegetative and reproductive organs. Larger leaf size was positively associated with number of florets per head and had a negative relationship with number of inflorescence per plant. Seed weight showed low heritability but this might have been due to low variability for this trait in the parents. Medeiros and Steiner (2000) in Oregon, USA, also found that intermediate leaf size cultivars produced more flowers than Ladino type large leaved cultivars

2.2.9 Molecular analysis

Field selection for seed production is time-consuming, expensive and often inconclusive due to environmental effects (Barrett et al., 2004a). Efforts have been made to provide molecular tools, such as genomic maps and QTLs, to improve the efficiency of this selection. Barrett et al. (2004b) developed simple sequence repeats (SSR) genetic markers of white clover by analysing hybrids of highly heterozygous parents. They identified 493 loci and eight homoeologous pairs of linkage groups. Using the same plant material, Barrett et al. (2005) discovered 23 quantitative trait loci (QTL) involved in seed production. Most of them were dominant in nature and suitable for marker assisted selection (MAS) to improve seed production. They also indicated genomic regions involved in seed production. Cogan et al. (2006) identified 10 QTLs related to seed production of white clover. Two QTLs, one on LG3 and other on LG18 (linked with flowering time) were shown to have strong influences on seed yield. Reproductive traits showed higher heritabilities than vegetative traits. Barrett et al. (2009) successfully applied MAS to seed yield of white clover. Up to three microsatellite markers associated with QTL SY03-D2 were applied to 12 multi-parent complex breeding pools. High phenotypic variation existed for the studied traits of seed yield (SY) and inflorescence density (ID). Marker-trait associations ranged from non-significant to 69%. However, two third of the breeding pools showed significant associations. This association accounted for an average of 38% (30-69%) difference in seed yield.

2.3 WIDE HYBRIDIZATION

Life is an ever evolving process. Individuals of a population acquire random heritable changes in their genetic material caused by factors like mutation, transposons, viral infection, etc. These changes become refined by recombination at the chromosomal level by sexual mating and at the gene level by crossing over during gamete formation. This creates random variation among individuals of a population. Factors like geographical isolation, natural selection, genetic drift, etc., promote accumulation of genetic variability which ultimately makes the organisms genetically so distinct that they can no longer interbreed with their original population. Reproductive isolation makes such individuals a distinct species. A species can become a source of further new species by the processes involved in its own creation, or by hybridization with other species (wide hybridization). Hancock (2004) explains that, despite having reproductive barriers, many plant species retain the ability to hybridize with their closely related species. Many areas of inter-species contact have been identified where closely related species can hybridize and form hybrid swarms. The fertility barriers between parental species may result in inviability, sterility or very low fertility of F_1 hybrids. Genome duplication or genetic recombination of relatively infertile hybrids can create new genetic variation. Some of these new segregates may have improved fertility along with adaptive divergence from their parent species. Success of new interspecific hybrids depends on their adaptive potential. One way to enhance this potential is by backcrossing with or introgression of the parental species. Alternatively, evolution of hybrids can occur by genetic differentiation and genome reorganization.

Wide hybridization is a natural phenomenon. For example, it is estimated that about 45% of the British flora was alien and 7% of these aliens were involved in production of hybrids with native flora. These hybrids have adapted and became part of the British flora (Abbott, 1992). This reviewer also provided a list of plant species like *Helianthus annuus*, *Senecio vulgaris*, *Iris nelsonii*, *Senecio cambrensis*, *Spartina anglica*, *Tragopogon mirus*, etc., having wide hybridization as their origin. Mallet (2005) surveyed studies of natural interspecific hybridization in plants and animals. He found that at least 25% of plant species and 10% of animal species are involved in wide hybridization. This process is rare on a per individual basis but common on per species basis. The hybrids, despite being rare, have important evolutionary importance as they can serve as a bridge for gene flow between the parental species. Many evolved plant species like *Triticum aestivum*, *Gossypium hirsutum*, *Brassica napus*, *Brassica juncea*, etc are products of natural wide hybridization. For example, bread wheat (*Triticum aestivum*) has evolved by interspecific crossing of three different species. First, *T. monococcum* crossed with an unknown related diploid species. The chromosomes of the hybrid became doubled, producing a tetraploid species, *T. turgidum*. This tetraploid hybrid then hybridized with diploid *T. tauschii*. Doubling of chromosome of this later hybrid resulted in production of *T. aestivum* (2n=6x=42). The basic set of 21 chromosomes of this species can be differentiated into seven homoeologous groups representing the three ancestral species.

2.3.1 Biology of wide hybridization

Hawkes (1977) discussed the importance of wild germplasm in plant breeding. He emphasized that in cases where a desired trait is not present in current and primitive cultivars; wild species can serve as a wide gene pool for most crops. The ease of gene transfer from wild relatives into cultivars depends on the numbers of genes involved, geographical distribution and ploidy levels of the two sources. Wide hybridization is easier among species having similar distribution areas, ploidy levels and genome formula. In the same distribution area, wide hybridization is a natural phenomenon and genes can get exchanged among cultivars, weeds and wild species. For example, cultivated potatoes *Solanum tuberosum* sub species *andigena* received resistance for potato cyst nematode (*Heterodera rostochiensis*) from a wild species *S. oplocense* via a weed species *S. sucrense* through natural crossing. Similarly, frost tolerance in *S. ajanhuiri*, *S. juzepczukii* and *S. curtilobum* were transferred from their wild relative species by natural wide hybridization.

Rieseberg and Carney (1998) reviewed plant hybridization phenomena. They discussed hindrance to wide hybridization at prezygotic and postzygotic levels. Figure 2.5 summarizes reproductive barriers at pre- and postfertilization levels.

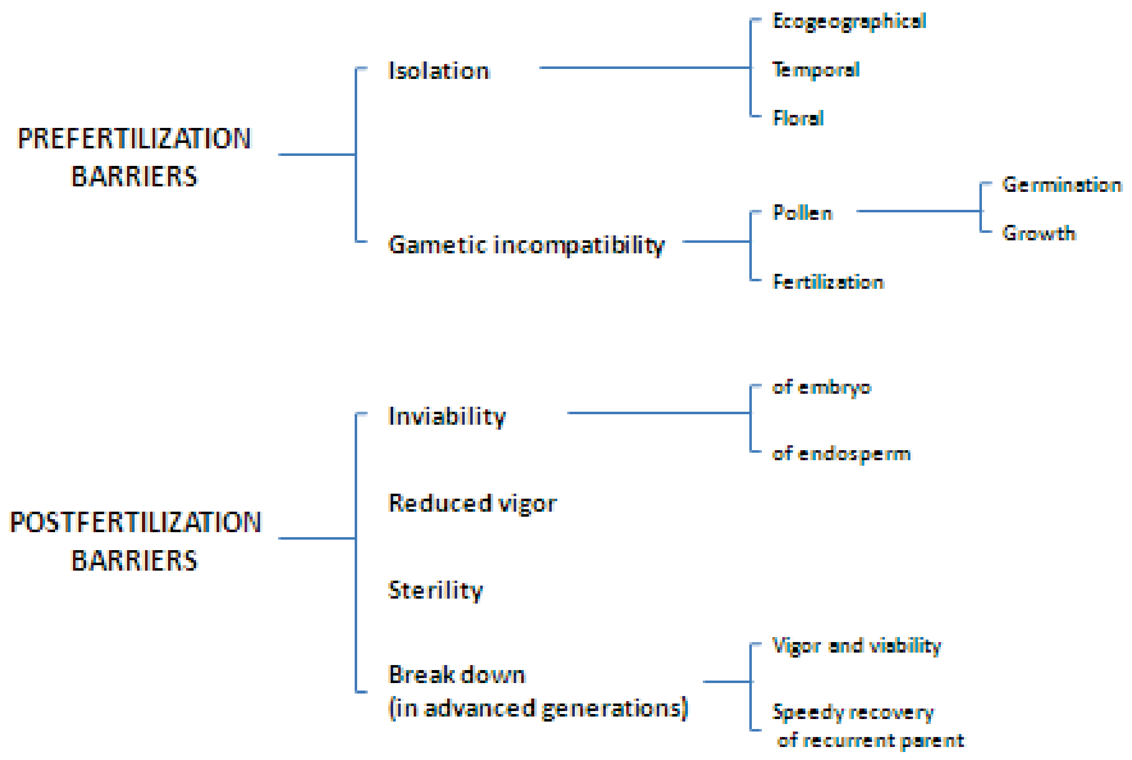


Figure 2.5. Schematic diagram showing pre-fertilization and post-fertilization barriers in wide hybridization.

2.3.2 Prefertilization barriers

Factors like habitat isolation, temporal differences, floral differentiation, and gametic competition or incompatibility resist hybridization of species before fertilization. Ecogeographical and temporal isolations prohibit overlapping of habitats and flowering times, respectively, of two species thus reducing their chances of hybridization. For example, the blueberry species *Vaccinium angustifolium* and *V. canaliculata* are isolated by both habitat and temporal barriers (Hancock, 2004). Differences in floral attributes like color, shape, size, nectar production, etc., can restrict cross breeding between two species by requiring different pollinators. For example, two closely related and cross compatible species of monkeyflowers *Mimulus lewisii* and *M. cardinalis* attract different pollinators, resulting in reproductive isolation caused by floral isolation. *M. lewisii* is pollinated by bees, whereas, *M. cardinalis* is preferentially pollinated by hummingbirds. Schemske and Bradshaw (1999) studied these two species, and the F_1 and F_2 generations to find the genetic basis of pollinator discrimination. Hummingbirds appeared to like flowers rich in nectar and anthocyanin content. Bees preferred large and low pigmented flowers. The researchers identified an allele that favoured petal carotenoid concentration and which reduced bee visitation by 80%. Another allele doubled hummingbird's visitation by increasing nectar production.

Gametic incompatibility is very important barrier for cross compatibility of two species. Smith (1970) compared pollens of different species and subspecies of *Haplopappus* (Compositae) for their ability to successfully fuse with an egg. He studied two species, *H. divaricatus* (n=4) and *H. validus* and three subspecies of *H. validus* i.e., granitius (n=7), torreyi (n=6) and validus (n=5). He used the interval required to competitively equalize domestic and foreign pollen in double pollination as an estimate of natural hybridization frequency. In a mixed application of domestic and foreign pollen, he delayed the application of domestic pollen for 0-180 minutes. The delayed interval resulting in production of 50% hybrids was taken as an indication that the foreign pollen had become capable of pollination equal to the domestic pollen, while other factors were assumed to be constant. Results showed weak correlation between chromosome balance and interval required to produce 50% hybrids. Chromosome balance contributes in pollen tube growth. Duplication of genetic material in pollen causes changes in genetic balance of incompatibility system of the species. It affects pollen tube growth.

No such change is associated with aneuploid change in chromosome number. The comparison of domestic versus foreign pollen for successful fertilization revealed that domestic pollen is always pre-potent over foreign pollen. More delay in application of domestic pollen results in more success of fertilization by foreign pollen. Cross fertilization success is also directly proportional to morphological and genetic relatedness. Plants having long styles were better pollen competitors than plants having small styles. This may be due to adaptation of pollen to grow faster in long styles. There was a clear difference in results of reciprocal crosses, which showed the asymmetrical nature of pollen competition barriers to hybridization. Evans (1962) identified that pollen tube growth inhibition in the style is the important pre-fertilization barrier in interspecific hybridization of *Trifolium* species. The incompatibility between the pollen tube and style tissues results in slower growth of pollen tubes in interspecific crosses than intraspecific crosses. The pollen tube growth in such crosses was found to be dependent on species as well as genotypes within species. Chen and Gibson (1972a) also found marked superiority of pollen germination, pollen tube growth and fertilization in intraspecific hybridization, compared to interspecific crosses of *Trifolium* genus. Nagy (1997) studied the reproductive dynamics that may limit gene flow between two divergent subspecies of *Gilia capitata* (Polemoniaceae). They found that the immigration frequency, pollinator behavior, the physical environment and compatibility status are important factors involved in introgression between these two closely related subspecies.

2.3.3 Post fertilization barriers

After successful fertilization, hybrid inviability, weakness, sterility or breakdown is caused by chromosomes, genes and/or their interactions with their micro and macro environments. Generally, interspecific hybrids show high variation for viability and fertility both within and between different hybrid generations (Rieseberg and Carney, 1998). This variability is at its extreme during the initial generations i.e., F_1, F_2 and BC_1. Grant (1966) showed that selection and drift reduces this variability in advanced generations. He selected hybrids of *Gilia mulior* and *G. moducensis* for vigor and fertility from F_2 to F_9. Both species are vigorous, fertile and allotetraploid with the same chromosome numbers but different genomes. The F_1 hybrids were vigorous but sterile. The average pollen fertility was 2% due to lack of bivalent chromosome pairing mainly caused by differences in chromosomal structures of the parental species. About 75% of germinable seeds were lost due to inviability at the embryonic or seedling stages. Selection produced a rapid response from F_2 to F_6 to restore fertility and vigor. However, only 2% of progenies from F_1 to F_6 were both vigorous and fertile. Artificial selection for vigorous and fertile plants was coupled with natural selection that occurred from pollination through to the seedling establishment stage. The fertile vigorous plants were progenies of eight lines out of an initial 28 F_2 lines. The families or their subsequent lines that showed constitutional defects in the early generations could not provide vigorous fertile plants in the most advanced generations. The stability of vigor and fertility of plants was associated with cytogenetical structural homozygosity fixed in the advanced inbred generations by selection and drift.

2.3.4 Chromosomal contribution

Imbalanced chromosome numbers, including both polyploidy and aneuploidy, resulting from interploidy crosses or meiotic abnormalities during gamete formation frequently contribute to sterility of interspecific hybrids. For example, seedless watermelon and most banana cultivars are triploid and cannot produce viable seeds. Liu et al. (2012) studied microsporogenesis of three populations of *Pinellia ternata*. This species has two basic chromosome numbers (x = 9, 14) and provides an extensive range of both polyploidy and aneuploidy i.e., 2n=26-114. Abnormalities in meiosis and cytokinesis resulted in 50% reduction in pollen production and only 2% fertility among the other 50% pollen grains that produced from normal meiosis. The researchers suggested that imbalanced chromosomal complements in the pollen may be an important contributor in low seed setting, thus making the species vegetatively propagated.

Henry et al. (2005) crossed *Arabidopsis thaliana* (Col-0) with its synthetically produced tetraploid derivative (4x-Col) and a natural autotetraploid ecotype (Wa-1) to develop triploids CCC and CWW, respectively. Both types of triploid had similar fertility related traits. Pollen production, viability and normal seed formation of triploids were significantly lower than in the parents. Inbreeding of the triploids produced a swarm of aneuploids and euploids. Although most of the aneuploids produced some viable seeds, the number was lower than that produced by euploids. The viability of seeds was more in aneuploids derived from CWW than CCC. The study of advanced segregating generations i.e., F_6 and F_8, of CWW showed a gradual improvement in survival and fertility, and a shift from aneuploidy to euploidy. The sterility and lethality of chromosomally imbalanced lines resulted in their elimination from population. The aneuploidy resulting from inbreeding of triploid in initial stages got settled into near-diploids and near-tetraploids genotypes in advanced inbred lines.

Hussain et al. (1997b) generated a sterile triploid (2n=3x=24) by interspecific cross between white clover (2n=4x=32) and *T. nigrescens* (2n=2x=16). Its fertility was restored by chromosome doubling with colchicine treatment. A series of ploidies, along with some aneuploids, was developed by backcrossing and interbreeding of the backcross derivatives of this hexaploid. Except for the triploid, all derived hybrids had reasonably high fertility and pollen stainability. However, the bivalent formation percentages for 3x, 4x, 5x, 6x and 7x were 53, 54, 54, 78 and 32, respectively, in pollen mother cells at metaphase-I. This indicated presence of different levels of meiotic abnormalities in these synthetic polyploids.

Chromosomal rearrangement is considered to be one of the causal factors for inviability, sterility and breakdown of interspecific hybrids. Chandler et al. (1986) investigated chromosomal relationships among *Helianthus* species. Complex interspecific variations in chromosomal structure were found. Species differed from each other by 0-6 translocations and 0-8 paracentric inversions. Meiotic abnormalities appeared to be the primary causes of sterility. The cytogenetic analysis of *H. annuus* and *H. argophyllus* revealed that these species differ by two reciprocal translocations. Quillet et al. (1995) analysed 48 segregating genetic markers in BC_1 hybrids of these two species. These markers were assigned to eight linkage groups. Three genomic groups located on linkage group 1, 2, and 3 were able to explain about 80% of the high variation (27-93%) in pollen viability. Chromosomal rearrangements (translocations) caused abnormality in meiotic behavior, resulting in inviability of pollen. Rieseberg et al. (1995) analysed introgression in rearranged chromosomal regions. They followed the movement of 197 molecular markers in backcrossed hybrids of *H. annus* and its divergent wild relative *H. petiolaris*. Introgression was significantly higher (94%) in collinear than in rearranged portions of the genome. This reduced recombination rate within the rearranged chromosome portions showed selection or barriers against introgression. However, the introgression in collinear portion was also not perfect. Sixty percent of the markers from that region could not introgress in the hybrids. This led the researchers to suggest that chromosomal rearrangements are not the sole barrier to introgression. Genic factors are also involved in creating barriers to introgression and recombination in interspecific hybrids.

2.3.5 Genic contribution

A gene is selected for its interaction not only with the external environment but also with other genes present in the genome (Hancock, 2004). Phenomena like pleiotropy, epistasis and special combining ability show important intragenomic interactions. Due to these interactions, the whole genome, instead of a particular gene(s), is a selection entity. However, the harmonious interactions among genes due to co-adaptation are disturbed by wide hybridization, which leads to hybrid breakdown. This disturbance increases with increase in developmental complexity of the organism. For example, hybrid breakdown is reported more in animals than in plants. The co-adapted evolution of nuclear-organelle interactions, for example in fertility restoration, is also sensitive to any change in this relationship. Hanson and Bentolila (2004) reviewed the role of interactions between nuclear and mitochondrial genomes in plants affecting male gametophytic development. Presence of different nuclear restorer loci indicates the possibility of multiple forms of nuclear cytoplasmic interaction involved in pollen viability and fertility. Interspecific hybridization may result in cytoplasmic male sterility not only due to disturbed nuclear-cytoplasmic interaction but also by making changes in the mitochondrial genome (Hanson and Conde, 1985).

Wan et al. (1996) discussed allelic interactions causing gamete abortion in rice hybrids. The loci S-5 and S-8 present on chromosome 6 and the S-7 locus present on chromosome 7 were found to be involved in female gamete abortion of hybrid rice. The neutral S-5n allele is a wide compatibility gene, capable of maintaining fertility of hybrids. The researchers reported presence of two additional loci i.e. S-9 and S-15, along with their neutral alleles, S-9n and S-15n, involved in fertility of rice hybrids. These neutral alleles combined with S-5n in a breeding line resolved the issue of hybrid sterility in wide hybridization of rice.

Li et al. (1997) studied quantitative trait loci (QTL) involved in hybrid sterility and hybrid breakdown in rice. They studied hybridization of rice cultivars Lemont and Teqing. Lemont, belonging to subspecies Japonica, is highly compatible with subspecies Indica. Cultivar Teqing, in spite of belonging to subspecies Indica, results in hybrid sterility when crossed with Japonica varieties. Reciprocal crosses showed that cytoplasmic gene(s) influence hybrid fertility. Lemont cytoplasmic gene(s) were supportive to the fertility of the F_1. QTL analysis showed that incompatibility at an epistatic locus led to sterility. An interaction between a pair of putative complementary genes was found to cause spikelet sterility. These genes influenced recombination frequencies between homologous chromosomes. The researchers identified five genomic regions having groups of tightly linked genes affecting plant fitness. Recombination in these so-called "supergenes" resulted in reduced fertility, plant height and grain numbers per panicle of F_4 hybrids. These supergenes have become co-adapted during the evolutionary selection process and have epistatic genetic effects. The recombination in these genes disturbs their interactions and results in breakdown of hybrids. This disturbance in co-adapted complexes has little effect on F_1 fertility. In conclusion the researchers proposed that cytoplasmic gene(s), putative complementary gene pairs and a co-adapted gene complex were involved in the success of hybridization of Lemont and Teqing cultivars of rice.

Shaw et al. (1986) were able to isolate and independently assess the relative chromosomal and genic contribution in reproductive isolation. They investigated three subspecies of grasshopper (*Caledia captiva*) i.e., Moreton, Torresian and Lake Entrance. Moreton and Torresian differ chromosomally by having a different series of pericentric rearrangements and genically by showing different allozyme patterns. Their BC_1 and F_2 hybrid generations have viability of 47% and 0%, respectively. Moreton and Lake Entrance were genically similar but have chromosomal differences. Their BC_1 and F_2 generations had 61% and 58% viability, respectively. In contrary, Lake Entrance and Torresian were chromosomally similar but genically different. The viabilities of their BC_1 and F_2 were 69% and 46% respectively. Inviability in F_2 generation due to chromosomal and genic divergence was 58% and 54%, respectively. However, if parents were divergent on both, chromosomal and genic basis, the viability of F_2 offspring drastically declined to 0%.

2.3.6 Endospermic contribution

The inviability or sterility of a hybrid caused by a post fertilization barrier can be due to failure of either the endosperm or zygote. Endosperm plays an important role in the development of an embryo as it is the source of nutrition to the developing embryo, and in many cases to the young seedlings. During pollen tube growth into the pistil, the generative nucleus of the pollen grain divides mitotically into two male gametes or sperms. One sperm unites with the egg and other fuses with two polar nuclei produced by the female parent. This union of two sperms with separate female cells is called double fertilization. In most flowering plants this double fertilization results in a diploid zygote and a triploid endosperm. A normal endosperm possesses maternal to paternal genome ratio of 2:1. This ratio is crucial for normal development of endosperm, and consequently of the embryo, according to the endosperm balance number (EBN) hypothesis. In this hypothesis the genome of each species is assigned an EBN based on results of its crosses with a standard species of known or given EBN. Johnston et al. (1980) explain this theory by assigning EBN of 2 to 2x *S. chacoense* species and taking it as a standard species to determine the EBN for other *Solanum* species. As 4x *S. acaule* successfully crossed with 2x *S. chacoense* and produced viable seeds, it was also given the same EBN i.e., 2. In another case, 4x *S. tuberosum* successfully crossed with colchicine-induced 4x S. *chacoense* but could not cross with 2x *S. chacoense* or 4x *S. acaule*, hence 4x *S. tuberosum* received an EBN of 4. The 2x *S. tuberosum* having EBN 2 (half of EBN of its 4x form) predicted to be cross compatible with 2x *S. chacoense* and 4x *S. acaule*. This theory made researchers able to predict and use a wide range of species in interploidy-intraspecific and interspecific hybridization, based on their EBN.

Parrott and Smith (1986) surveyed the reported results of interspecific crosses at different ploidy levels in *Trifolium*. The EBN hypothesis successfully explained the outcomes of these crosses. The researchers suggested haploid (2n=1x) *T. occidentale* as a reference species with EBN of 1. They then assigned EBN to *Trifolium* species e.g. 2 to 2x *T. occidentale*, 4 to 4x *T. occidentale*, 4x white clover and 2x *T. nigrescens*, 8 to 4x *T. nigrescens*, etc. According to Carputo et al. (1999), EBN is a powerful isolating mechanism causing incompatibility between sympatric species and preserving genomic integrity of *Solanum* species. They developed triploid and pentaploid hybrids between haploids *S. tuberosum* and *S. commersonii*. These odd ploidy hybrids were used in inter- and intra-EBN crosses. The seed production results of these crosses were explainable according to the EBN theory. The researchers concluded that EBN is a crucial determinant in the success of intra- and inter-ploid interspecific via normal endospermic and embryonic developments.

Hussain and Williams (2008) successfully applied gametic EBN to explain outcomes of complex polyploid crosses involving uneven genomes i.e., 5x and 7x. They used EBN at gamete/haploid levels i.e., r (for white clover) = 1 and n (for *T. nigrescens*) = 2. They studied different backcrosses of a colchicine-induced 6x interspecific hybrid between white clover and *T. nigrescens*. The backcross of the 6x hybrid (rrrrnn) with white clover (rrrr) resulted in a 5x BC_1F_1 hybrid (rrrrn). This 5x BC_1F_1 hybrid produced gametes of three types i.e., rr (EBN = 2), rrn (EBN = 4) or rr 0.5n (EBN= ~3). The meiosis of most pollen mother cells (PMC) of this 5x BC_1F_1 showed a 20-20 disjunction of chromosomes confirming formation of rr 0.5n (EBN= ~3) pollen. As the pollen is more abundantly produced by a plant than eggs, there are more chances of successful seed setting if this 5x BC_1F_1 plant is used as a male parent with a female parent having the same gametic EBN i.e., 3 or ~3. This explains the production of 8 seeds from crossing white clover (rrrr) as female parent with 5x BC_1F_1 (rrrrn) as male parent as compared to 1 seed produced by reciprocal crosses, from same the number (160) of crosses in both directions. Similarly, about 67% of the seed produced by hybridization between 4x BC_1F_1 (rrnn) and 5x BC_1F_1 (rrrrn) were from crosses when the 5x BC_1F_1 was used as the male parent. Moreover, the surviving mature plants were aneuploid with 2n = 36. This confirmed the preferential union of rr 0.5n (EBN = 3) pollen, despite availability of rr (EBN=2) and rrn (EBN=4), with rn (EBN = 3) due to similarity of EBN. This demonstrates the functioning of gametic filters in breeding of complex genotypes. Similar EBN at gametic level can be provided by similar ploidy or EBN of the parents or unreduced gametes of a parent having an EBN that is half of the other parent.

2.3.7 Genomic imprinting

Genomic imprinting is an epigenetic phenomenon in which expression of a gene is dependent on its maternal or paternal origin. Gutierrez-Marcos et al. (2003) used modified Allelic Message Display (AMD) technique to identify biallelic, dosage dependent, exclusively paternal and exclusively maternal allelic expression in endosperm. The observations showed that expression of a PEG1 sequence is paternally imprinted in endosperm. Its expression is normal (biallelic) in the embryo. It was also discovered that a small gene family having maternal imprinting expressed in the endosperm. Endosperm, being the product of two female and one male gametic nuclei, has a disturbed genomic ratio of male and female parents, compared to embryo. Development of the endosperm was found to be highly sensitive to any change in allelic copy number, sequence or epigenetic imprints (Gutierrez-Marcos et al., 2003). Haig and Westoby (1991) built up their model on the assumption that genomic imprinting patterns evolve during adaption by natural selection and become fixed for a population. Such patterns differ from species to species. An interspecific cross is expected to combine different imprinting patterns in the hybrid. This results in imbalanced and abnormal expression of imprinted genes, especially in the endosperm. The cross between a diploid and its autotetraploid disturbs the natural balance of imprinted genes and results in incompatibility and failure. Indeterminate gametophyte (*ig*) is a mutant allele in maize that generates different numbers of polar nuclei for secondary fertilization. Lin (1984) used this mutant to generate 12 classes of embryos having different composition of maternal to paternal genome in endosperm. The observation showed that embryo having 2x:1x composition of maternal: paternal genomes in endosperm, developed normally. The 3x:1x maternal : paternal ratio in endosperm produced half sized kernels. However, this abnormal endospermic genomic composition supported normal growth of embryo resulting in normal seed germination. The diploids having 1x:1x and tetraploids having 2x:2x maternal : paternal genomic ration in endosperm were both severely defective, resulting in embryo abortion. These results show adverse effects of presence of an extra genome in endosperm. Tetraploid endosperms were also produced by crossing wild-type diploid females to tetraploid males having maternal to paternal ratio of 2x:2x. In this case, the tetraploid kernels were small, shriveled, mostly inviable and weighed much lesser than kernels having 3x:1x maternal to paternal ratio. This indicated that parental source was effective in kernel development of the tetraploid endosperms.

2.3.8 Rapid recovery of the recurrent parent

In backcrossing, interspecific hybrids often revert back to their recurrent parents much faster than expected. Stephens (1949) followed segregation patterns of eight independent marker genes in an interspecific hybrid between two cotton species. The line of *Gossypium hirsutum* was maintained to carry the six dominant and two recessive independent marker genes. The inbred line of *G. barbadense* carried contrasting alleles to those carried by *G. hirsutum*. The interspecific hybrids of these species were heterozygous for the eight marker genes. The backcross of their hybrid was expected to result in 1:1 phenotypic expression of hybrid to recurrent parent. But the actual ratios were significantly skewed towards the recurrent parent in most of the cases. For example seven segregates of the backcross having *G. hirsutum* as recurrent parent were studied. Five of them showed higher than expected expression of *G. hirsutum* genotype, whereas, two segregates showed higher than expected phenotypic expression of *G. barbadense*. Similarly, in the BC_1 with *G. barbadense* as recurrent parent, four out of five segregates showed excess expression of alleles from the *G. barbadense* parent. In that study, selective elimination of alleles from the donor parent was expressed by alleles present on only seven out of total 26 chromosome pairs. The cumulative recombinant effect on differentiated chromosome segments present on all 26 chromosome pairs was expected to be effective in the rapid elimination of most of the donor genotype in backcrossing. The direction of cross did not affect the results. It showed that at that stage parental sex did not contribute in the distorted segregation. However, F_1 plants proved to be much better male contributors rather than female contributors in making viable BC_1 seeds. The researchers hypothesized that the co-adapted polygenic complexes of the donor parent and any other recombination involving them faced rapid selective elimination during backcrossing. It was suggested that different mechanisms like seed abortion and inappropriate flowering time, create selection pressure against the recombined chromosome segments, resulting in unexpectedly high rate of elimination of the donor genotype and recovery of the recurrent parent in backcrossing of an interspecific hybrid in cotton.

Irrespective of rapid loss of the donor parent genome in backcrossing, interspecific hybridization has been proved a useful technique for stable transfer of desired traits from one species to another species. Jena et al. (1992) analysed the BC_2F_8 generation of a hybrid between *Oryza sativa* and its wild relative *O. officinalis* for introgression of *O. officinalis* genome segments into the genome of *O. sativa*. Selection was made for insect resistance and general agronomic traits at each generation during development of the BC_2F_8. The RFLP analysis showed that 16.1% of total alleles in hybrids were of *O. officinalis* origin. These introgressed segments were present on 11 of the 12 chromosomes. In most cases the alleles of *O. officinalis* appeared to have replaced alleles of *O. sativa*. Comparison of early and late generations showed similar patterns of introgression. Resistance to brown plant hopper was successfully introgressed from the wild species (*O. officinalis*) into some introgressed hybrid lines. Mao et al. (1995) analysed RFLPs for F_1 and F_2 progenies of a hybrid between *O. alta* (2n=48) and *O. sativa* (2n=24). It was observed that about one third of chromosomes were lost in partially fertile plants. One of the two semi-fertile plants showed presence of awn traits from the wild parent in these early generations. LeRoy et al. (1991) reported that seed weight of soybean (*Glycine max*) can be reduced by interspecific hybridization. The seed weight of cultivated soybean is more than 100 mg per seed which is undesirable for some soybean derived food products. One of its wild relatives, *G. soja*, has a seed weight of less than 30 mg. The researchers backcrossed hybrids of *G. max* and *G. soja* to *G. max* as recurrent parent to produced BC_3. Selection for low seed weight was imposed at each generation. This backcrossed generation was then selfed for two seasons to produce F_2. The resultant BC_3F_2 generation showed seed weight of $\leq$ 100 mg per seed for 37% of its population.

2.3.9 Contribution of mating designs

Viable, fertile and recombinant hybrids are required for a successful interspecific hybridization program. Wall (1970) investigated the effect of mating systems on three features of interspecific hybrids of *Phaseolus vulgaris* and *P. coccineus*. Sib-mating and selfing resulted in introgression as a result of improved recombination between parental genomes. This enhanced recombination was directly proportional not only to introgression but also to improved viability and fertility. Selfing produced ill-balanced embryos resulting in hybrid breakdown. In contrast, backcrossing improved viability and fertility but reduced introgression due to decrease in recombination. Sib-mating in alternation with backcrossing gave optimum recombination, viability and fertility, resulting in a heterospecific genome. Haghighi and Ascher (1988) hybridized *P. vulgaris* and *P. acutifolius* and observed the typical outcomes of backcrossing i.e., improvement in viability and fertility and loss of traits of the donor parent. However, when the interspecific hybrids were backcrossed to each of their parental species in alternate generations i.e., congruity backcrossing, new traits appeared along with retention of parental traits in the hybrids. Congruity backcrossing also showed marked improvement of developmental abnormalities and hybrid breakdown, compared to conventional backcrossing. The intermediate plants obtained from congruity backcrossing were similar to F_1 hybrids but with increased fertility. Backcrossing increased viability and fertility by providing fully functional gametes from the recurrent parent. In contrast, alternating parents in backcrossing resulted in more recombined advanced interspecific hybrids. Compatibility of these hybrids was so improved that they crossed more readily with other *Phaseolus* accessions than with their parents. This feature made these hybrids a possible bridging means for gene transfer from other *Phaseolus* species.

Rieseberg et al. (1996) employed Randomly Amplified Polymorphic DNA (RAPD) markers to follow introgression of *H. petiolaris* genes into *H. annus* in three mating designs. One or two generations of sib-mating prior to backcrossing gave more efficient introgression (both in collinear and rearranged positions of chromosomes) than backcrossing followed by sib-mating.

2.4 WIDE HYBRIDIZATION IN AGRICULTURE

The evolutionary journey of crops has been directed by conscious and unconscious human efforts based on persistent selection, national and international propagation and hybridization (Ulukan, 2009). Interspecific hybridization with wild relatives is a focused breeding method to improve tolerance to diseases, pests, unfavourable environmental effects and to increase persistence (Repkova et al., 2006). It can introgress desirable genes from wild species to cultivars of interest. Interspecific hybrids can also be extremely useful sources of new genetic combinations produced as a result of crossing over and breakage of linkage groups. Heslop-Harrison (2002) discussed the possibility of exploiting novel germplasm. The cultivated food plant species are about 200 and there are about 250,000 available flowering plant species. A huge genetic resource remains unused. This untapped genetic resource can be directly bred into new crops or can also be a source of desirable genes to improve existing crops.

According to Pimentel et al. (1997), usage of wild relatives of crops in genetic modification and breeding has led to a 30-50% yield increase since 1945. This contribution was worth $ 115 billion per year worldwide. Hajjar and Hodgkin (2007) reviewed use of wild relatives in crop improvement from the mid-1980s to 2005. They focused on 30 main crops. Wide hybridization appeared to be a focused and active research area. There was a steady increase in numbers of improvements developed by using wild relatives in wide hybridization. The main traits improved through this process were pest and disease resistance, yield, quality, male sterility or fertility restoration and abiotic stress tolerance. Pest and disease resistance was the main area of improvement, whereas yield was the least successful trait. The reason for this might be the relatively poor agronomic potential of wild relatives. Despite considerable wild germplasm collection and techniques to overcome crossability, wide hybridization has not achieved its full potential. Intense efforts, time and expenses required to remove undesired traits of wild parents from hybrids is a big issue. However, modern genomics and genetic engineering tools have potential to facilitate wide hybridization.

2.4.1 Early history

Zirkle (1932) reviewed the contributions of early researchers in revealing the phenomenon of plant hybridization. According to this review, Cinerarias in 1694 experimentally showed that pollen grains are required for successful seed production. Mather in 1716 reported his observations on pollination by wind and the phenomenon of hybridization. Spontaneous hybridization was first reported by Bradley in 1717 in several varieties of *Pyrus, Malus* and *Primula auricula*. In the same year, Fairchild reported production of the first artificial hybrid. He crossed two species of *Dianthus* i.e., *D. caryophyllus* (carnation) and *D. barbatus* (Sweet William), to produce an interspecific hybrid. That hybrid was dubbed as ‘Fairchild’s mule’ due to its infertility and interspecific nature. In 1721, Philip Miller’s observations on spontaneous hybridization in *Brassica*, insect pollination in tulips and sexual reproduction of *Spinacio* and *Cucumis*, were published. Carolus Linnaeus wrote ‘nullae dantur species novae’ (there are no new species). However, in 1742, after observing the hybrid nature of a fertile mutant of *Linaria*, he changed his opinion and proposed that new species could arise via hybridization (Baack and Rieseberg, 2007). Koelreuter, in 1761, presented his work on hybridization in the genera *Nicotiana*, *Kedmia*, *Biathus*, *Mattiola*, *Hyoseyamus*, etc. He systematically studied the process of hybridization and proved that both male and female parents contribute to the offspring. He showed that human manipulation or disturbance of habitat can facilitate interspecific hybridization.

Winge (1917) proposed allopolyploidy as a possible mechanism of restoring the fertility of an interspecific hybrid. He described how interspecific hybrids can become fertile by two means. In the first case, the chromosomes of both parents are alike and in harmony. They unite in a zygote to form something entirely new. In the second scenario, less harmony between chromosomes of two species restricts their paring in gametogenesis of the hybrid, resulting in its sterility. To overcome this problem, each chromosome has to get doubled to produce its own homologous pair. The hybrids will then have 4x chromosomes instead of 2x. In the reduction division each chromosome will have a pairing partner. The hybrid will produce 2x gametes and will become fertile. Templeton (1981) discussed two routes of speciation through interspecific hybridization. In one way, the interspecific hybrids co-exist with their parents. They gain time by developing mechanisms of asexual propagation, e.g. parthenogenesis. Mutations and karyotypic evolution continue to improve adaptability of the hybrids. Selection for genes favouring diploidization also takes place. Eventually, polyploid hybrids become fertile by becoming structurally diploidized. In the other way, inbreeding results in genetic rearrangements coupled with mutations, and nature selects favourable recombinants. In both cases, interspecific hybrids become reproductively isolated from their parents through genomic and habitat changes.

2.4.2 Artificial wide hybridization

Many successful efforts have been made to use the wide hybridization process for the creation of beneficial products. Burk and Heggestad (1966) reviewed transfer of mosaic and wildfire resistance from *Nicotiana longiflora* and black rot resistance from *N. debneyi* into *N. tabacum* (common tobacco) by doubling the chromosomes of the species involved and then backcrossing them with autotetraploid *N. tabacum* as the recurrent parent. Poehlman and Sleper (1995) described that chromosomes of *Agropyron cristatum* ($2n = 14$) were doubled to make its cross compatible with naturally occurring tetraploid *A. desertorum* ($2n = 28$). An interspecific cross between autotetraploid *A. cristatum* and *A. desertorum* resulted in production of the crested forage wheatgrass ‘Hycrest’. Diploid *Gossypium thurberi* ($2n = 26$) was crossed with diploid *G. arborium* ($2n = 26$). The chromosomes of the hybrid were doubled to make it cross compatible with tetraploid cultivated cotton *G. hirsutum* ($2n = 52$). This interspecific hybridization was used to transfer lint strength from *G. arborium* into *G. hirsutum*. Brar and Khush (1997) discussed the successful transfer of cytoplasmic male sterility, resistance to grassy stunt virus and bacterial blight from wild rice species into cultivated species. Molecular techniques like RFLP and RAPD confirmed introgression from wild species into *O. sativa*. Coman and Popescu (2009) described how the flavor of strawberry (*Fragaria ananassa*) fruit could be improved by crosses with wild relatives like *F. vesca* and *F. moschata*. The linkage of better flavor trait with undesired traits of the wild species could be broken by increasing the numbers and complexity of interspecific hybrids.

Triticale (*Triticosecale*), a man-made allopolyploid, is a commercially successful example of artificial wide hybridization. The general aim of this hybridization was to combine the hardiness of rye into the wheat genome. Lukaszewski and Gustafson (1987) reviewed the cytogenetics of triticale. Initially, the triticale hybrid was a cross between hexaploid wheat (2n = 6x = 42) and diploid rye (2n = 2x = 14) resulting in a vigorous but sterile hybrid. Later, a spontaneous chromosome doubling resulted in the first true breeding octaploid triticale hybrid. The discovery of chromosome doubling with colchicine made possible the production of tetraploid, hexaploid, octaploid and decaploid triticales by using various ploidy levels and species of wheat and rye. Wheat is usually used as the female parent in this cross. In addition to many other factors, presence of two crossability genes Kr1 and Kr2 on wheat chromosomes in dominant form facilitates this hybridization. The octaploid triticale combined the quality of wheat with hardiness and disease resistance of rye. However, the fertility level of this hybrid was low due to meiotic abnormalities. Hexaploid triticale (2n = 6x = 42) was produced by crosses between tetraploid wheat (2n = 4x = 28) and diploid rye followed by doubling the chromosomes of the F1 hybrid. These were termed as primary hexaploid triticales. They had high vegetative vigor but low fertility and shriveled grain production. Secondary hexaploid triticales were generated by backcrossing these primary hexaploid hybrids with hexaploid wheat or crossing with octaploid triticale to overcome chromosomal pairing difficulties. ‘Armadillo’ is a secondary hexaploid triticale being early maturing, fertile and good in kernel characteristics.

2.5 WIDE HYBRIDIZATION AMONG *TRIFOLIUM* SPECIES

2.5.1 The genus *Trifolium*

Trifolium L. is one of the largest genera of the family Fabaceae (subfamily Papilionoideae), comprising of around 250 herbaceous species which are commonly called clovers. This is the most widely spread, distinct and evolved genus of its tribe *Trifolieae*. According to the old taxonomy, this genus consists of 8 sections (Zohary and Heller, 1984). Section *Lotoidea* is the oldest, largest and widest in its distribution. It includes the important perennials *T. repens* (white clover), *T. hybridum* and *T. ambiguum*. The second largest section is *Trifolium* having important species like *T. pratense* (red clover), *T. incarnatum*, *T. alexandrinum*, etc. Ellison et al. (2006) proposed a new infrageneric classification of the genus based on results of phylogenetic study. They placed white clover in a new section *Trifoliastrum* of subgenus *Trifolium*. Their DNA sequences analysis showed that similarities among species are influenced by their geographic distributions. White clover was grouped with 14 species of European and Eurasian distribution in a clade. The genus *Trifolium* is widely distributed in temperate and sub-tropical parts of the world. The Mediterranean and Californian regions are its main distribution centers. Especially Mediterranean region is considered to be the centre of the origin of the genus and is the source of origin of cultivars by domestication and breeding.

Badr et al. (2002) analysed isozyme polymorphism in seven *Trifolium* species to investigate their ancestral relationships. Their analysis revealed that white clover was close to *T. uniflorum* and *T. nigrescens*. However, shared alleles were also detected between white clover and both *T. occidentale* and *T. isthmocarpum*. Pairwise distances indicated that there were strong relationships between white clover, *T. nigrescens* and *T. uniflorum*. They also suggested that *T. occidentale* might have contributed to the genome of white clover but via *T. nigrescens* and *T. uniflorum.* Ellison et al. (2006) comprehensively studied the phylogeny of the *Trifolium* genus. They analysed 218 species representing a wide geographic and taxonomic range. Internal transcribed spacers from nuclear DNA and chloroplast (*trnL* intron) DNA were employed to construct a phylogenetic tree. Their results confirmed a monophyletic and Mediterranean origin of the genus during the Early Miocene period. They found that 16 is the ancestral diploid chromosome number. In the phylogenetic tree of section *Trifoliastrum*, *T. occidentale* and *T. uniflorum* appeared as close perennial relatives to white clover. This study showed that *T. pallescens* (in chloroplast DNA analysis) and *T. occidentale* (in nuclear DNA analysis) are the likely diploid progenitors of white clover. However, combined analysis did not pool these two species in one group.

Williams et al. (2006) explored the section *Trifoliastrum* and extracted a group of eight species which appeared to be closely related to white clover on experimental phylogenetic basis. They named this group as the “white clover complex”. It consists of

T. ambiguum, T. montanum, T. nigrescens, T. isthmocarpum, T. uniflorum, T. occidentale, white clover, *T. pallescens and T. thalii*. The white clover complex has significant diversity in terms of geographic and environmental adaptation, plant morphology, life cycle and tolerance to various biotic and abiotic stresses. The researchers developed a comprehensive interspecific hybridization programme to exploit this valuable genetic diversity.

The study of Williams et al. (2008) showed that 2*x* *T. nigrescens* (both subspecies *nigrescens* and *petrisavii*) and 2*x* *T. occidentale* can be hybridized without much difficulty. These hybrids produced normal F_2, F_3 and BC_1F_1 progeny (with *T. nigrescens* as recurrent parent). Plants of the F_2 and F_3 generations lacked the stoloniferous, perennial nature of *T. occidentale*. There was complete bivalent chromosomal pairing and evidence of introgression from *T. occidentale* into *T. nigrescens* genome in the backcrossed hybrids. This showed incomplete and relatively recent reproductive isolation of these species. Taxonomic closeness, success of hybridization and chromosomal homology showed that *T. nigrescens* and *T. occidentale* were close relatives of white clover. However, based on the nonappearance of stoloniferous perennial plants among hybrids of *T. nigrescens* and *T. occidentale* and the results of DNA analysis by Ellison et al. (2006), the researchers concluded that *T. occidentale* could be a parent of white clover but *T. nigrescens* was probably not. However, this species clearly was a close relative of *T. occidentale*.

Abberton and Thomas (2010) highlighted the importance of conservation of *Trifolium* germplasm from diverse origins. They emphasized the importance of conservation of wild *Trifolium* species for agriculturally important species like *T. pallescens* and *T. occidentale* for white clover, and *T. diffusum* for *T. pratense* breeding programs. The gene banks were found to have largest collections of accessions from wild populations. This could be highly valuable source for future efforts to transfer desired genes from wild types into cultivated species. Williams and Nichols (2011) have also comprehensively reviewed the distribution, diversity and usage of *Trifolium* species.

2.5.2 Wide hybridization

Pandey (1957) suggested that speciation in *Trifolium* has resulted from mutation and chromosomal changes leading to cytological incompatibility among species.

Consequently hybridization among *Trifolium* species is difficult. This general absence of interspecific hybridization has evolutionary value. Zohary and Heller (1984) suggested that a predominance of selfing is the cause of the near-absence of interspecific hybridization. Ellison et al. (2006) found only 5–6 cases of interspecific hybridization among a large sample of 218 *Trifolium* species. This extremely low rate of natural hybridization shows strong genetic barriers for interspecific hybridization in this genus. Attempts at artificial wide hybridization among *Trifolium* species have been done with two wide perspectives, i.e. to understand evolutionary relationships within the genus and to introgress desirable traits into agriculturally important species, mainly red clover and white clover. The success of these efforts, especially for intersectional hybridization, has been low.

Williams (1987b) reviewed interspecific hybridization in pasture legumes with special emphasis to *Trifolium, Lotus and Ornithopus*. The reviewer indicates that prefertilization and postfertilization barriers similar to other plant families prevent interspecific hybridization in these legumes. The genotypes of individual plants involved in interspecific hybridization affect the intensity of incompatibility. Normal pollen tube growth and pod enlargement is taken as an indication of absence of prefertilization barriers. Endospermic inviability can be an active postfertilization barrier for interspecific hybridization in these legumes. During its early development endosperm form a haustorium at the chalazal end of the embryo sac. Failure in formation or attachment of the haustorium with maternal tissues results in failure in development of endosperm. Embryo abortion results due to starvation in absence of endosperm and disturbance in normal water potential gradients of the seed. Embryo rescue and in vitro development can overcome these problems. Normally developing hybrid embryos, usually at early heart stage, are excised from the enlarged pods and placed in normal endosperm (nurse culture technique) or in carefully composed growth media for further growth. Embryos of white clover can be normally grown on EC6 medium without the aid of nurse endosperm.

Red clover (*T. pratense* L.) is a perennial diploid (2n=14) species widely cultivated in most temperate regions. It has high seedling vigor, rapid growth, salinity tolerance and high nutritive value. It is much shorter lived and less grazing tolerant than white clover. Improvement in persistence is one of the major areas of active research for *T. pratense*. Travin (1930) reported success of crosses between *T. pratense* and *T. medium*. Taylor et al. (1963) used *T. pratense* as the female parent and crossed it with 47 *Trifolium* species to explore transferability of longevity of growth period into *T. pratense*. They successfully generated an amphidiploid hybrid (2n=30) by a cross between colchicine-induced tetraploid *T. diffusum* (2n=32) and tetraploid *T. pratense* (2n=28). This hybrid was backcross compatible with both parents. It was intermediate between its parents in most respects but was annual in growth habit. This hybrid was projected as a valuable bridging source for future interspecific hybridization. Taylor and Collins (1989) reported a hybrid between strongly perennial *T. sarosiense* and the weakly perennial *T. pratense* ('Kenstar'). This hybrid had 31 chromosomes, 24 from *T. sarosiense* and 7 from *T. pratense* and expressed complete absence of meiosis and fertility. The hybrid was perennial in nature and could be a useful source of improvement for *T. pratense* if its fertility could have been restored. Phillips et al. (1992) could recover only one F_1 hybrid from crosses between *T. alpestre* and *T. pratense*. That hybrid had completely dysfunctional gametogenesis. A successful attempt to transfer the rhizomatous growth habit of *T. medium* (2n=80) into *T. pratense* (2n=28) was made by Sawai et al. (1995). Chromosomes were doubled by colchicine treatment of the hybrid embryo. The third backcross progeny, using *T. pratense* as recurrent parent, had a range of chromosome numbers (2n= 32 – 42), acceptable fertility and rhizomes. Isobe et al. (2002) observed seed production and vigor of four backcrossed generations (having *T. pratense* as recurrent parent) of these hybrids under field conditions. Fertility and survival improved with advancement in backcrossing. The BC_4 generation had survival rates similar to *T. pratense*. However, the rhizomatous trait did not appear in hybrids after the BC_1 generation. Most of the BC_4 plants had more than 28 chromosomes, and were different from *T. pratense* in leaf size and shape. This indicated the presence of *T. medium* genome in these hybrids. The genomic diversity, acceptable longevity and considerable seed production made these BC_4 hybrids a potentially valuable breeding resource. Kouame et al. (1997) screened *T. pratense* and seven related species against four root-knot nematode species i.e., *Meloidogyne arenaria*, *M. hapla*, *M. incognita* and *M. javanica*. The various host and pest species combinations contributed to the appearance of a wide range of resistance responses. The host response seemed to be associated with plant species life form and root habit. The majority (98%) of *T. pratense* were intermediately or highly susceptible to all four nematode types. More than 50% of *T. alpestre* accessions expressed high resistance to all nematodes species. About 33% of *T. medium* samples were resistant or highly resistant to three nematode species. *T. medium* provided the greatest variation, from highly resistant to highly susceptible. This analysis demonstrated that transfer of root-knot nematode resistance from wild relatives into *T. pratense* by interspecific hybridization might be possible.

Evans (1962) showed the existence of a high degree of incompatibility between *Trifolium* species. Grafting as well as crossing techniques were used to screen for compatibility among nine agronomically important species and eight taxonomically related species of *Trifolium*. Hybridization was significantly greater for graft compatible genotypes than for graft incompatible genotypes. Twenty eight hybridizations between naturally occurring diploids and polyploids resulted in seed production for five combinations. Induced polyploids of white clover, *T. nigrescens*, *T. hybridum*, *T. pratense*, *T. incarnatum* and *T. alexandrinum* were tested in 10 reciprocal combinations. Five hybridizations produced seeds but only one hybrid i.e., white clover (8*x*) x *T. nigrescens* (4*x*) matured into plants.

Chen and Gibson (1972) studied pollen germination, pollen tube growth and fertilization in *Trifolium*. They used white clover as the maternal parent in intra- and interspecific crosses with *T. nigrescens* (2*x*), *T. uniflorum* (4*x*), *T. occidentale* (2*x*), *T. uniflorum* x *T. occidentale* (4*x*), *T. hybridum* (2*x*) and *T. ambiguum* (2*x*). Intraspecific crosses performed clearly much better than interspecific crosses for all studied traits. Pollen germination during interspecific crosses was very slow, compared to intraspecific crosses. In interspecific crosses, pollen of *T. occidentale* (4*x*) germinated faster than other species. However, little difference was observed among other species for pollen growth. The most different pattern from white clover was shown by *T. uniflorum*, while *T. occidentale* showed the least deviated pattern of pollen tube growth. A direct relationship was found between pollen tube growth and fertilization. Species like *T. nigrescens* and *T. occidentale* (2*x*) having normal pollen tube growth similar to that of white clover, fertilized more ovules than species like *T. uniflorum* and *T. ambiguum*, which had abnormal and slow pollen tube growth. The researchers proposed that as pollen tubes contacted only stigmatoid tissues of the pistil during their growth, the compatibility of these tissues with pollen is a crucial factor for pollen tube growth. Once the pollen tube enters into the embryo sac, there is no barrier to prevent fertilization. All interspecific crosses involving different species remained successful in fertilization after 24 hours from pollination. Post-fertilization barriers appeared to be responsible for incompatibility of some interspecific crosses. In an earlier study (Chen and Gibson, 1971a), they observed that starvation of embryos was caused by abnormal development and disintegration of endosperm. This starvation eventually led to embryo abortion.

Ferguson et al. (1990) studied the potential of in vitro embryo and ovule rescue for *Trifolium* hybrids. The cross between *T. isthmocarpum* (2x) and white clover (4x) produced highly infertile triploid (3x) hybrids. A *T. ambiguum* x *T. occidentale* hybrid was produced, but it did not flower. A hybrid of white clover with *T. occidentale* failed to produce viable embryos when crossed with *T. ambiguum*. Similarly, cross between white clover and *T. ambiguum* (2*x*, 4*x* and 6*x*) failed either to produce embryos or to regenerate plantlets. An important successful plant regeneration obtained was for the products of crosses between a trihybrid (RUO) of white clover, *T. uniflorum* and *T. occidentale*, backcrossed to *T. occidentale* (as maternal parent) and hexapoloid *T. ambiguum* (as paternal parent) i.e., {[(white clover x *T. uniflorum*) x *T. occidentale* (2n=32)] x *T. occidentale* (2n = 32)} x *T. ambiguum* (2n = 48).

2.6 INTERSPECIFIC HYBRIDIZATION OF WHITE CLOVER

2.6.1 White clover

White clover (white clover) is one of the most important species in the genus *Trifolium*. It is a perennial legume and major companion species of forage grasses in temperate pastures. It has an established contribution in complementing the grass sward, fixing atmospheric nitrogen and, providing nutritious food to livestock and honey bees. It arrived in New Zealand with early settlers in the 1800s and adapted well to different environments, geography and grazing systems of this country (Mather et al., 1996).

According to the classification of Ellison et al., (2006), white clover belongs to section *Trifoliastrum*. Pandey (1957) described white clover as a species with worldwide distribution. Zohary and Heller (1984) described white clover as a rhizomatous perennial species (in fact it is stoloniferous), occurring as several botanical varieties with 2n=16, 28, 32, 48 and 64. It produces nodal roots and bears more than 20 florets per inflorescence. It has long peduncles which are equal or longer in length than the subtending leaves. Its natural habitat includes lawns, grassy places, damp and swampy soils, etc. According to Morris and Greene (2001), this species exists naturally in most of the Europe, Middle East, the Mediterranean region, Asia Minor and West Asia. The agricultural forms of white clover are tetraploid with 2n=32 somatic chromosomes, and two satellites. There is karyotypic similarity among white clover, *T. occidentale*, *T. nigrescens* and *T. petrisavii* suggesting close phylogenetic relationship among these species (Chen and Gibson, 1971b).

White clover has many inbuilt deficiencies like shallow roots, lack of rhizomes, slow seedling development, susceptibility to biotic stresses (e.g. viruses and nematodes), and abiotic stresses, (e.g. heat, drought, and salt). It is adapted to moist temperate climates, and its primary gene pool is deficient in tolerance to drought and related stresses. However, the wide geographical distribution of *Trifolium* species provides an opportunity to improve white clover traits by interspecific hybridization by utilizing its secondary and tertiary gene pools (Williams et al., 2010). Other species of *Trifolium* can serve as possible sources to overcome these deficiencies. For example, *T. ambiguum* can provide rhizomes, deep tap roots and tolerance to drought, root-knot nematodes and viruses. Interspecific hybridization can potentially improve adaptation of white clover to semi-arid and other marginal environments (Williams et al., 2007). The genetic base of white clover has limited potential to improve seed production due to its morphology and high proportion of non-reproductive nodes (Marshall et al., 2005).

2.6.2 Hybridization with *T. ambiguum*

T. ambiguum (Kura clover or Caucasian clover) is a long lived perennial species having rhizomes, thick deep roots and vigorous stems. Its large rhizomatous root mass provides drought tolerance, insect resistance and virus resistance. Its flowers produce a very pleasant scent and abundant nectar. It lacks nodal roots and is a slow to establish clover type. It exists in a series of ploidy levels i.e., diploid, tetraploid and hexaploid adapted to a continuum from high altitude to low altitude respectively (Williams and Nichols, 2011). This species has been hybridized with white clover to introgress its persistency, drought tolerance and biotic stress tolerance into white clover (Abberton, 2007).

Williams (1978) produced mature hybrids between white clover and *T. ambiguum* with an embryo rescue technique using transplanted nurse endosperm. Both parents were tetraploid but their hybrids were highly sterile. Although the hybrids showed potentially good agronomic trends, especially a stoloniferous nature and the leaflet texture of white clover, they were generally intermediate to their parents. In a further attempt (Williams and Verry, 1981), a partly fertile hybrid (H-435) between these two species was produced. Using this hybrid, they also produced F_2 and backcrossed generations with white clover as recurrent male parent. One backcrossed progeny had normal (2n=32) chromosomes, while another appeared to be a pentaploid (2n=42). Both plants showed improved resemblance to their recurrent parent and irregular chromosome pairing during meiosis.

Anderson et al. (1991) assessed the possibility of introgression between white clover and *T. ambiguum* by backcrossing 4*x* and 8*x* H-435 plants. The tetraploid hybrids were self-incompatible, whereas selfing of the octaploid hybrid resulted in an F_2 generation. The backcrossing of 4*x* H-435 with *T. ambiguum* remained unsuccessful, but backcrosses to white clover resulted in BC_1 populations consisting of 4*x*, 6*x* and aneuploid plants. The backcrossing of 8*x* H-435 with white clover and *T. ambiguum* produced hybrids with expected ploidy level of 6*x*. Both 4*x* and 8*x* levels of H-435 showed frequent existence of uni-, bi- and quadrivalent chromosomal associations. The researchers predicted the possibility of successful selection in advanced generations of 8*x* H-435 for improved fertility and persistence.

Pederson and Windham (1989) found 38% of *T. ambiguum* plants resistant to *Meloidogyne incognita*. Difficulty in producing fertile hybrids with white clover was a potential hurdle in transferring this resistance into the latter species. Meredith et al. (1995) produced interspecific hybrids between white clover and *T. ambiguum* (2n = 4*x* = 32) and backcrossed generations with white clover as the recurrent male parent. Most of the BC_1 plants had 48 chromosomes but some were normal i.e., 32 chromosomes, indicating production of both normal and unreduced gametes from the hybrids. Frequent bivalent chromosomal association during meiosis of BC_1 plant with 48 chromosomes showed a stable chromosome balance. Morphology of the BC_1F_1 plants was generally similar to F_1 plants. They were stoloniferous with some evidence of rhizomatous growth. Plants of BC_2F_1 showed more variation in expression of *T. ambiguum* traits than BC_1F_1. Hussain and Williams (1997) overcame the difficulty of hybridizing white clover with *T. ambiguum* by creating a ‘fertile bridge’ between them. The 8x H-435 produced backcrossed seeds with white clover. However, it failed to backcross with the *T. ambiguum* parent. Cytological analysis of BC_1F_1 showed high frequencies of multivalents at metaphase-1. This indicated occurrence of both autosyndetic and allosyndetic pairing and chances of recombination in this hybrid generation. Selfing of these hybrids resulted in appearance of improved traits like increased fertility, nodal rooting, nodulation and appearance of combined stoloniferous and rhizomatous growth habit in BC_1F_2 plants. One of these plants was found to be cross compatible with *T. ambiguum* resulting in a 6x BC_2 generation. Being cross compatible with white clover and *T. ambiguum*, the plants of the BC_1F_2 or BC_2F_2 generation could be an efficient 'fertile bridge' between these two species.

Marshall et al. (2001) investigated the performance of white clover, *T. ambiguum* and their hybrids under drought stress. The purpose of hybridization and backcrossing was to in-corporate the rhizomatous characteristics of *T. ambiguum* into white clover genotypes. They studied parents and backcrossed generations in monocultures as well as in mixed culture with perennial ryegrass. The monoculture experiment presented clear results with better drought tolerance indications for *T. ambiguum* in terms of relative water content (RWC), leaf water content (LWC) and leaf water potential (LWP), compared to those of white clover. The performances of BC_1 and BC_2, with white clover as recurrent parent, were generally in-between the parents. However, dissimilarity between results of the monocultures and mixed cultures was observed. Mixed culture produced complex results. For example, BC_1 and BC_2 retained lower LWC even than white clover in mixed culture. Differences were also apparent for LWP, with *T. ambiguum* and white clover having the highest and lowest values, respectively and BC_1 and BC_2 in-between them in monoculture, however in mixed culture, the order was BC_2 > white clover > *T. ambiguum* > BC_1. In particular the BC_2 made a dramatic shift from the lowest to highest rank during the third week of high water stress. The difference between the results of monoculture and mixed culture could be linked with the poor competitiveness of rhizomatous development in *T. ambiguum*. As the BC_1 and BC_2 generations also had some rhizomes, along with stolon characteristics, they behaved differently in mixed culture (competition with grass) as compared to monoculture. The dry matter yield of the BC_1 followed the behavior of *T. ambiguum*, and increased gradually with increase in water deficiency. However, the BC_2 generation showed similarity to white clover and did not show increase in dry matter production with increasing water stress. This response was only studied in monoculture. In general, BC_1 and BC_2 combined the rhizomatous trait with stolons in a white clover genetic background. They showed improved RWC and LWP over white clover under water deficit conditions in monoculture. The production of dry matter by the BC_1 was near to that of white clover with improved drought tolerance. But this combination did not follow into the BC_2 population. The researchers emphasized the need to improve dry matter yield under water deficit conditions. This work showed the importance of study under mixed culture to have results with application to field conditions and to study seedling vigor for better competitiveness under natural mixed cultures. The *T. ambiguum* parent used in this study was tetraploid (Meredith et al., 1995).

Williams and Hussain (2008) explored suitable breeding strategies to combine desirable traits of *T. ambiguum* and white clover. They employed a colchicine doubled 8x hybrid of these species to create four backcrossed (white clover as recurrent parent) and four segregating generations. The intercrossed BC_1 generations i.e., BC_1F_1 – BC_1F_4, showed predominantly *T. ambiguum* traits. They had stable chromosome numbers (6*x*). Their roots were better in thickness and nodulation than white clover but lacked rhizomes. They were inferior to white clover in production of dry matter, nodes, stolons and inflorescences. Fertility was lower in the BC_2F_1 generation due to the 5*x* chromosome number which would lead to abnormal chromosome pairing in subsequent generations. Advanced generations backcrossed with white clover were comparable with white clover in terms of nodulation, stolon thickness, nodes and internodal length. However, they showed inferior dry weight, root thickness, numbers of stolons, nodal rooting and inflorescence production. Based on this experiment, the researchers recommended advancing generations of BC_1 by intercrossing and selecting for combinations of desired traits. They also suggested that selection, especially for vegetative traits, should be delayed for at least two years.

2.6.3 Hybridization with *T. occidentale*

T. occidentale (Western clover) is a wild perennial diploid species with a stoloniferous growth habit. It is pre-dominantly self-pollinated and has little genetic diversity. However, populations from north-western Spain are cross pollinated and have high genetic diversity. Due to its adaptation to a narrow range of maritime habitats and lack of genetic diversity, it has no significant contribution to agriculture. Its self-pollinated diploid nature, crossability with white clover, and closeness to white clover on a phylogenetic basis has made it a model species for genetic and genomic research related to the white clover complex (Williams and Nichols, 2011). *T. occidentale* is probably a parent of white clover (Ellison et al., 2006). A meiotic study of hybrids between white clover and *T. occidentale* (diploid and autotetraploid) showed homology between chromosomes of the two species (Chen and Gibson, 1971b). *T. occidentale* may be a source of persistence, resistance to some viruses, tolerant to salinity and drought (Gibson et al., 1971; Williams, 1987b).

Being identified as an ancestor of white clover, *T. occidentale* can potentially serve as a bridge to transfer genes of interest into white clover from other species like *T. ambiguum*, which are not easily crossable with white clover (Ellison et al., 2006; Williams et al., 2010). Gibson and Beinhart (1969) studied crossability of *T. occidentale* with white clover and *T. nigrescens* subspecies *nigrescens*. They observed that these three species are directly crossable with each other, indicating their close phylogenetic relationship. In particular, crossing between white clover and *T. occidentale* was relatively easy and could be used to exploit hybridization between these two species. Crossing between *T. nigrescens* (2n=16) and colchicine-induced tetraploid *T. occidentale* (2n=32) was much easier and successful than crosses involving diploid *T. occidentale* (2n=16). Three F_1 hybrids were successfully produced by *T. occidentale* (2n=32) x *T. nigrescens* (2n=16). They were triploid (2n=24), expressed meiotic abnormities and were highly sterile. They had perennial growth with non-rooting prostrate stems instead of stolons, and were morphologically more like *T. occidentale* than *T. nigrescens*. These triploid hybrids were backcrossed with *T. occidentale* (2n=32) and diploid *T. nigrescens* (2n=16). The BC_1F_1 having *T. nigrescens* as the recurrent parent was tetraploid (2n=32), self-fertile, weakly perennial with procumbent stems and crossable with its all three parents. Although the researchers could not develop an allotetraploid form (a synthetic white clover) as a result of crossing between *T. occidentale* (2n=32) and *T. nigrescens* (2n=32) to verify that these two species were parents of white clover, they suggested that other subspecies of *T. nigrescens* might be more successful for such efforts.

Williams et al. (2010) crossed *T. pallescens* and *T. occidentale* as they were identified as the probable maternal and paternal ancestors, respectively, of white clover. Three normal diploid plants were produced by embryo rescue. These plants were open pollinated in the presence of white clover, and resulted in production of tetraploid hybrids. These hybrids had a white clover appearance and were inter-fertile with it. This demonstrated the possibility of generating ‘synthetic white clover’ from its proposed parental species. These researcher also effectively progressed the concept of ‘genetic bridging' in white clover breeding. Four different interspecific hybrids were successfully made involving *T. occidentale* as one parent. Cytological analysis showed chromosome pairing and thus chances of recombination in these hybrids.

These researchers also reported hybrids between 2*x* and 4*x* *T. occidentale* and 4*x* white clover. Chromosomes of 2x *T. occidentale* were doubled and fertile hybrids with white clover were produced. The evidence of interspecific chromosome pairing showed the possibility of introgression of drought and salt tolerance into white clover by backcrossing of these hybrids with white clover as the recurrent parent.

2.6.4 Hybridization with *T. nigrescens*

T. nigrescens is a diploid (2n=16) annual Mediterranean species producing extensive long stems from a single crown. It has at least three subspecies i.e., *nigrescens*, *petrisavii* (ball clover) *and meneghinianum*. Only subspecies *petrisavii* has commercial value due to its high protein forage. It also expresses high seed production potential (Williams and Nichols, 2011). It has a close ancestral relationship with white clover. It has profuse flowering and resistance to clover cyst nematode, valuable traits desired to be transferred into white clover (Abberton, 2007).

Brewbaker and Keim (1953) reported production of hybrids from reciprocal crosses between colchicine-doubled white clover and *T. nigrescens*. These hybrids had 48 chromosomes, were fertile and crossable with each other and with their doubled parents. Gibson and Beinhart (1969) made F_1 hybrids of *T. nigrescens* and *T. occidentale* and then successfully backcrossed them with white clover. Pederson and Windham (1989) found that *T. nigrescens* was a potentially valuable source to transfer resistance against southern root-knot nematode (*M. incognita*) into white clover. They screened white clover, *T. uniflorum, T. occidentale, T. nigrescens, T. isthmocarpum,* T. *hybridum, T. ambiguum*, and *T. argutum* and seven interspecific hybrids involving these species, for resistance against this nematode. The mean gall indexes of 32% of *T. nigrescens* plants were 0 to 1, showing their resistance against this pest. This species and its backcrossed hybrids with white clover showed the highest resistance with mean gall indexes of 0.8 and 0.0, respectively. These hybrids were fertile and expected to be a good source for further improvement. Hussain et al. (1997a) successfully transferred resistance to clover cyst nematode from *T. nigrescens* into its hybrids with white clover. They produced a sterile triploid hybrid by the embryo rescue technique and doubled its chromosome number to improve its fertility and utility in a breeding scheme. Both 3*x* and 6*x* hybrids showed similar resistance to clover cyst nematodes as that of *T. nigrescens*. Their meiotic studies (Hussain et al., 1997a) of F_1 and BC_1F_1 hybrids showed presence of allosyndetic pairing, indicating possible genetic exchange between these two species.

Marshall et al. (1998) attempted to transfer high intensity and less intermittence of inflorescence production from *T. nigrescens* into white clover by interspecific hybridization. They observed that plant growth during the flowering stage was an influential factor in seed yield of white clover as it causes an indeterminate and intermittent pattern of inflorescence development. The F_1 hybrids were successful in expressing more inflorescences than white clover but they lost the nodal root formation and also produced no seeds. However, backcrossed generations regained higher florets per head and nodal root production and sustained almost double the production of inflorescences of that of their recurrent parent, white clover. The suppression of nodal roots in F_{1s} (F_1 plants with small leaves) generation and their recovery in BC_{1s} generation indicated the recessive nature of genes involved in this trait. Both BC_1 and BC_2 generations produced numbers of seeds per floret intermediate to their parents. They also showed lower reserves of water soluble carbohydrates and lower root dry weight, indicating their lower persistence than white clover. The researchers observed a wide range of variation for all observed traits in the BC_2 generation. They concluded that BC_2 is an appropriate generation in an interspecific hybridization program to apply selection pressure to gain economic as well as reproductive trait improvement.

Marshall et al. (2002) conducted a field evaluation of second and third backcrossed generations of these hybrids. The backcrossed generations produced more inflorescences and florets per inflorescence than white clover, but this did not make them more productive in terms of seed yield, compared to white clover. Higher number of seeds per floret, seeds per inflorescence and seed weight of white clover made it a significantly higher seed producer than its backcrossed progenies. There were highly significant correlations between seed yield and all studied reproductive characteristic, except floret numbers per inflorescence. Peduncle length was found to be independent of seed yield but positively correlated with plant fresh weight. Significant variation was observed for reproductive and vegetative traits in the BC_3 generation. Peduncle length and growth were improved in the BC_3. These plants retained the pattern of axillary bud production of white clover, resulting in similar persistence to white clover. The study showed that increase in number of florets per inflorescence and inflorescences per plant did not improve seed yield per plant. Rather, it was the combination of seed components with seed harvestability that effected overall seed yield. Marshall et al. (2005) grew these hybrids under rotational sheep grazing with perennial ryegrass as a companion over three harvest years. They compared their persistency and forage yield with small, medium and large leaved white clover varieties. In general, the forage yields of BC_2 and BC_3 were comparable with small and medium leaved varieties and better than large leaved control varieties. For number of stolon and growing point density traits, the backcrossed generations were inferior, comparable and superior to small, medium and large leaved white clover varieties, respectively. The backcrossed generations maintained an acceptable clover proportion (0.3/year) and did not affect the growth of companion perennial ryegrass. In a later field experiment, Marshall et al. (2008) found that BC_3 plants were less intermittent in flowering pattern, had a higher proportion of consecutive reproductive nodes, more inflorescences and a higher potential seed yield than the medium leaved white clover variety ‘Aberherald’. No significant differences in most of the stolon traits were evident. The researchers classified BC_3 plants on the basis of leaf size. They intercrossed within each category and produced populations of small [BC_3 $(F_2)_s$], medium [BC_3 $(F_2)_m$] and large [BC_3 $(F_2)_l$] leaved plants. They compared these BC_3 groups with small, medium and large leaved white clover varieties. Both [BC_3 $(F_2)_m$] and [BC_3 $(F_2)_l$] produced more inflorescences and potential seed yield as compared to their respective control varieties. However, [BC_3 $(F_2)_s$] could not supersede its control white clover variety Aberdale. This was thought to be due to the fact that Aberdale was developed for high seed yield. There were no differences in onset or peak of flowering between the different groups of BC_3 and their respective white clover control varieties.

The above mentioned work of Marshall et al. (2008) showed that, through backcrossing and selection, reproductive traits can be introgressed from *T. nigrescens* into white clover without sacrificing dry matter yield and persistence. Williams et al. (2010) developed genetically stable backcrossed populations showing introgression of *T. nigrescens* into white clover. They subjected them to screening for tolerance to diseases and pests, and agronomic performance.

2.6.5 Hybridization with *T. uniflorum*

T. uniflorum is a tetraploid (2n = 32), single crowned wild species of Mediterranean origin. It has small plant size and does not have the stoloniferous spreading nature and productivity of white clover. It is adapted to dry environments, has no economic importance and is highly variable (Williams and Nichols, 2011). Zohary and Heller (1984) described *T. uniflorum* as a perennial species with a woody taproot, producing 1–3 (-5) flowered inflorescences. It has a short peduncle which is shorter than the subtending leaves. It grows in fields, roadsides, maquis and mountainous slopes from sea level to 1300 meters altitude. Generally, it is found in South France, South Italy, Sicily, Greece, Crete, Libya and Turkey. Pandey (1957) described *T. uniflorum* as a localized species of alpine regions of the Mediterranean area. Morris and Greene (2001) placed *T. uniflorum* along with *T. argutum* and *T. nigrescens*, in the secondary gene pool of white clover on the basis of their hybridizing potential with white clover. Theodoridis and Koukoura (2006) reported that *T. uniflorum* has been found from 1500 – 2000 m altitude in the mountains of Greece. Cocks (2001) indicated that *T. uniflorum* was distributed in south Europe, Turkey and North Africa (Libya) and identified it as a potential perennial pasture legume species. The Mediterranean origin and adaptation to dry conditions was expected to make this species useful in phase farming systems in southern Australia.

Zohary and Heller (1984) described *T. uniflorum* as a tetraploid species with 32 as the somatic chromosome number. Gibson and Chen (1971) studied the cytology and reproduction of 36 plants of *T. uniflorum*. This species appeared to be mostly self-incompatible, having an average of 7.9 ovules per pod, producing 3.2 seeds per pod by crossing. The occurrence of frequent quadrivalents at meiosis-1 and four satellites, led the researchers to suggest an autotetraploid origin for this species.

Pandey (1957) crossed *T. uniflorum* (as female parent) with white clover to investigate the evolution of self-incompatibility. He successfully obtained one hybrid plant out of 30 seeds sown and this plant was self-fertile. To explain this self-fertile outcome of a cross between self-incompatible parents, he proposed that S alleles might be present on different chromosomes or loosely linked on the same chromosome of the two species. The self-fertile plant was reciprocally crossed with both parents. Potential value of this F_1 hybrid was emphasized, and it was later on (Pandey et al., 1987) predicted that the larger seed size of *T. uniflorum* might improve the seedling vigor of the hybrids. The work of Pandey et al. (1987) showed the potential of interspecific hybridization for generating desirable genetic variation and exchange between white clover and *T. uniflorum*. They observed quadrivalent associations of chromosomes in pollen mother cells of hybrids. Their backcrosses with white clover as recurrent parent showed inter-genomic pairing of white clover chromosomes. Hybrids were found to be intermediate between the parents for recorded parameters. The most vigorous hybrids showed stronger and deeper main tap roots, compared to that of white clover. It is generally believed that the female parent is more influential than the male parent due to cytoplasmic effects in hybrid production. This study indicated that the hybrids of RAZ-3 Huia line of white clover crossed with lines 520 and 521 of *T. uniflorum* as male parent were generally more inclined towards *T. uniflorum*. Complexity was evident, as line 442-2 of *T. uniflorum* crossed with line 642-5 of white clover and line 848-10 of *T. uniflorum* crossed with line 642-5 of white clover, produced hybrids more resemblene to white clover in the former cross and *T. uniflorum* in the latter cross. This showed influence of both factors i.e., parental genotype and the direction of crosses, in the phenotypes of hybrids. This also showed the importance of the individual genotypes being used as parents. However, the results showed an inclination of hybrids towards their male parents in the majority of recorded traits that were not intermediate between the parents. Their backcrossing results, using white clover as recurrent parent, showed effective recovery of most of the vegetative traits of white clover. Use of *T. uniflorum* as the recurrent parent was effective in restoration of its own floral traits like head size, pedicel and peduncle length. This indicated that directing the variation created by interspecific hybridization toward an ideotype having strong roots, and improved disease and pest resistance traits while retaining and/or improving the reproductive characteristics, might be challenging. Although the researchers did not show detailed data for BC_1F_1, the information given showed the effectiveness of backcrossing. For example, the pollen fertility of BC_1F_1 was 0–84%, F_1 was 0–58% and F_2 was 0–13%. Potential for selection within a BC_1F_1 generation base population was evident to combine improved fertility with the other desired traits. However, the small sample size, resulting from recovery of low numbers of embryos per plant by the embryo rescue technique, limited the conclusions that could be reached from their experiment.

Gibson et al. (1971) made crosses among *T. uniflorum, T. occidentale,* white clover and their hybrids. One hybrid plant was obtained from 2160 florets of *T. occidentale* (including both 2x=16 and 4x=32 *T. occidentale*) crossed with *T. uniflorum*. This hybrid was produced by using autotetraploid *T. occidentale*. Its pollen viability was considerably higher than other hybrids. The researchers thought it might be due to balanced dosage of genes from the parents and chromosomal homology during meiosis. Further crossing among the hybrids led them to suggest that crossing of white clover or *T. uniflorum* with *T. occidentale* weakens the barriers for crossability between white clover and *T. uniflorum*. These authors further discussed the potential contributions of the three *Trifolium* species for the improvement of white clover. Hybridization of white clover with *T. nigrescens* resulted in weak perenniality, infrequent rooting at the nodes, and more risk of susceptibility to viral diseases. However, *T. occidentale* was identified as a source of improved persistence and tolerance to some of the viral diseases. Along with general tolerance to viral diseases, *T. uniflorum* could also contribute tolerance to sooty blotch and powdery mildew in white clover. Moreover, its woody taproot, short internodes and larger seed size, could improve the persistence, strength and seedling vigor of white clover. Chen and Gibson (1971a) studied the growth of embryo and endosperm tissues in an interspecific cross between white clover and *T. uniflorum*. They found that growth of the embryo, endosperm and seed development were retarded. Retardation of endosperm growth began three days after pollination. This lack of endosperm development resulted in disintegration of the embryo. It was proposed that initially both embryo and endosperm grew normally at the expense of the inner integument and the endothelium. At the exhaustion of this food source, the endosperm was supposed to become cellular and serve as a food source for the growing embryo. In this interspecific cross, an unfavourable genetic balance or interaction of the endosperm with maternal tissues resulted in improper flow of nutrients from maternal tissues to the endosperm. This retarded the growth of the endosperm which failed to become cellular and a food source for a developing embryo. Consequently the embryo starved and was aborted. Chen and Gibson (1972) studied the chromosomal relationships between white clover, *T. uniflorum* and colchicine induced autotetraploid *T. occidentale* at metaphase-1. For crosses between white clover and *T. uniflorum* they studied four hybrids produced by different parental genotypes. They observed considerable association between chromosomes of these two species with a mean of 1.24 (range 0.58 to 2.25) quadrivalents. The chromosome associations were clearly affected by parental sources of the hybrids. The researchers suggested that white clover might be the source of this variation. Chromosome associations were observed between *T. uniflorum* and *T. occidentale* (4x) with mean frequency of 2.81 quadrivalents per PMC. This study showed the genomic similarity among white clover, *T. uniflorum* and *T. occidentale*.

Pederson and Windham (1989) evaluated *Trifolium* species and their hybrids for resistance against Southern root-knot nematode (*Meloidogyne incognita*). Out of two populations of *T. uniflorum* evaluated, one appeared to be more resistant, while the other was susceptible. White clover was also found to be susceptible to *M. incognita*. Hybrids of white clover with *T. occidentale* were consistently susceptible, whereas, hybrids of white clover with *T. uniflorum* showed most variation in their resistance with mean gall indices ranging from 0.2 to 4.6. The researchers proposed that these hybrids tolerated nematodes by maintaining a high proportion of fibrous roots. Dymock et al. (1989) observed significantly more restricted growth of grass grub (*Costelytra zealandica*) on *T. uniflorum* than on white clover. One seedling of *T. uniflorum* showed resistance against this grub similar to that of *L. pedunculatus* (a known resistant *species*). Backcrossing of the hybrids to *T. uniflorum* further improved this resistance. However, this improvement against grass grub was accompanied by the poor agronomic traits of *T. uniflorum*. It was expected that the restricted growth of grass grub on *T. uniflorum* was due to poor nutritional quality and an unfavourable overall balance of stimulants and deterrents in roots. The researchers expressed the possibility that, after reliable screening of *T. uniflorum* parents, the resistance against grass grub might be transferred to white clover by interspecific hybridization. Howieson et al. (2005) isolated rhizobial strain WSM1313 from *T. uniflorum* (collected from Greece) showing effective nodulation with many annual clovers from the same region. Effective and partially effective associations of *T. uniflorum* with rhizobial strains WSM 1689, WSM 1313, TA1, WSM 409, CC 4335 and CC 2483 were observed. All these strains had a Euro-Mediterranean origin. Three of these strains (TA1, CC4335 and CC 2483) had partially effective relationships with white clover.

Williams et al. (2010) formed hybrids involving *T. occidentale* as male and *T. uniflorum*, as female parents. These hybrids having genomes from two drought tolerant species may become a good source of drought tolerance. Selfing and sib-mating produced F_2 populations. These hybrids were crossed with white clover to produce three way hybrids. The researchers also made hybrids of colchicine doubled *T.*

occidentale with white clover. An early backcrossed population was also produced using white clover as the recurrent parent. Interspecific chromosome pairing at meiosis was observed in these hybrids. This showed the possibility of introgression of drought and salt tolerance from these hybrids into white clover.

T. uniflorum produces big flowers and seeds. It has resistance to sooty blotch (*Cymadothea trifolii*), powdery mildew (*Erisyphe polygoni* DC) and a range of viruses (Gibson et al., 1971). Interspecific hybridization between white clover and *T. uniflorum* has the potential to improve white clover by transfer of drought and grass-grub tolerances and enhanced ranges of viral tolerance and nutrient uptake. The transferability of these desired traits from *T. uniflorum* into white clover is feasible, as both species have the same chromosome numbers and produce fertile hybrids. There is good chromosomal pairing and crossing over, giving chances of genetic exchange between these two species and creation of new and useful genetic variations (Williams and Nichols, 2011). Nichols (2012) comprehensively studied white clover, *T. uniflorum* and their BC_1F_1 and BC_2F_1 (having white clover as the recurrent parent) hybrids. *T. uniflorum* produced thicker roots than white clover. It had a herringbone-like root system indicating its adaptation to low soil fertility. Some hybrid families were found tolerant to low soil fertility. The BC_1F_1 hybrids had intermediate dry matter production, growth habit, stolon morphology and leaf size than that of their parents. Both *T. uniflorum* and BC_1F_1 showed better tap root survival than white clover. *T. uniflorum* and both hybrid generations showed more root to shoot mass production and, faster root penetration to depth than that of white clover cultivars under dry conditions. This showed their potential for better establishment and root development to access deeper soil water. The analysis of dry matter production, stolon morphology, plant shape, leaf and stolon senescence under water stress conditions proved hybrids more drought tolerant than white clover. Physiological and biochemical studies also confirmed this finding. The hybrids showed variation for traits like lateral spread, stolon density, nutrient uptake, resistance to shoot fungal diseases and viruses, etc. This showed potential for selecting desired hybrid genotypes.

Williams and Nichols (2011) are of the view that many *Trifolium* species are under threat due to their very narrow distributions. For example, there are only five accessions of *T. uniflorum*, preserved in gene banks worldwide. As being a highly diversified wild species, it needs better conservation efforts. Snogerup and Snogerup (1987) recorded the dynamics of plant species present on 11 different islets in the Aegean, taking four repeated observations over 14 years. They observed changes in many plant species. *T. uniflorum* occurred on Theotokos Vrakhos East, Theotokos Vrakhos West and Makedhona. On Makedhona islet, the *T. uniflorum* population of 1001 – 10,000 plants found during the 1971 visit had become extinct by 1985. High diversity, adaptation to dry climate and ability to transfer desired traits into hybrids by successful hybridization with white clover, justify better conservation and utilization of *T. uniflorum*.

2.7 SUMMARY

White clover contributes in New Zealand economy by feeding livestock, improving nitrogen content of soil and by seed export sales. Cultivars with improved genetics and reasonable seed production are required to keep New Zealand competitive in international seed markets and to enhance adaptability and performance of this important pasture legume. Agronomic practices and genetic selection have been developed that can achieve high seed yields i.e., up to two tons per hectare with an average of one ton/ha in a commercial seed crop. However, seed production in white clover is generally considered unreliable due to intermittent flowering pattern, low seed set per floret and unfavourable environmental conditions at harvesting time. Approximately half of its available ovules develop into seeds and again only half of these seeds may become harvestable due to rains at harvesting time and losses due to lodging of the peduncles. Numbers of heads, florets per head and seeds per floret, all have moderate heritabilities and are correlated with seed yield. Heads per plant is usually taken as the most important trait in improvement of seed production because of its direct association with seed production, easy measurability and high heritability. On the other hand, this trait has a strong negative association with stolon density and slight negative association with dry matter yield. Florets per head and seeds per floret, although more laborious to measure, have high heritabilities, and have no direct negative associations with stolon density and persistence, except for resource consumption. Peduncle length and strength are very important heritable traits to provide improvements in seed harvest, especially if the florets per head and seeds per floret are also increased to improve seed production.

Interspecific hybridization is a promising strategy to transfer desired traits from wild relatives into white clover. The available germplasm of white clover is deficient in some important traits like drought tolerance, persistence, root pests resistance, profuse flowering, etc. These traits are available in the secondary and tertiary gene pools of white clover. Very few efforts are underway to utilize these precious sources for white clover improvement. *T. uniflorum*, with a deep woody tap root system and adaptation to dry areas, is a promising source for the improvement of white clover by hybridization. The complex biology involved in interspecific hybridization and presence of undesired traits of *T. uniflorum* like low seed yield, low vegetative vigor, poor stolon formation, etc, require intensive breeding efforts to develop this hybrid as an improved product. The defective seed production ability received especially from *T. uniflorum* demands special focus on this attribute of the hybrid to make it a commercially viable product.

Chapter 3

EXPLORING VARIABILITY

3.1 INTRODUCTION

Primary (F_1) hybrids between white clover and *T. uniflorum* were achieved using the embryo rescue technique. However, these hybrids had very poor seed production. The BC_1F_1 generation was developed by using a diverse range of F_1 and white clover parents. The wide variation created in this interspecific hybridization provided an opportunity to start a breeding effort with an aim of restoring the seed production of these highly valuable hybrids. Plant cuttings of the BC_1F_1 generation (grown at Lincoln from September 2007) were transferred to Palmerston North and planted during the last week of November 2008. These cuttings were used for this preliminary study to:

1. Explore the existing variation in BC_1F_1 for seed production traits.
2. Develop selection criteria to select the better performing genotypes.
3. Create the next level generations for future use.

3.2 MATERIALS AND METHODS

The starting material of this study was seventy five BC_1F_1 plants involving nine different white clover varieties as their recurrent parents in backcrossing. Previously, different F_1 hybrids were crossed with a single white clover variety to produce different BC_1F_1 plants (Table 3.1). These BC_1F_1 plants were named according to their recurrent white clover parent variety. The numbers used as suffix of the variety name showed identity of each genotype. As there were nine white clover varieties involved as recurrent parents, nine such different BC_1F_1 groups made the starting material of this experiment. All white clover cultivars were represented with two capital letters. Most of the BC_1F_1 hybrids were shown by a single capital letter. Only BC_1F_1 hybrids of Crau and Trophy were represented by two letters. The second letters are small for both of them.

Table 3.1. Pedigree and naming procedure for BC_1F_1 plants. Selected genotypes of only two white clover varieties (Aran and Barblanca), used as recurrent parent in backcrossing, are mentioned as an example.

F_1	Recurrent Parent	BC_1F_1	Code
(white clover x *T. uniflorum*)	**(white clover)**	**(white clover x {white clover x *T. uniflorum*})**	**(BC_1F_1)**
(KopuII$_2$, Pitau$_1$) x T66-6	Aran-1	Aran-1 x {(KopuII$_2$, Pitau$_1$) x T66-6)}	A_1
(Aran, Tribute$_1$) x T66-3	Aran-2	Aran-2 x {(Aran, Tribute$_1$ x T66-3)}	A_2
KopuII$_2$ x Az4383-11	Aran-2	Aran-2 x {(KopuII$_2$ x Az4383-11)}	A_3
Sustain$_1$ x Az4437-3	Barblanca-b	Barblanca-b x {(Sustain$_1$ x Az4437-3)}	B_1
(KopuII$_2$, Pitau$_1$) x T66-6	Barblanca-b	Barblanca-b x {(KopuII$_2$, Pitau$_1$ x T66-6)}	B_2
(Tribute$_1$, Aran$_1$, Sustain$_1$ x T66-3)	Barblanca-a	Barblanca-a x {(Tribute$_1$, Aran$_1$, Sustain$_1$ x T66-3)}	B_6

An unreplicated trial was established in a greenhouse at 25^{o}C and under normal light conditions between November 2008 and February 2009. Cuttings were planted in a peat/sand potting mix in tapered 5.7 L cylindrical plastic pots (205 mm height × 221 mm top diameter × 170 mm bottom diameter). The BC_1F_1 plants were grown along with four cultivars of white clover and a Chinese ecotype 'Tienshan' (Table 3.2).

Table 3.2. Details of plant material used in experiment-1. White clover was used as recurrent parent. The recurrent parents mentioned for the BC_1F_1 generation were used in creation of this generation, whereas white clover cultivars mentioned as recurrent parents were crossed with plants of BC_1F_1 generation to produce BC_2F_1 generation.

S. No.	GENERATION	Recurrent parent cultivar	PLANTS USED	ABBREVIATION USED (to represent plants of a family)
	BC_1F_1			
1		Aran	1-15	A_{1-15}
2		Barblanca	1-9	B_{1-9}
3		Crusader	1-8	C_{1-8}
4		Crau	1-4	Cu_{1-4}
5		Durana	1-2	D_{1-2}
6		Kopu II	1-4	K_{1-4}
7		Sustain	1-19	S_{1-19}
8		Tribute	1-8	T_{1-8}
9		Trophy	1-6	Tf_{1-6}
	Parent (white clover)		45	WC
1		Tienshan	1-15	CH_{1-15}
2		Ladino	1-8	LA_{1-8}
3		Prestige	1-3	PR_{1-3}
4		Aran	28-30	AR_{28-30}
5		Durana	21, 25, 42, 44, 45, 46, 49, 57, 59, 60, 846, 849, 859	DU_{21}, DU_{25}, DU_{42}, DU_{44}, DU_{45}, DU_{46}, DU_{49}, DU_{57}, DU_{59}, DU_{60}, DU_{846}, DU_{849}, DU_{859}

3.2.1 Measurements

Data were collected from all plants, as follows:

3.2.1.1 Reproductive traits

a. Pollen stainability

All plants of the BC_1F_1 generation were assessed for pollen stainability from 18 December 2008 to 29 December 2008. The calyx and corolla of a fully opened flower were removed and anthers were dehisced over a glass slide. A drop of 2% acetocarmine solution was added to stain the viable pollen grains. Viability of pollen grains, as indicated by their stainability, was counted under a compound microscope at 100x magnification. At least 300 pollen grains of each flower and two flowers of a plant were sampled. Data were compiled as pollen stainability percentage (Ps %).

b. Bud and inflorescence emergence per plant

All inflorescences having a visible corolla were counted on 11 January 2009. Visible green flower buds were also recorded separately.

c. Per inflorescence measurements

Suitable maturity of inflorescence for harvesting was defined by browning of the upper part of the peduncle. A random sample of 10 mature inflorescences was removed from each plant on 7 February 2009. Florets per inflorescence and peduncle length were recorded.

d. Seed data

All crossing were done manually. Crosses were made with completely emasculated female inflorescences by the method of Williams (1954). Seed setting by selfing of each plant was assessed by gently rubbing the inflorescence between the thumb and fingers every day until all florets on the inflorescence were deflexed (Williams 1987). Inflorescences harvested on 7th of February 2009 were used to record seeds produced by each plant as:

1. Female parent, pollinated by other BC_1F_1 plants.
2. Male parent, crossed to other BC_1F_1 plants.
3. Male parent, crossed to white clover plants.
4. Self pollinated plant.

Total number of florets and seeds were counted. Seeds production ability of 100 florets was derived and used as percentage seed setting ability of the plant.

3.2.1.2 Vegetative traits

a. Leaf Traits

Leaf area and petiole length were recorded for 5 leaves per plant on 26 February 2009. Petiole length of intact leaves adjacent to fully deflexed flower heads was measured.

Leaf shape was considered as an ellipse (Figure 3.1).

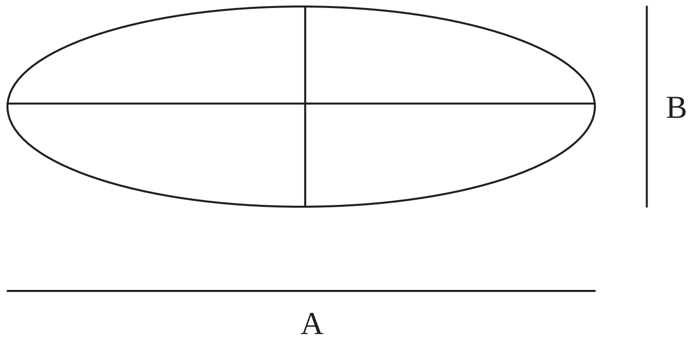


Figure 3.1. Geometric shape of a typical ellipse.

The area of a leaf was calculated by formula:

$$\text{Area of an ellipse} = \pi * (A/2) * (B/2)$$

b. Plant growth

Foliage biomass, including the reproductive parts, was harvested from plants on 28 February 2009 and weighed within approximately two hours after the harvest.

3.2.1.3 Root traits

Roots were washed on 12th of April 2009 to record the following parameters.

a. Nodal root thickness

Diameters of the 10 thickest nodal roots at 1 mm depth were measured by using a Vernier Caliper (Mitutoyo digital Caliper- 500.550.551).

b. Roots dry weight

Roots were removed from stolons, oven dried at 80^{o}C for 24 hours and weighed.

3.2.2 Selection criteria

Four different indices were developed to sort the plants based on their performances for different traits. The following was the general order of preference for allocating weightage to different traits.

Seed setting in intercrossing > Selfing and pollen stainability > Florets per head > Heads per plant > Peduncle length > Seed setting in backcrossing > Foliage weight

Index 1 carried lower weights than index 2 and 3 for most of the traits. Exceptions were traits related to seed setting, for which index 1 had more weights as compared to the weights in index 2. Index 3 was carrying double weights than index-2 (Table 3.3). The quartile index sub-grouped the data for each trait on a quartile range basis (Table 3.4). It followed the scoring pattern of index 2 with the exception that peduncle length and heads per plant were given more weightage in quartile index than in index 2. All four indices were considered in deciding the final score of each plant. Plants were also sorted on the basis of total number of florets per plant. The final arrangement was based on consensus between outcome of indices and florets per plant. This final arrangement became available only at the end of the experiment.

Crosses were made during experiment-1 on phenotypic expression of head production ability of the plants. At the end of this experiment the outcome of the selection process was used to select the available crosses for future use.

Table 3.3. Details of Index 1, 2 and 3. The range represents grouping of data to allocate them different scores. The scores represent the weightages given to each trait in each index.

	Index ↓	Range →							
Foliage Weight (gm)		Range	77-140	141-204	205-268	269-332	333-396	> 396		
	1	score	0.1	0.2	0.4	0.6	0.8	1		
	2	score	0.5	1	2	3	4	5		
	3	score	1	2	4	6	8	10		
Peduncle Length (mm)		Range	< 34	35-50	51-70	71-160	161-200	>200		
	1	score	0.5	1	2	4	6	8		
	2	score	1.5	3	4	6	7.5	9		
	3	score	3	6	8	12	15	18		
Heads / plant (No.)		Range	2-17	18-33	34-49	50-65	66-81	82-97	98-113	> 113
	1	score	1	2	3	4	5	6	7	8
	2	score	2	3	4	5	6	7	8	9
	3	score	4	6	8	10	12	14	16	18
Florets / head (No.)		Range	< 10	10-20	21-35	36-50	> 50			
	1	score	1.5	3.5	6	9	10.5			
	2	score	2	5	10	15	18			
	3	score	4	10	20	30	36			
Pollen Stainability (%)		Range	< 9	9-20	21-30	31-55	56-70	> 70		
	1	score	0.5	4	6.5	11.5	14	16.5		
	2	score	2.5	5	7.5	10	12.5	15		
	3	score	5	10	14	20	25	30		
Selfing (%)		Range	< 8	9-20	21-40	41-80	81-150	151-200	> 200	
	1	score	1	2.5	3.7	5	6.2	7.5	8.7	
	2	score	0.5	2.5	5	7.5	10	12.5	15	
	3	score	1	5	10	15	20	25	30	
Seed setting as ♀ with BC_1F_1 (No. of seeds)		Range	< 8	9-20	21-40	41-80	81-150	151-200	> 200	
	1	score	5	10	15	20	25	30	35	
	2	score	1	5	10	15	20	25	30	
	3	score	2	10	20	30	40	50	60	
Seed setting as ♂ with BC_1F_1 (No. of seeds)		Range	< 8	9-20	21-40	41-80	81-150	151-200	> 200	
	1	score	2.5	5	7.5	10	12.5	15	17.5	
	2	score	0	1.5	3	4.5	6	7.5	9	
	3	score	0	3	6	9	12	15	18	
Seed setting as ♂ with white clover (No. of seeds)		Range	< 8	9-20	21-40	41-80	81-150	151-200	> 200	
	1	score	2.5	5	7.5	10	12.5	15	17.5	
	2	score	0	1.5	3	4.5	6	7.5	9	
	3	score	0	3	6	9	12	15	18	

Table 3.4. Details of Quartile Index. The ranges represent the four quartiles and the scores represent their respective weights.

Shoot weight	Range	77-165	166-239	240-300	> 300
	score	1.5	3	6	9
Peduncle length	Range	41-78	79-103	104-141	> 141
	score	3	6	9	12
Heads / plant	Range	3-18	19-26	27-42	> 42
	score	2.5	5	10	15
Florets / head	Range	11-17	18-19	20-24	> 24
	score	5	10	15	20
Pollen stainability	Range	11-46	47-60	61-67	> 67
	score	5	10	15	20
Selfing	Range	< 5	6-21	22-86	> 86
	score	5	10	15	20
Seed setting	Range	1-37	38-84	85-107	> 107
as ♀ with BC_1F_1	score	10	20	30	40
Seed setting	Range	0-50	51-75	76-120	> 120
as ♂ with BC_1F_1	score	3	6	9	12
Seed setting	Range	5-69	70-106	107-131	> 131
as ♂ with white clover	score	3	6	9	12

3.3 ANALYSES AND RESULTS

This feasibility study was based on an unreplicated experiment having completely randomized arrangements of the plants. Analysis of this preliminary study was based on general comparisons and Pearson correlations of the original data.

3.3.1 Exploring the existing variation

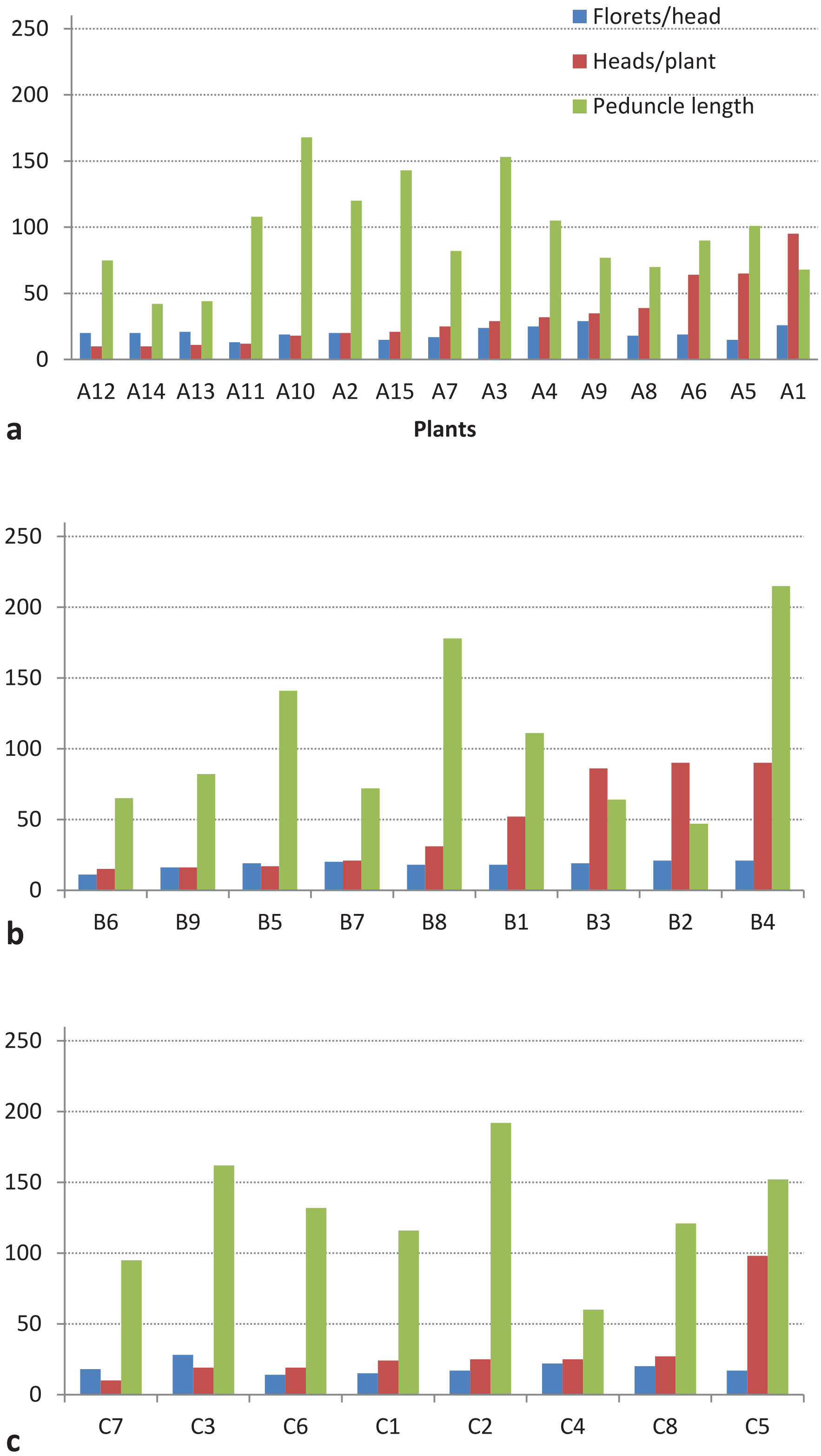
Considerable phenotypic variation was observed within and among families for most of the traits in this BC_1F_1 generation (Table 3.5). Numbers of florets per head (head size) of the hybrids were about half, compared to their white clover parent. The hybrids showed less variation than white clover for this trait. Their pollen viability was lower than the reported pollen stainability (87.8%) of the white clover cultivar Grasslands Huia at 20^{o}C under controlled conditions (Thomas, 1987). In a follow-up study by the current author, it was observed that the peduncle in *T. uniflorum* was very short, remained hidden under stipule, and did not elongate enough to take the flower head above the foliage canopy. Thus only the pedicel facilitated exposure of the flower above the foliage cover. The peduncle plus pedicel to petiole ratio for *T. uniflorum* (Accession Az 4383) was found to be 0.35, whereas, the average peduncle to petiole ratio of white clover cultivars (Tahora and Aran) was 1.54. The current study showed that the BC_1F_1 hybrids were like their *T. uniflorum* parent, and all hybrid families other than Cu and D produced longer petioles than the adjacent peduncles. This indicated that the peduncles in the majority of the BC_1F_1 hybrids were short, and hence unable to extend the flower heads above the foliage canopy, unless their head size was large enough to compensate for the difference between the petiole and peduncle lengths or the petiole was less vertically oriented than the adjacent peduncle. The BC_1F_1 hybrids showed higher self compatibility than white clover. However, they were inferior to white clover in seed setting as the result of crossing.

Table 3.5. Basic statistic of plant material used in experiment-1. Number of plants observed (N), mean values (X) and Coefficient of variation (CV) of different BC_1F_1 families and white clover varieties (WC) for different traits involved in the study.

		Families										
TRAITS		**A**	**B**	**C**	**Cu**	**D**	**K**	**S**	**T**	**Tf**	**Average**	**WC**
Florets / head	N	15	9	8	4	2	4	17	7	8	8	38
	X	20	18	19	28	21	24	26	17	19	21	39
	CV	22	17	24	53	17	16	38	15	34	26	39
Heads / plant	N	15	9	8	4	2	4	17	7	8	8	.
	X	32	46	31	72	14	33	25	29	41	36	.
	CV	76	72	90	53	5	40	79	63	71	61	
Peduncle length (mm)	N	15	9	8	4	2	4	17	7	8	8	.
	X	96	108	129	158	160	91	120	114	99	119	.
	CV	39	54	32	23	31	33	51	29	32	36	
Pollen stainability %	N	14	8	7	4	2	4	15	6	8	8	.
	X	62	32	51	62	49	48	66	54	61	54	.
	CV	20	56	30	17	34	28	25	38	25	30	
Selfing %	N	14	9	8	4	2	4	15	7	8	8	8
	X	39	31	38	71	63	30	53	47	63	48	5
	CV	177	157	117	66	129	93	133	111	87	119	94
Seed setting % in backcrossing	N	11	7	8	4	2	3	11	5	7	6	9
	X	114	64	106	115	94	104	78	145	127	105	188
	CV	46	80	26	37	53	10	98	51	26	47	45
Seed setting % in intercrossing	N	7	6	6	4	0	2	7	3	5	4	9
	X	95	38	143	89	.	136	95	35	75	88	185
	CV	36	64	44	61	.	0	81	65	43	49	45
Foliage weight (gm)	N	15	9	8	4	2	4	17	6	8	8	.
	X	230	210	232	254	411	241	230	255	245	256	.
	CV	37	32	43	32	15	57	37	40	26	35	.
Peduncle length/petiole length	N	15	9	8	4	2	4	17	6	6	8	.
	X	0.64	0.63	0.90	1.0	1.5	0.66	0.68	0.69	0.58	0.81	.
	CV	45	48	41	37	73	10	51	31	21	40	.
Nodal roots diameter (mm)	N	13	9	8	4	2	4	17	6	8	8	.
	X	1.45	1.70	1.65	1.58	1.50	1.13	1.52	1.63	1.29	1.51	.
	CV	30	27	17	47	47	17	28	23	23	29	
Roots dry weight (gm)	N	14	9	8	4	2	4	17	6	8	8	.
	X	7	8	8	9	8	4	8	9	6	7	.
	CV	65	50	37	86	110	74	44	69	42	64	

3.3.1.1 Variation within a family

Considerable variation was observed among plants of a family for florets per head, heads per plant and peduncle length (Figure 3.2).



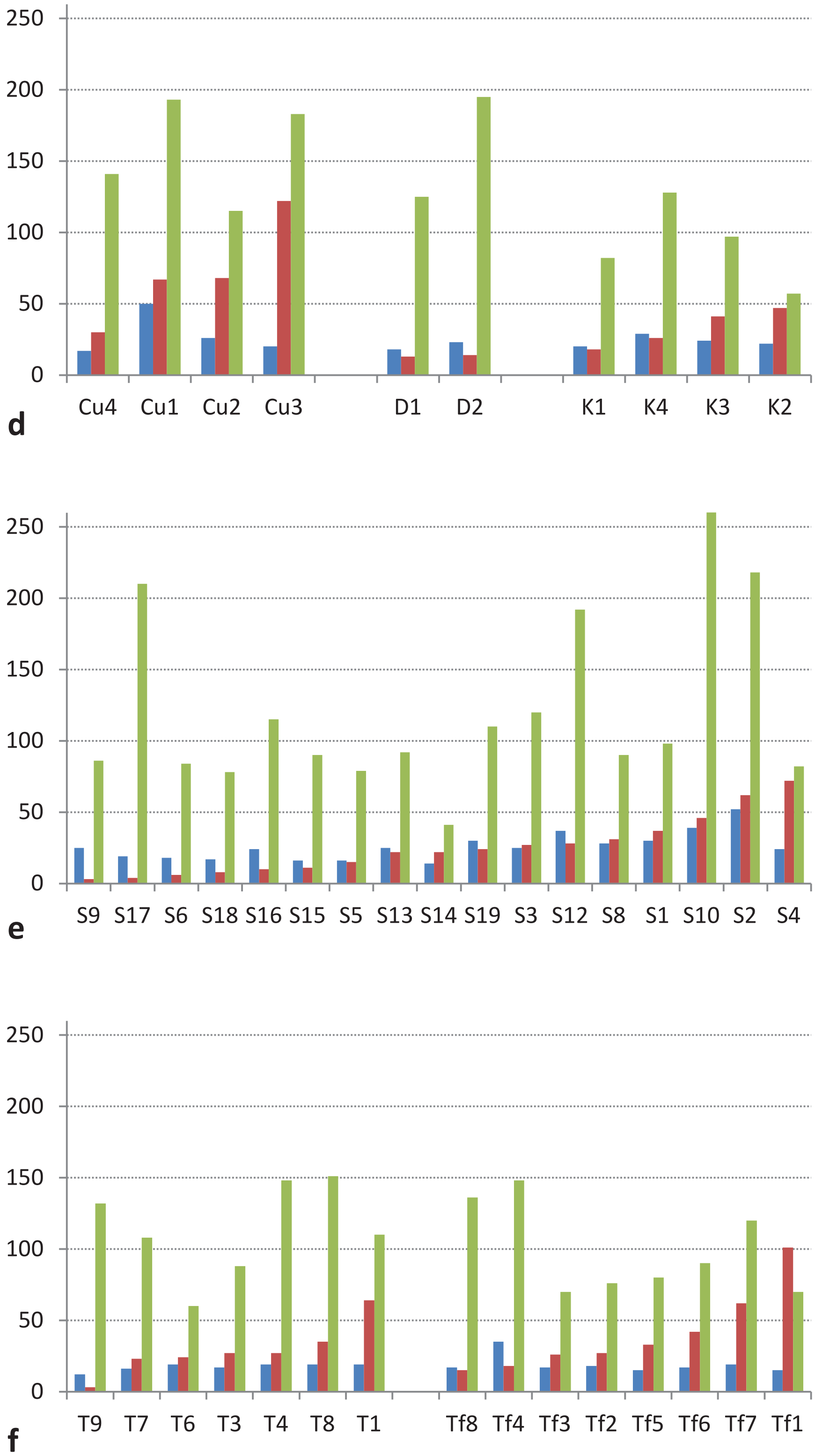


Figure 3.2. Variation among plants of family A (a), B (b), C (c); Cu, D and K (d), S (e), and T and Tf (f) , for florets per head, heads per plant and peduncle length.

Box plots were used to express variation present both among and within families (Figure 3.3).

3.3.1.2 Variation among families

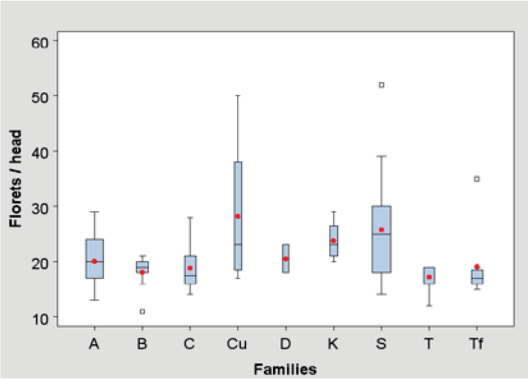
There was considerable variation for most of the traits among families. The family Cu had higher mean values and ranges for florets per head, heads per plant and florets per plant than other backcrosses to other white clovers (Figures 3.3a, 3.3b and 3.3c, respectively). Plants from backcrosses to S also produced more florets per head, but they were not high performers for heads and florets per plant. Plants of family D produced low numbers of heads per plant and florets per plant.

Most families had moderate average pollen stainability percentage (Figure 3.3d). Only the family B had low average pollen stainability. Plants representing family Cu and D had longer peduncles than other families (Figure 3.3e). A few plants of family S also produced long peduncles. Total number of florets and seeds produced by a plant were counted. Seed production ability of 100 florets was derived and used as percentage seed setting ability of the plant. There was a greater range of seed setting ability from selfing among BC_1F_1 families than for white clover (Figure 3.3f). The average selfing success of BC_1F_1 hybrids was 48%, compared to that of 5% shown by white clover. Five families i.e., Cu, D, S, T and Tf, showed potential of more than 100 % seed setting as result of selfing for their most self fertile plants. For some plants there were extremes of high self compatibility, e.g. a few A and S plants set seeds more than 200%.

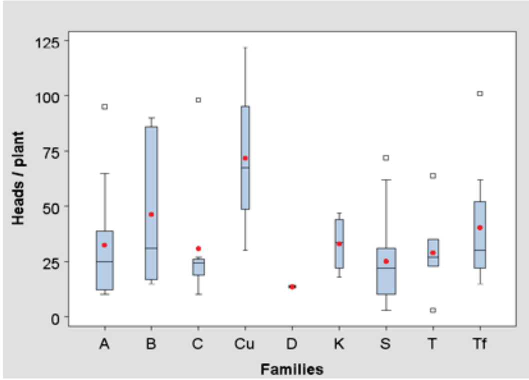
The BC_1F_1 plants were crossed with each other (intercrossing) and backcrossed with white clover plants. White clover performed the best for seed setting as a result of the intercrossing (Figure 3.3g) and backcrossing (Figure 3.3h). Family B expressed lowest means for these two traits. In intercrossing, there were some plants in families C and S which produced seeds near to the upper limit of white clover (if the white clover outlier is ignored). As a result of backcrossing, some plants of families S and T produced more seeds than average seeds produced by white clover.

For leaf traits i.e., petiole length and leaf area, relatively small variation was observed among average values for families (Figures 3.3i and 3.3j). However, variability did appear within families.

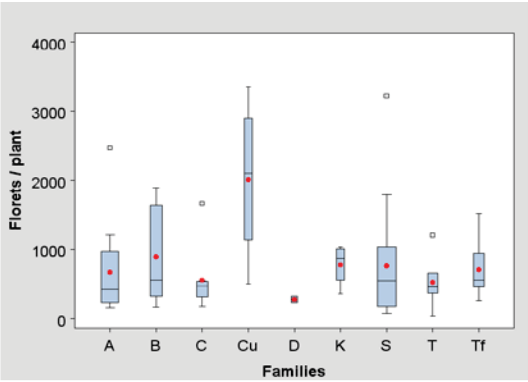
The family D produced considerably higher foliage biomass than other families (Figure 3.3k). Variation was observed for root traits (Plate 3.1). For root biomass and nodal root diameter, plants of family Cu exhibited a higher range as compared to the plants of other families (Figures 3.3l and 3.3m). Family K possessed the lowest root weight and nodal root diameter.


Florets / head
60
50
40
30
20
10
A B C Cu D K S T Tf
Families

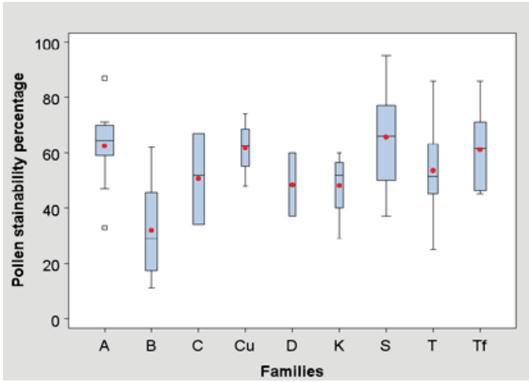
a


Heads / plant
125
100
75
50
25
0
A B C Cu D K S T Tf
Families

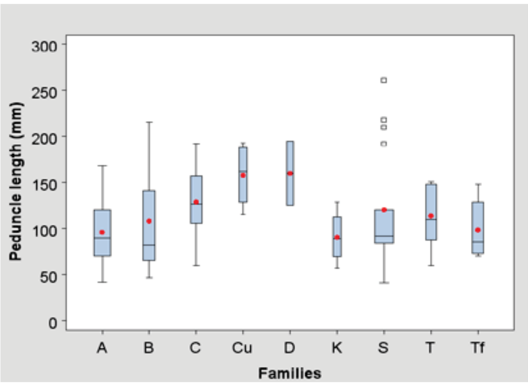
b


Florets / plant
4000
3000
2000
1000
0
A B C Cu D K S T Tf
Families

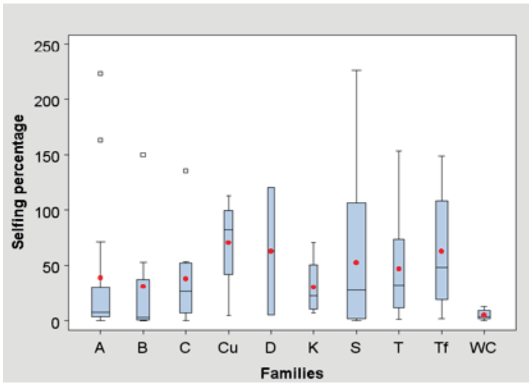
c


Pollen stainability percentage
100
80
60
40
20
0
A B C Cu D K S T Tf
Families

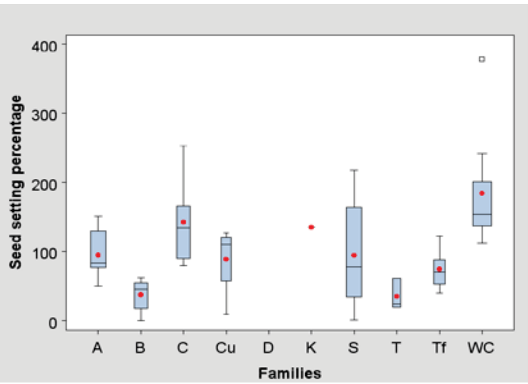
d


Peduncle length (mm)
300
250
200
150
100
50
0
A B C Cu D K S T Tf
Families

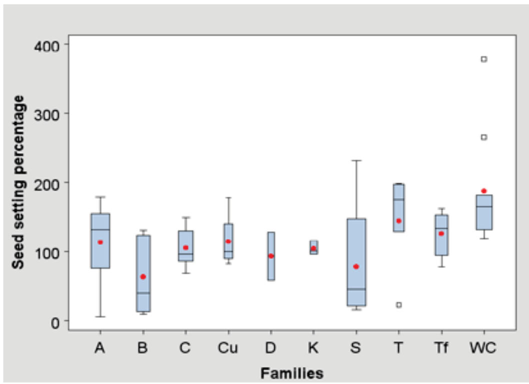
e


Selfing percentage
250
200
150
100
50
0
A B C Cu D K S T Tf WC
Families

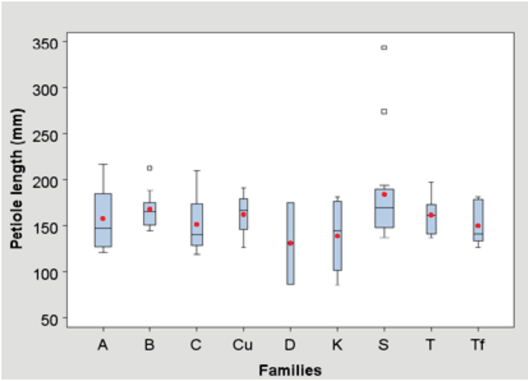
f

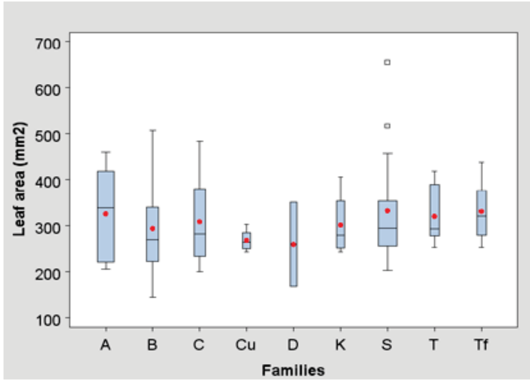

Seed setting percentage
400
300
200
100
0
A B C Cu D K S T Tf WC
Families

g


Seed setting percentage
400
300
200
100
0
A B C Cu D K S T Tf WC
Families

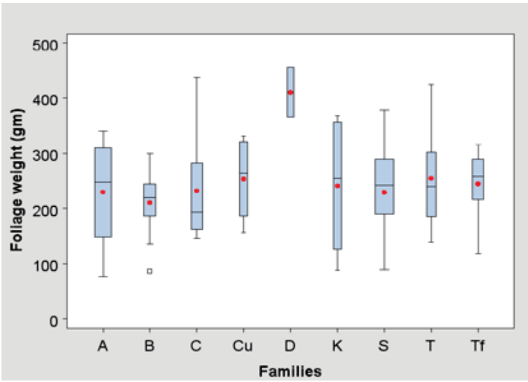
h

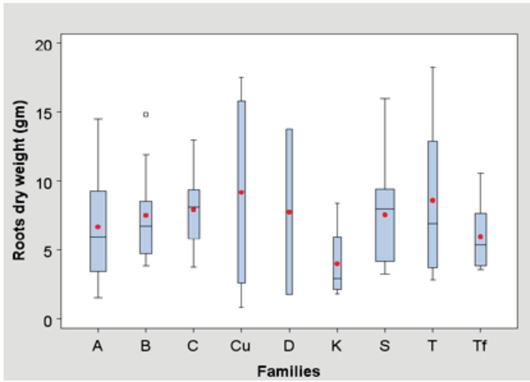




i

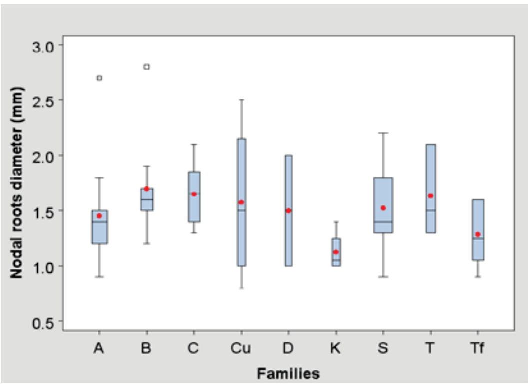
j





k

l



m

Figure 3.3. Comparison of families for florets / head (a), heads / plant (b), florets / plant (c), pollen stainability (d), peduncle length (e), selfing performance (f), seed setting in intercrosssing (g), seed setting in backcrossing (h), petiole length (i), leaf area (j), foliage weight (k), roots dry weight (l) and nodal root diameter (m) . The width of each box shows the number of observations. The upper and lower edges of the box represent first and third quartiles, respectively. The horizontal line drawn within the box marks the median. The red dot shows the sample mean. The whiskers are drawn to the most extreme points that lie within the fences. The upper fence is defined as the third quartile plus 1.5 times the interquartile range and the lower fence is first quartile minus 1.5 times the interquartile range. Observations outside the fences are marked as squares.

a

b

c

Plate 3.1. Variation in root traits of BC_1F_1 hybrids. Plant "a" has an intact and thick tap root, plant "b" has white clover type roots and plant "c" shows development of thick nodal roots.

3.3.2 Differences in parental effects

The variation in seed setting efficiency as the result of backcrossing was contributed by both the BC_1F_1 hybrids and the white clover recurrent parents. In general, the BC_1F_1 hybrids appeared to be more influential than white clover parents. For example, A_7 always gave low seed set while A_1 gave high seed set with all three white clover plants (Figure 3.4). An exception was B_9 which had higher seeding than B_2 and B_4, except where AR28 was the white clover tester (Figure 3.5). Differences were also evident in performance of plants within white clover cultivars. For example, Figures 3.6 and 3.7 show differences between plants of white clover varieties AR and PR, respectively.

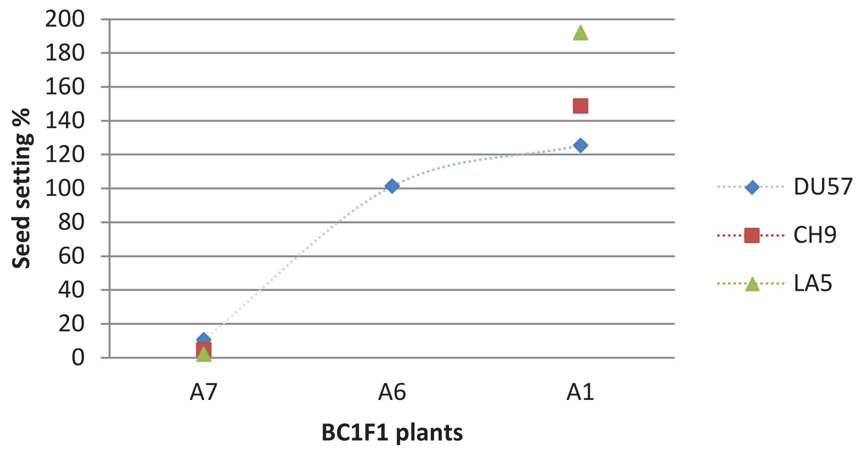


Figure 3.4. Seed setting as result of backcrossing of different plants of BC_1F_1 family A with different white clover varieties.

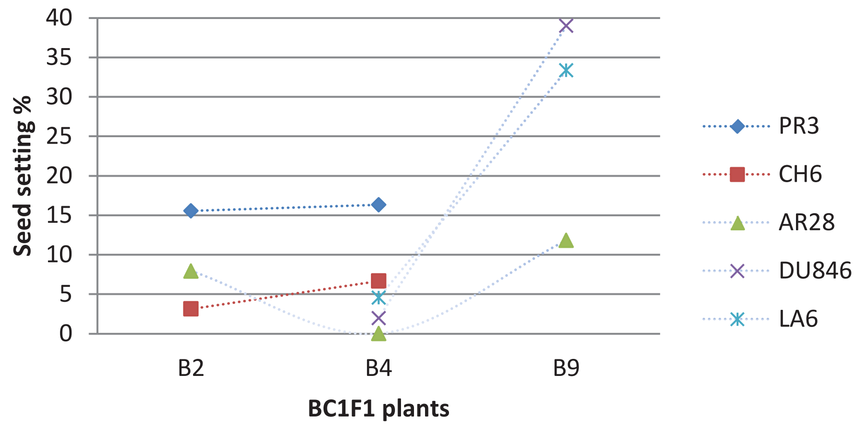


Figure 3.5. Seed setting as result of backcrossing of different plants of BC_1F_1 family B with different white clover varieties.

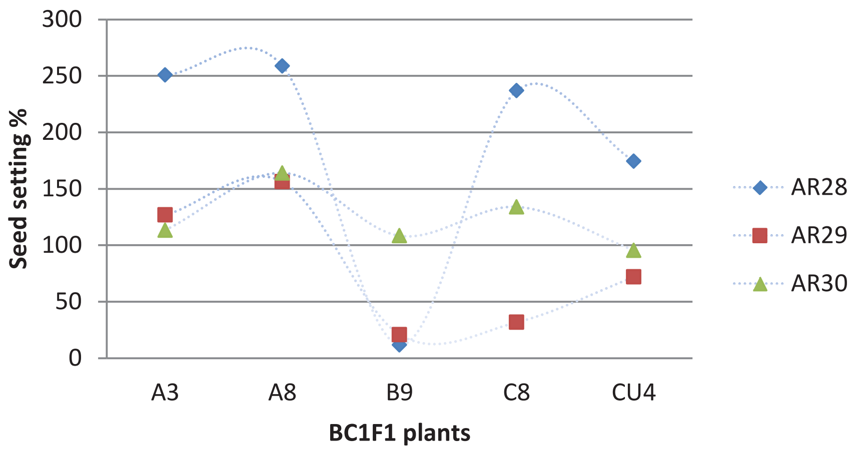


Figure 3.6. Seed setting as result of backcrossing using different plants of white clover cultivar AR as recurrent parent with different BC_1F_1 families.

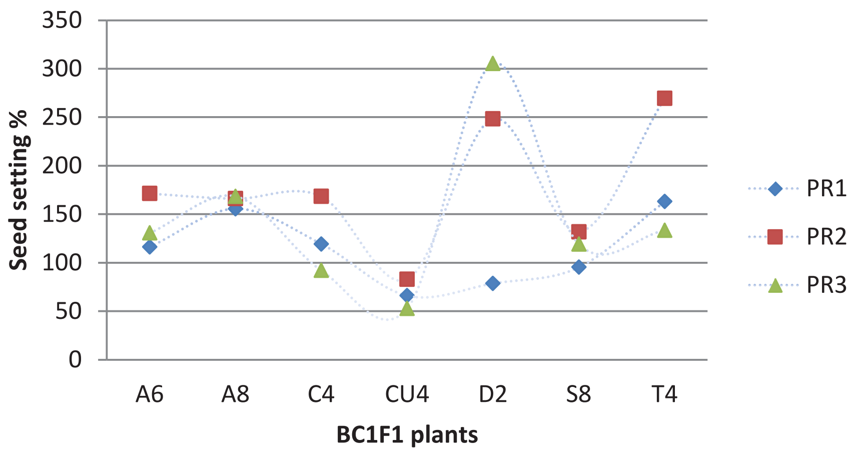


Figure 3.7. Seed setting as result of backcrossing of different plants of white clover cultivar PR with different BC_1F_1 families.

3.3.3 Associations among the traits

Associations among traits were studied by using Pearson correlation analyses (Table 3.6). Head size showed moderate correlation with peduncle length. Florets per plant were derived from heads per plant and florets per head. This association was evident from high correlation coefficients between florets per plant and the two later traits. Seed setting after intercrossing proved to be associated with selfing ability of the plant. In backcrossing, pollen stainability also appeared influential, along with selfing ability. Foliage weight was moderately linked with root dry weight. Petiole length showed a moderate positive link with leaf area. There were no significant negative associations between root traits (root dry weight and nodal root diameter) and any of the seed production traits.

An overall negligible correlation (r = 0.18) between florets per head (head size) and heads per plant was observed. However, families showed variation for association between head size and number of heads produced by their plants (Figure 3.8). The BC_1F_1 family D showed more average florets per head than number of heads present on the plant, whereas family Cu expressed much lower but adequate florets per head than heads per plant.

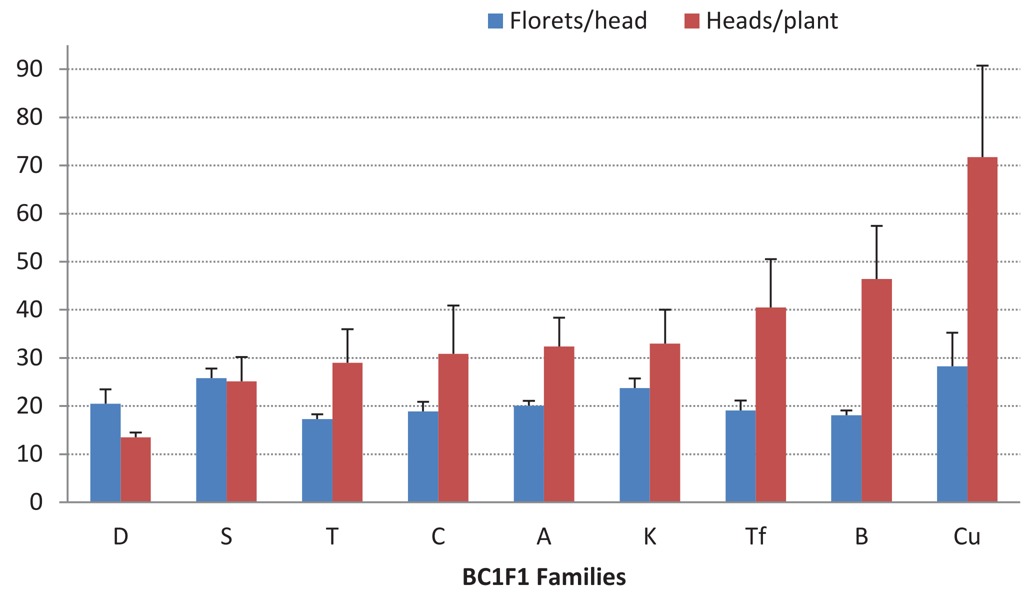


Figure 3.8. Association between florets per head and heads per plant as indicated by their average values, in different BC_1F_1 families. Lines on bars represent standard error.

Table 3.6. Associations among traits as expressed by Pearson correlation coefficients. Cells containing significant correlations at P ≤ 0.05, are highlighted.

Traits		2	3	4	5	6	7	8	9	10	11	12	13
Peduncle length	1	0.10	0.43	0.30	-0.02	0.00	0.09	0.02	0.13	0.21	0.25	0.06	0.09
Heads/plant	2		0.18	0.85	0.03	0.12	0.00	-0.10	0.16	0.09	-0.21	-0.15	0.07
Florets/head	3			0.62	0.16	0.19	-0.03	0.11	-0.07	0.26	0.10	0.14	0.13
Florets/plant	4				0.09	0.16	-0.07	-0.11	0.08	0.25	-0.08	-0.10	0.02
Pollen stainability	5					0.22	0.33	0.01	-0.02	-0.13	-0.08	0.11	0.02
Selfing	6						0.33	0.41	-0.11	-0.16	0.01	-0.11	0.04
Backcrossing	7							0.21	-0.05	-0.23	0.00	-0.06	0.20
Intercrossing	8								0.31	-0.09	0.06	-0.01	0.09
Roots weight	9									0.22	0.32	0.27	0.09
Nodal roots diameter	10										0.11	-0.03	-0.07
Foliage weight	11											0.16	-0.12
Petiole length	12												0.38
Leaf area	13												

3.3.4 Developing selection criteria

This preliminary study was not in replicated form and heritability information regarding the plants was therefore not available. Moreover, being early stage interspecific hybrids, the heritability estimates and trait association results available for white clover could not be applied on this plant material. The results displayed variation for almost all traits both within and among families. Phenotypic data of all traits was considered in deciding the comparative performance of a plant. Four different indices were developed for successive selection of plants. Traits were given weightage according to their importance in seed production (Table 3.4 and 3.4). For example, in all indices shoot growth received lesser weightage than traits related to direct seed setting. In addition to the index selection, florets per plant were considered to assess performance of the plants of each family.

3.3.4.1 Index selection

Four different empirical selection indices were developed to assist in future planning (Table 3.3 and 3.4). The traits directly involved in seed production were given more weightage than others. The highest weightage was given to seed setting ability as female plant in all indices. The general order of preference for allocating weightage to different traits (T1–T9) was:

Seed setting in intercrossing as male (T8) and female (T9)
Selfing (T5) and pollen stainability (T6)
Florets per head (T4)
Heads per plant (T3)
Peduncle length (T1)
Seed setting in backcrossing (T7)
Shoot growth (T2)

Indices 2 and 3 carried higher weights than index-1 for most of the traits. Exceptions were traits related to seed setting, for which index-1 had higher weights than index-2. Index 3 carried double the weights of index-2. The quartile index was based on the arrangement of the data into four groups based on quartile distribution (Table 3.4). The scoring pattern was the same as that of index-2, except that peduncle length and heads per plant were give more weightage in the quartile index than in index-2. The index selection procedure is described here for family B and index-1 as an example. The data of all genotypes were arranged for all traits (Table 3.7).

Table 3.7. Original data arranged per plant for nine traits. The abbreviations used are T1 for peduncle length, T2 for vegetative growth, T3 for heads per plant, T4 for florets per head, T5 for pollen stainability percentage, T6 for seed setting percentage in selfing, T7 for seed setting percentage as male parent in backcrossing, T8 for seed setting percentage as male parent in intercrossing and T9 for seed setting percentage as female parent in intercrossing. The order of traits is based on the availability of data for each trait. The blank cells show unavailability of the data for corresponding traits.

	T1	T2	T3	T4	T5	T6	T7	T8	T9
B_1	111	245	52	18	53	35	94	55	
B_2	47	135	90	21	38	1	9	10	68
B_3	64	87	86	19	62	150	131	120	5
B_4	215	230	90	21	28	53	13	14	21
B_5	141	186	17	19	11	3			
B_6	65	220	15	11		0	38	0	
B_7	72	207	21	20	15	0			
B_8	178	300	31	18	20	2	123	97	4
B_9	82	284	16	16	30	37	39		

For each trait, the original data was replaced by its respective weightage according to the under discussion index i.e., index 1, (Table 3.8). For example, first value of the trait peduncle length (T1) was 111 mm. In index 1, this value existed in the range of 71-160 having weightage of 4. So the value 111 was replaced by 4 in the next step.

Table 3.8. Original data replaced by respective weighted based on index-1.

	Weightage								
	T1	**T2**	**T3**	**T4**	**T5**	**T6**	**T7**	**T8**	**T9**
B_1	4	0.4	4	3.5	11.5	3.7	12.5	10	
B_2	1	0.1	6	6	11.5	1	5	5	20
B_3	2	0.1	6	3.5	14	6.2	12.5	12.5	5
B_4	8	0.4	6	6	6.5	5	5	5	15
B_5	4	0.2	1	3.5	4	1			
B_6	2	0.4	1	3.5		1	7.5	2.5	
B_7	4	0.4	2	3.5	4	1			
B_8	6	0.6	2	3.5	4	1	12.5	12.5	5
B_9	4	0.6	1	3.5	6.5	3.7	7.5		

Cumulative marks were obtained by adding marks of all available traits for each genotype (Table 3.9). For example, the second column showed the cumulative weightage of first (of T1) and second (of T2) columns for each plant.

Table 3.9. Cumulating the weightage.

Genotype	**T1**	**T1 T2**	**T1 T2 T3**	**T1 T2 T3 T4**	**T1 T2 T3 T4 T5**	**T1 T2 T3 T4 T5 T6**	**T1 T2 T3 T4 T5 T6 T7**	**T1 T2 T3 T4 T5 T6 T7 T8**	**T1 T2 T3 T4 T5 T6 T7 T8 T9**
$\mathbf{B_1}$	4	4.4	8.4	11.9	23.4	27.1	39.6	49.6	
$\mathbf{B_2}$	1	1.1	7.1	13.1	24.6	25.6	30.6	35.6	55.6
$\mathbf{B_3}$	2	2.1	8.1	11.6	25.6	31.8	44.3	56.8	61.8
$\mathbf{B_4}$	8	8.4	14.4	20.4	26.9	31.9	36.9	41.9	56.9
$\mathbf{B_5}$	4	4.2	5.2	8.7	12.7	13.7			
$\mathbf{B_6}$	2	2.4	3.4	6.9					
$\mathbf{B_7}$	4	4.4	6.4	9.9	13.9	14.9			
$\mathbf{B_8}$	6	6.6	8.6	12.1	16.1	17.1	29.6	42.1	47.1
$\mathbf{B_9}$	4	4.6	5.6	9.1	15.6	19.3	26.8		

The first three columns were removed as their weightage for all plants was accumulated in column 4. Columns onward from 4 had missing data for a few plants. So they were all considered individually. The plants based on their weightage in each column were sorted in descending order (Table 3.10). This aligned all plants in descending order on the basis of their cumulative weightage for each column. Due to accumulation of different weightage at each step, plant alignments differed from column to column. For example, the first five alignments in descending order shown by column 1 of table 3.10 were B_4, B_2, B_8, B_1 and B_3. In contrast, these first five alignments were B_4, B_3, B_2, B_1 and B_8 in the second column. Differences of less than 0.6 were considered negligible and plants having such little difference were considered to have the same position. For example, B_9 and B_5 differed by 0.4 according to the first column in table 3.10. This difference in weightage was ignored and both plants were given the same position for further analysis.

Table 3.10. Alignments of genotypes on cumulative weightage basis. The highlighted cells show plants considered at same position as the difference in their weightage is less than 0.6.

	T1 T2 T3 T4		**T1 T2 T3 T4 T5**		**T1 T2 T3 T4 T5 T6**		**T1 T2 T3 T4 T5 T6 T7**		**T1 T2 T3 T4 T5 T6 T7 T8**		**T1 T2 T3 T4 T5 T6 T7 T8 T9**
B_4	20.4	**B_4**	26.9	**B_4**	31.9	**B_3**	44.3	**B_3**	56.8	**B_3**	61.8
B_2	13.1	**B_3**	25.6	**B_3**	31.8	**B_1**	39.6	**B_1**	49.6	**B_4**	56.9
B_8	12.1	**B_2**	24.6	**B_1**	27.1	**B_4**	36.9	**B_8**	42.1	**B_2**	55.6
B_1	11.9	**B_1**	23.4	**B_2**	25.6	**B_2**	30.6	**B_4**	41.9	**B_8**	47.1
B_3	11.6	**B_8**	16.1	**B_9**	19.3	**B_8**	29.6	**B_2**	35.6		
B_7	9.9	**B_9**	15.6	**B_8**	17.1	**B_9**	26.8				
B_9	9.1	**B_7**	13.9	**B_7**	14.9						
B_5	8.7	**B_5**	12.7	**B_5**	13.7						
B_6	6.9										

The weightage in each column was replaced by its respective plant identity (Table 3.11). In making final decisions, the overall cumulative weightage of all alignments for a genotype was not exclusively considered because:

Complete data for all genotypes were available for only the first four traits i.e., peduncle length, vegetative growth, inflorescences per plant and florets per inflorescence. Combined weightage could be obtained satisfactorily for only these four traits.

Each trait was given due consideration by considering each alignment individually according to its importance for seed setting potential.

The overall position of a genotype within a family was considered by visual observation of its position in seven available alignments (Table 3.11). The plants changed positions as more data was accumulated. e.g. B_4 dropped slightly as more fertility data were added, whereas, B3 improved as fertility data were added. The importance of alignment increased in the direction of column 1 to column 7 i.e., giving importance to traits on the basis of their likely potential contribution in seed setting.

Table 3.11. Final alignments and outcome for index-1 and family B.

T1 **T2** **T3** **T4**	**T1** **T2** **T3** **T4** **T5**	**T1** **T2** **T3** **T4** **T5** **T6**	**T1** **T2** **T3** **T4** **T5** **T6** **T7**	**T1** **T2** **T3** **T4** **T5** **T6** **T7** **T8**	**T2** **T3** **T4** **T5** **T6** **T7** **T8** **T9**	outcomes (ranked order)
$\mathbf{B_4}$	B_4	B_4, B_3	B_3	B_3	B_3	B_3
$\mathbf{B_2}$	B_3		B_1	B_1	B_4	B_1,B_4
$\mathbf{B_8}$, $\mathbf{B_1}$, $\mathbf{B_3}$	B_2	B_1	B_4	B_8, B_4	B_2	
	B_1	B_2	B_2		B_4	B_2
	B_8, B_9	B_9	B_8	B_2		B_8
$\mathbf{B_7}$		B_8	B_9			B_9
$\mathbf{B_9}$, $\mathbf{B_5}$	B_7	B_7				
	B_5	B_5				B_7
$\mathbf{B_6}$						B_5,B_6

The above mentioned procedure was followed with all four indices. The outcomes of these different indices were compared to obtain a final outcome for index selection (Table 3.12). As an outcome of index selection, three categories i.e., positive, medium and negative, were established consisting of genotypes carrying higher, medium and lower weightage, respectively.

Table 3.12. Comparison of the ranked orders for nine genotypes of family B using four indices to get final outcome for index selection.

Index-1	Index-2	Index-3	Index-quartile	outcome	
B_3	B_3	B_4,B_3	B_3,B_1	B_3	Positive
B_1,B_4	B_1, B_4	B_1	B_4	B_1,B_4	
B_2	B_8				
B_8	B_2	B_2,B_8,B_9	B_8	B_2,B_8	Medium
B_9	B_9		B_2	B_9	
			B_9,B_7		
B_7		B_7	B_5	B_7	Negative
B_5,B_6	B_5,B_6,B_7	B_5,B_6	B_6	B_5,B_6	

Data were also separately arranged on the basis of florets per plant. The outcomes of index selection were compared with results of florets per plant (Table 3.13). Those plants agreeing in both arrangements were finally selected. They were categorized as superior, medium and inferior on combined evaluation by index selection and florets per plant.

Table 3.13. Final outcome of index selection compared with florets per plant to get the overall final outcome.

Index selection	Florets/plant	Final outcome	
B_3	B_2, B_4	B_3,B_4	Superior
B_1,B_4	B_3	B_2	
	B_1		
B_2,B_8	B_8	B_8	Medium
B_9	B_7	B_7	
B_7	B_5		
B_5,B_6	B_9	B_5,B_6	Inferior
	B_6		

The above mentioned selection procedure was used to align plants of all BC_1F_1 families based on their performance (Table 3.14). Plants of each family were sorted independently. For example, plants 9, 6 and 1 of family A and plants 3 and 4 of family B may or may not have had different index values and florets per plant, but they were the top performer of their respective families.

Table 3.14. Alignment of genotypes of BC_1F_1 families on the basis of their performance as predicted by the index selection and florets per plant.

		BC_1F_1 families								
	Performance	**A**	**B**	**C**	**Cu**	**D**	**K**	**S**	**T**	**Tf**
Genotypes	**High**	9,6,1	3,4	5,4	1	2	2	2,10	8	1
		10	2	3	2	1	3	1	6	6
		3,15,11	8	2	3		4	19	9	4
		12,14	7	8	4		1	13		5
			5,6	6,7				14		3
	Low							6		8

3.3.5 Improving variation for future use

The results of intercrossing and backcrossing were important components of index selection. The outcomes of these crosses (ripened seeds) were expected to be available at or near the end of the season. Moreover, variation in flower emergence also delayed establishing of florets per plant criteria. Efforts were made to involve maximum possible numbers of BC_1F_1 genotypes in intercrossing and backcrossing. Priority was given to the plants showing better head production ability. These crosses created:

a Seed production data to be used in index selection.

b. Next generations (BC_1F_2 and BC_2F_1) to be used in subsequent experiment-2.

3.4 SUMMARY

This experiment was a pilot study to establish the feasibility of a full fledged breeding effort, with an aim to improve seed production of the interspecific hybrids of white clover and *T. uniflorum*. The BC_1F_1 hybrids showed lower pollen viability, head size and seed setting compared to their recurrent parent, white clover. They were more self compatible than white clover. The BC_1F_1 families expressed a range of variation for most of the traits both within and among families. For example, family Cu was efficient in florets per head, heads per plant and florets per plant, whereas family D produced fewer heads. Both, the white clover and the F_1 hybrid parents appeared to be effective in creating variation in seed setting efficiency in the BC_1F_1 plants. Plants of a white clover cultivar differed in their compatibility with F_1 hybrids to produce seeds in BC_1F_1 plants. The correlation studies showed that seed setting was associated with self compatibility. There was a weak positive correlation between head size and number of heads. Peduncle length was moderately associated with head size. Most of the hybrid families had peduncles shorter than the subtending petioles. No marked negative associations were found between root traits and any seed production trait.

The considerable range of variation for all seed production traits showed that there was potential for breeding work to improve seed production of these BC_1F_1 hybrids. No heritability estimates relating to these hybrids were available. Associations among the traits were moderate to weak and unreliable due to the early generational stage of these interspecific hybrids. Index selection, coupled with the florets per plant trait, was adopted to consider all traits likely to have importance in seed production. The BC_1F_1 plants were backcrossed with white clover varieties of diverse origin, and intercrossed to create BC_2F_1 and BC_1F_2 generations, respectively, for use in successive stages of the study.

Chapter 4

MANAGING VARIABILITY

4.1 INTRODUCTION

The reasonably high level of variation, improved self-compatibility and weak correlations among seed production traits found in BC_1F_1 during experiment-1 supported further research to explore and improve seed production of this interspecific hybrid. Seventy five different genotypes consisting of two generations, i.e. BC_1F_2 and BC_2F_1 were selected for this experiment-2. Following questions were posed in this study:

4.2 RESEARCH QUESTIONS

1. **Potential and actual seed setting**
   The number of ovules in an ovary shows the potential for high seed production. Very low harvestability of the available ovules as mature seeds is a much highlighted issue in white clover. The hybrids were also expected to have this problem due to their interspecific hybrid nature, the expected low adaptation of the *T. uniflorum* parent to a temperate humid climate and the known difference between potential and actual seed yield of the white clover parent.
2. **Associations among the traits**
   Relationships among traits with relevance to seed production highlight their possible importance in any effort to improve seed production of a plant. It indicates the benefits and compromises attached with each trait. Identification of composite traits having cumulative positive effects of several primary traits was expected to save time and reduce the efforts required in selecting the primary traits individually.
3. **Difference in parental effect**
   The hybrids were outcrossed and expected to have heterozygous genetics to perform well. There were two parental sources of variation, i.e. the white clover cultivars used as recurrent parents in backcrossing and the hybrids used in intercrossing. The difference in level and effect of the

heterozygosity contributed by these two parental sources was important to determine their importance in further hybridization.

4. **Differences in family effects**

   Differences in white clover cultivars involved in the creation of BC_1F_1 hybrids were effective in creating differences among families of this generation. It was important to know whether further intercrossing within a family or its backcrossing with a common recurrent parent, facilitated maintaining the identity of a family. This analysis was also expected to reveal the differences in performances of the families derived from different white clover cultivars and with different breeding strategies.

5. **Inheritance of the traits**

   The heritability of a trait is a very important attribute from a breeding point of view. Selection for an important but non-heritable trait cannot result in any improvement in the next generation. An index selection and florets per plant were used as selection criteria in BC_1F_1 generation due to unavailability of heritability estimates and the early generational stage of the hybrids. The analysis of the effectiveness of these selection criteria was important in designing future selection strategies.

6. **Differences in generation effects**

   Backcrossing recovers the phenotype of the recurrent parent. However, reduction of the genomic proportion of the donor parent in the hybrids reduces the chances of introgression. Intercrossing facilitates introgression but stable recovery of the desired phenotype is expected to be slower. Comparison between BC_2F_1 and BC_1F_2 was expected to show the difference between the efficiencies of these two breeding approaches to improve seed production of the hybrids.

4.3 MATERIALS AND METHODS

4.3.1 Plant material

The BC_1F_1 hybrids of each family were crossed with each other to produce BC_1F_2 generation and with white clover to create BC_2F_1 plants at the end of experiment-1 (Table 4.1).

Table 4.1. Details of plant material used in experiment-2.Code numbers represent the crosses.

Family	BC_1F_2 Pedigree	BC_1F_2 Code number	BC_2F_1 Pedigree	BC_2F_1 Code number
A	A_{10} x A_6	1	DU_{57} x A_1	69
	A_9 x A_6	3	DU_{846} x A_1	70
	A_{11} x A_6	4	L_1 x A_1	71
	A_6 x A1	5	L_8 x A_1	72
	A_6 x A_9	6	L_4 x A_1	73
	A_6 x A_{11}	7	CH_5 x A_1	74
	A_6 x A_{15}	8	CH_2 x A_1	75
	A_6 x A_{10}	9	CH_4 x A_1	82
	A_{15} x A_6	10	DU_{45} x A_1	63
	A_3 x A_6	31	DU_{21} x A_{10}	64
			DU_{44} x A_{11}	66
			DU_{44} x A_6	65
S	S_{10} x S_{12}	11	CH_{13} x S_{10}	45
	S_{10} x S_1	12	L_4 x S_{10}	46
	S_{10} x S_{14}	13	CH_{11} x S_{13}	47
	S_{10} x S_8	14	CH_{13} x S_{13}	48
	S_{12} x S_{10}	15	L_4 x S_{13}	49
	S_8 x S_{10}	16	CH_6 x S_{14}	51
	S_1 x S_{10}	17	CH_{11} x S_{14}	52
	S_{14} x S_{10}	18	L_1 x S_{14}	53
			L_4 x S_{14}	54
B	B_2 x B_3	19	P_3 x B_4	60
	B_2 x B_4	20	P_2 x B_6	62
	B_2 x B_8	21	P_2 x B_8	61
	B_4 x B_2	22		
	B_3 x B_2	23		
	B_8 x B_2	24		

C	C_4 x C_8	25			
	C_5 x C_8	26			
	C_8 x C_6	27			
	C_8 x C_5	28			
	C_8 x C_4	29			
	C_8 x C_7	30			
CU				CH_{13} x Cu_1	36
				AR_{28} x Cu_1	37
				L_8 x Cu_3	38
				L_8 x Cu_2	39
				CH_5 x Cu_2	40
				AR_{28} x Cu_2	41
				L_3 x Cu_4	42
				CH_{13} x Cu_4	43
				AR_{28} x Cu_4	44
				AR_{29} x Cu_4	76
				AR_{30} x Cu_4	77
				CH_5 x Cu_2	78
				CH_6 x Cu_4	79
				L_2 x Cu_4	80
				L_8 x Cu_4	81
TF				L_8 x Tf_6	2
				L_5 x Tf_4	56
				L_5 x Tf_1	57
				L_5 x Tf_5	59
				L_5 x Tf_3	67
				L_3 x Tf_3	68

The plant material was used in experiment-2. The intercrossed (BC_1F_2) and backcrossed (BC_2F_1) generations were considered separately (Table 4.1). The family name of BC_1F_1 hybrid parent, used to produce second backcrossed or intercrossed generations was used as their family identity (Table 4.2).

Table 4.2. Pedigree and naming procedure for BC_1F_2 and BC_2F_1 plants used in experiment-2. A selected genotype of only white clover variety Aran is mentioned as an example.

Hybridization	**1st backcrossing**	**Experiment-1**		**Experiment-2**		
F_1	Recurrent Parent	BC_1F_1	Code			Family
white clover x *T. uniflorum*	**white clover**	**white clover x {white clover x *T. uniflorum*}**	**BC_1F_1**	**BC_1F_2**	**BC_2F_1**	
$KopuII_2$, x T66-6	$Aran_1$	$Aran_1$ x ($KopuII_2$ x T66-6)	**A_1**	**A_1** x A_1	CH x A_1	A

The BC_1F_1 plants belonging to six families i.e., A, B, C, S, Cu and Tf, were used in crossing. The white clover varieties used as female parent in backcrossing to produce BC_2F_1 plant were Tienshan (CH), Aran (AR), Ladino (LA), Durana (DU) and Prestige (PR).

Seeds were scarified by rubbing them gently with sand paper. Seeds were placed on filter papers moistened with deionized water on 9th of June 2009. Seeds took about 2 hours to imbibe. The imbibed seeds were placed in a refrigerator at 10°C for 2 nights. Then the seeds were transferred into an incubator (at 25°C) to stimulate their germination. Seedlings showing normal germination were transplanted into thoroughly moistened potting mixture in trays. Seedlings were allowed to grow and establish in glasshouse conditions. Later on, trays carrying plantlets were placed outside for vernalization. On 2nd of August 2009, plantlets showing normal growth were shifted into a tapered cylindrical plastic pot (150 mm height × 146 mm top diameter × 124 mm bottom diameter and approximately 1.9 L in volume) commonly used in greenhouse. The pots were placed outside 20 cm apart from one another.

4.3.2 Experimental plans

Plants of 75 families representing BC_1F_2 and BC_2F_1 generations were arranged in a randomized complete block design (RCBD) with three replications and five plants per family per replication (75 x 5 x 3). The pots were placed outside at eight weeks old from August 2009 until May 2010. This ensured the low temperature and changing daylength patterns lead to normal flower initiation and appearance. Following methodologies were adapted to address the research questions.

4.3.2.1 Potential and actual seed setting

Four selected families were examined to observe difference between potential and actual seed setting. Potential seed setting was determined by microscopic count of available ovules in an ovary. Average ovules per ovary were multiplied with florets per plant to derive potential seed yield of the plant. Actual seed yield of a plant was estimated by multiplying the average harvested seed yield of a ripened head with heads per plant.

4.3.2.2 Association among the traits

Principal component analysis (PCA) was used by following the methodology described by SAS support (SAS, 2012). Path coefficient analysis (ULCA, 2012) was used to explore association among different traits. Data were analysed by considering:

a. All traits. It includes root traits along with seed production traits.
b. Only traits directly related to seed production.

4.3.2.3 Differences in parental effects

Progenies were evaluated for effects created by white clover, BC_1F_1, and male or female parents.

a. White clover parents: Nine plans were used to observe inter-varietal and intra-varietal differences of the influence of white clover parent in backcrossing for recovery of seed production traits (Figure 4.1). Subscripts A, B and C represent different varieties of white clover and 1, 2 and 3 correspond to the different plants of each white clover variety.

$$\left.\begin{array}{l} WC_{A1}\ \ WC_{A2}\ \ WC_{A3}\ \ x \\ WC_{B1}\ \ WC_{B2}\ \ WC_{B3}\ \ x \\ WC_{C1}\ \ WC_{C2}\ \ WC_{C3}\ \ x \end{array}\right\} \qquad BC_1F_1$$

Figure 4.1. Crossing pattern of plans used to analyse effect of white clover parent on progenies. The letter "WC" represents white clover.

b. BC_1F_1 parents: Within family and between families effect of different BC_1F_1 parents were observed in intercrossing and backcrossing.

i. **Intercrossing:** Four plans were employed to observe the influence of BC_1F_1 as female parent (Figure 4.2). Four other plans investigated effect of BC_1F_1 parent on progenies as male parent (Figure 4.3).

$$\left.\begin{array}{l} BC_1F_{1b} \\ BC_1F_{1c} \\ BC_1F_{1d} \end{array}\right\} \begin{array}{l} x \\ x \\ x \end{array} \qquad BC_1F_{1a}$$

Figure 4.2. Crossing pattern of plans used to analyse effect of BC_1F_1 female parent on progenies.

$$BC_1F_{1a} \qquad \left.\begin{array}{l} \\ \\ \end{array}\right\} x \qquad \begin{array}{l} BC_1F_{1b} \\ \qquad BC_1F_{1c} \\ BC_1F_{1d} \end{array}$$

Figure 4.3. Crossing pattern of plans used to analyse effect of BC_1F_1 parents on progenies.

ii. **Backcrossing:** Three plans were used to explore the effect of BC_1F_1 as a male parent in backcrossing (Figure 4.4).

$$WC \qquad x \left.\begin{array}{l} \\ \\ \end{array}\right\} \qquad \begin{array}{l} BC_1F_{1a} \\ BC_1F_{1b} \\ BC_1F_{1c} \end{array}$$

Figure 4.4. Crossing pattern used to analyse effect of BC_1F_1 male parent on progenies. Letter "WC" represents white clover.

c. Male or female parent: Progenies of reciprocal intercrosses were compared to find out any differences of maternal or paternal influence on the traits. Thirteen different plans involving families A, S, B and C, were used for this analysis.

4.3.2.4 Differences in family effects

Four families i.e., A, S, B and C, of BC_1F_2 generation were used to evaluate difference within and among families. Similarly data of four families i.e., A, Cu, Tf and S, of BC_2F_1 generation was examined to explore differences present within and among these families.

4.3.2.5 Inheritance of the traits

Based on index selection and their florets per plant production capacity, plants of experiment-1 were classified as positive, medium and negative for their predicted high, medium and low seed production performance, respectively. These positive, medium and negative BC_1F_1 plants intercrossed with a single BC_1F_1 (Figure 4.5) or backcrossed with a single white clover (Figure 4.6), were grown to evaluate differences among their seed production abilities. The number of plans used for this study was 4 for intercrossing and 5 for backcrossing.

$$\left.\begin{array}{ll} \text{Positive } BC_1F_1 & \text{x} \\ \text{Medium } BC_1F_1 & \text{x} \\ \text{Negative } BC_1F_1 & \text{x} \end{array}\right\} \qquad BC_1F_1$$

Figure 4.5. Crossing pattern used to analyse inheritance of florets and seed production potential in intercrossing.

$$\left.\begin{array}{ll} \text{Positive } BC_1F_1 & \text{x} \\ \text{Medium } BC_1F_1 & \text{x} \\ \text{Negative } BC_1F_1 & \text{x} \end{array}\right\} \qquad \text{WC}$$

Figure 4.6. Crossing pattern used to analyse inheritance of florets and seed production potential in backcrossing. Letter "WC" represents white clover.

Crosses involving genotypes having different peduncle lengths were used to observe heritability of peduncle length trait (Figure 4.7). This study was done by using 4 separate plans for each of intercrossed and backcrossed pattern.

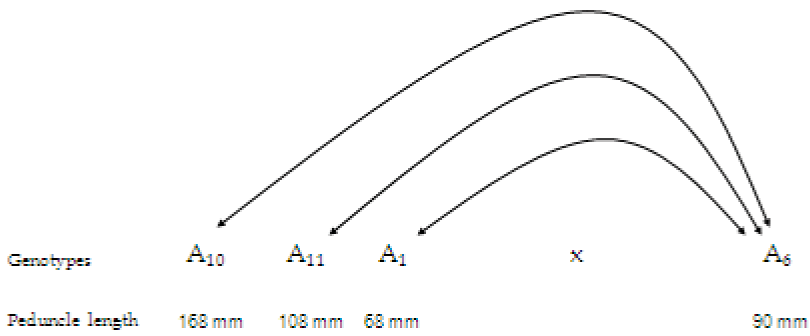


Figure 4.7. Schematic diagram of intercrosses of family A used to measure heritability of peduncle length.

4.3.2.6 Differences in generation effects

Overall performances of second backcrossed (BC_2F_1) and intercrossed (BC_1F_2) generations were compared for seed production traits.

4.3.3 Measurements

Data were collected for following parameters.

4.3.3.1 Reproductive traits

a. **Floral bud and head emergence per plant:** Ten counts for each plant were made for head production from 22nd of September 2009 to January 12, 2010. The first seven counts were taken at intervals of about seven days. This determined the starting date of flowering for all families. The last three counts were used to determine the dates of the flowering peak and seed harvest.

b. **Per head measurements:** Ten mature heads were removed from each plant during the second week of January 2010. Data for seeds per floret, florets per head and peduncle length were extracted and used to derive data for seeds per head.

c. **Per plant measurements:** Data for individual plants were derived by using head production data of the second to last count taken on 13th of December 2009 with per head data taken at the end of experiment i.e., during second week of January 2010.

d. **Pollens and ovules assessments:** Pollen stainability was determined by using 2% acetocarmine stain as described in experiment-1 (Section 3.3.1.1). Ovules with in an ovary were counted under dissecting microscopic (Plate 4.1). For each family, 2 heads were removed from 5 available genotypes. One floret from each bottom, middle and top part of the head was taken for ovule count. There involved 4 families, 5 genotypes per family, 2 heads per genotype and 3 florets per head. The carpel was removed from each floret and dipped into Carnoy's solution (60% ethanol, 30% chloroform and 10% glacial acetic acid) for 5-10 minutes. This fixative was used to remove chlorophyll from the ovarian tissues to make them transparent and improve the visibility of the ovules. After taking the carpel out of Carnoy's solution, it was lightly touched on a tissue paper to remove the excess solution. Then it was placed on a slide and one drop of 1% acetocarmine was added as a colouring agent. Being red, acetocarmine proved to be a good contrast to green. Ovules were counted directly under a dissecting microscope with 10x magnification and a red background.

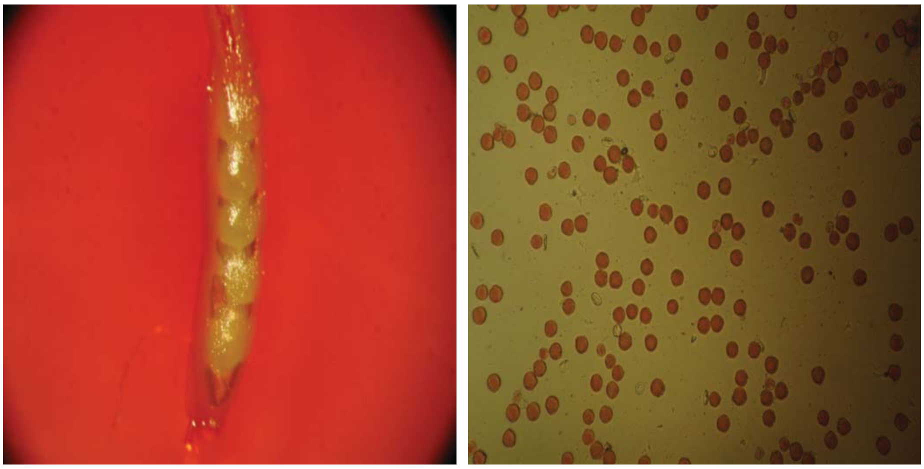


Plate 4.1. Ovule count and pollen stainability assessments. A, a transparent pod showing the contained ovules, B, pollen viability determined by staining with 2% acetocarmine. Viable pollens are fully expended spheres. Non-viable pollens are clear or shrunken spheres.

4.3.3.2 Vegetative traits

Foliage biomass was recorded on January 17, 2010. Foliage, including the reproductive parts, was harvested from all plants and weighed within approximately two hours after harvest.

4.3.3.3 Root traits

Roots of a subset of the plant population were washed on 13th of May 2010 to record the following statistics.

a Tap root diameter: *Vernier Caliper (Mitutoyo digital Caliper- 500.550.551)* was used to measure diameter of tap root at the base of the crown.

b Nodal root diameter: Diameter of 10 thickest nodal roots was recorded at 1 mm depth.

c. Woody Tap root length. Tap root produced thin branches at its terminal end. Length of intact woody portion of tap root was measured till the branched end.

d. Root dry weight: Root dry weight was recorded after separating the roots from stolons and oven drying at 80^{o}C for 24 hours.

4.4 ANALYSES AND RESULTS

4.4.1 General features of the data

Data for all traits were transformed to fulfil the assumption of the normal distributions required by all statistical procedures used in this study. Box-Cox transformation in GenStat v12 was employed to get lambda values that were used as powers for data transformation. General features of the plant population used in this experiment are summarized in Table 4.3.

Table 4.3. Summary statistics based on data of all plants of experiment-2. The abbreviations N, CV, SD, Min, Max and SE stand for number of plants observed, Coefficient of variation, standard deviation, minimum value, maximum value and standard error, respectively.

Variable	N	Mean	CV	SD	Variance	Min	Max	SE	Skewness*	Kurtosis*
Seeds / floret	838	1.00	65.93	0.66	0.43	0.00	3.89	0.02	-0.15	-0.09
Florets / head	839	36.57	39.32	14.38	207	10.00	100	0.50	0.18	-0.07
Seeds / head	839	38.61	85.38	32.96	1087	0.01	197	1.14	0.08	0.13
Heads / plant	832	33.34	84.10	28.04	786	1.00	167	0.97	0.18	-0.82
Florets / plant	817	1079	86	928	861158	14.50	5923	32.47	0.28	-0.33
Seeds / plant	817	1159	125	1450	2101104	0.01	11750	50.71	0.11	-0.68
Peduncle length (mm)	788	129	32.52	41.87	1753	35.00	339	1.49	-0.02	0.23
Foliage dry weight (gm)	800	125	28.57	35.78	1280	54.00	292	1.27	0.13	-0.24
Root dry weight (gm)	321	4.56	40.25	1.83	3.37	0.71	11.58	0.10	-0.02	0.27
Woody tap root length (mm)	300	33.06	56.91	18.81	354	4.00	120	1.09	-0.01	0.70
Tap root diameter (mm)	309	9.15	25.70	2.35	5.53	3.79	19	0.13	0.06	0.15
Nodal root diameter (mm)	322	1.94	26.67	0.52	0.27	0.92	3.67	0.03	0.12	-0.11

* **Derived from transformed data.**

The generalized linear model (GLM) was used for ANOVA to accommodate the missing values and unbalanced experimental design. Significant differences were observed between generations for all seed production traits, except for the seeds per floret trait (Table 4.4). The second backcrossed generation (BC_2F_1) was significantly superior in florets per head, seeds per head, peduncle length, foliage dry weight and tap root diameter. The second intercrossed generation (BC_1F_2) performed considerably better than BC_2F_1 in production of heads, florets and seeds per plant. The families within a generation significantly differed for all traits except for woody tap root length (Plate 4.2). The plants of a family were similar in all traits. Replication differences were present for most of the seed traits.

Table 4.4. P-values showing ANOVA results. Highlighted cells show significant difference (P ≤ 0.05).

	Observations	Replication	Generation	Family	Plant
Levels		3	2	76	5
Seeds / floret	838	0.0381	0.1103	<.0001	0.2673
Florets / head	839	0.0694	<.0001	<.0001	0.3408
Seeds / head	839	0.0055	<.0001	<.0001	0.2494
Heads / plant	832	<.0001	<.0001	<.0001	0.2288
Florets / plant	813	<.0001	<.0001	<.0001	0.3474
Seeds / plant	816	0.0082	0.0054	<.0001	0.1476
Peduncle length	786	0.0029	<.0001	<.0001	0.2639
Foliage dry weight	799	<.0001	<.0001	<.0001	0.9840
Root dry weight	321	0.2523	0.4149	<.0001	0.9690
Woody tap root length	300	0.6356	0.7304	0.1129	0.8536
Tap root diameter	309	0.4263	0.0016	<.0001	0.4528
Nodal root diameter	322	0.3634	0.7426	0.0001	0.4234

A

B

Plate 4.2. Family variation of the BC_1F_2 and BC_2F_1 generations in experiment-2. In A, the left row is a family of the BC_1F_2 generation. The other rows are BC_2F_1 families. In B, BC_2F_1 plants indicating difference in growth are shown.

4.4.2 Potential and actual seed set

Two pairs of families belonging to the BC_2F_1 generation were selected to estimate differences between potential and actual seed yield (Table 4.5). Pair one had one BC_1F_1 hybrid plant backcrossed with two different white clover cultivars. In pair two, two different BC_1F_1 plants were backcrossed to the same white clover plant. Numbers of ovules present in an ovary were multiplied by florets produced per plant to estimate potential seed yield. Florets were taken from three different positions i.e., top, middle and bottom of the head. It was found that position of a floret on the head had no significant affect (F = 0.1103) on its ovule production ability.

Table 4.5. Details of families used to study potential and actual seed settings.

Pair	Family	Cross	Average ovules / ovary
1	44	AR_{28} x Cu_4	6.0
	42	L_3 x Cu_4	5.4
2	56	L_5 x Tf_4	5.8
	57	L_5 x Tf_1	5.6

Considerable difference between potential and actual numbers of harvestable seeds was observed (Figure 4.8). This dissimilarity was affected by the parents involved. Both white clover and BC_1F_1 hybrid parents affected seed production potential. In case of the pair one, white clover cultivar L showed better compatibility with Cu_4 for seed production potential, compared to cultivar AR. In the second pair, the BC_1F_1 hybrid Tf_1 contributed better in seed production potential than the hybrid Tf_4. The range of ratios between potential and actual seed setting for the different parental combinations was considerably large i.e., 0.03 to 0.29.

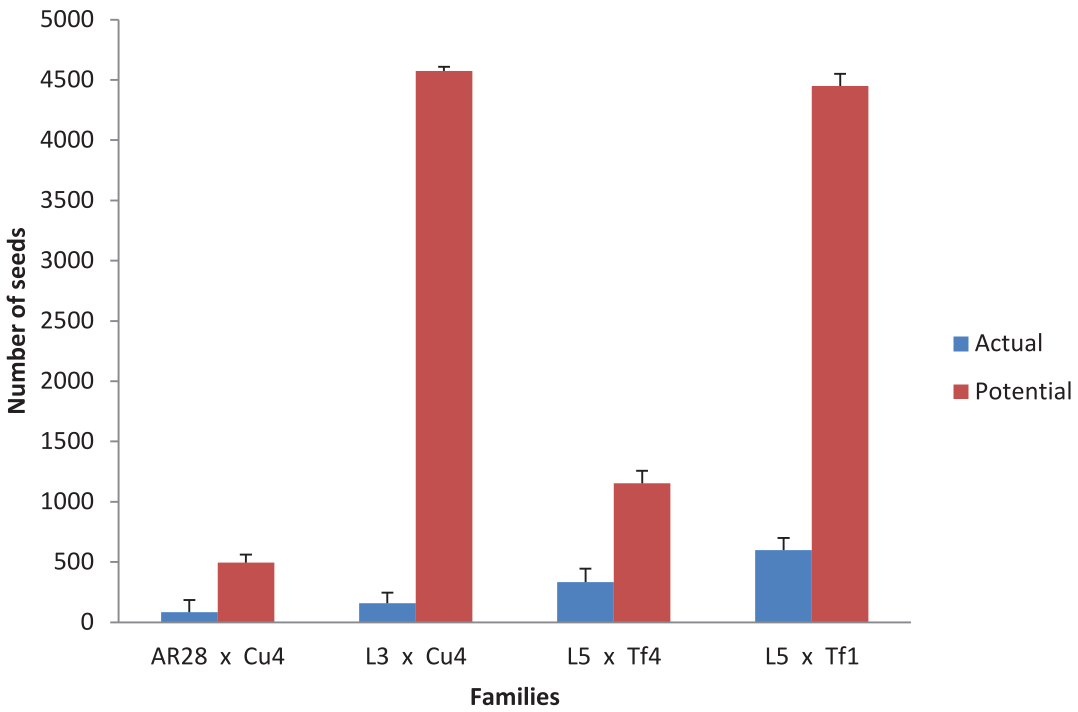


Figure 4.8. Difference between potential and actual seed setting. Coefficients of variation are shown by lines in the bars.

On average for all the four hybrid families studied, only a small proportion (11%) of seed production potential was harvested. However, the range for harvestable proportion of potential seed yield was considerably high, i.e. 0.2% to 39.2%.

4.4.3 Associations among the traits

Principal component analysis (PCA) and path analysis were used to explore relationships among the traits in relation to seed production.

4.4.3.1 PCA

The relationships among different traits were explored using PCA in SAS 9.2 (SAS, 2012). The factor procedure was used with principal axis methodology, having one as the prior communality estimate. Principal axes were adjusted by Varimax (orthogonal) rotation to refine the results. Analyses were done to investigate relationships among:

a. All traits.
b. Important seed traits only.
c. Generations and families.

a. **Relationship among all traits:** The PCA analysis considering all traits used only 250 observations, as fewer observations were taken for the root traits as compared to those for the seed traits. The first four factors having eigenvalues greater than one and appearing meaningful in a scree plot were considered for interpretation (Table 4.6). These factors explained 71% of the total variation. Factor one revealed strong positive association among seeds per floret, florets per head, seeds per head and overall seeds produced by a plant. According to factor two, number of heads produced by a plant proved to be an effective contributor to numbers of florets and seeds present on a plant. Relationships among root traits were expressed in factor three. These relationships were high among root dry weight, tap root diameter and nodal root diameter. Factor four showed strong positive associations among head size, peduncle length, foliage dry mass and woody tap root length. No major negative relationship was apparent between foliage and head production traits. No major negative association appeared between root and seed production traits.

Table 4.6. PCA results with four retained factors rotated with the varimax procedure showing relationship among all traits.

	Factor 1	Factor 2	Factor 3	Factor 4
Eigenvalue	3.31	2.49	1.51	1.23
Variance Proportion	0.28	0.21	0.13	0.1
Seeds/floret	90*	21	6	-15
Florets/head	73*	-15	2	44
Seeds/head	98*	9	5	6
Heads/plant	-13	96*	-10	-7
Florets/plant	16	93*	-9	9
Seeds/plant	60*	78*	1	-4
Peduncle length	38	-30	-17	56*
Foliage dry weight	5	2	11	76*
Roots dry weight	5	-7	68*	15
Tap root diameter	2	8	58*	23
Woody tap root length	-12	13	30	53*
Nodal root diameter	4	-22	76*	-18

*** coefficients of eigenvectors greater than 50.**

b. **Relationship among important seed traits:** PCA was also done by considering data for only seed traits. The removal of root traits made 721 observations available for this analysis. The first three factors having eigenvalues greater than one, explaining 84% of total variation and appearing meaningful in a scree plot were considered for explanation (Table 4.7). The associations among seed traits were the same as found earlier (Table 4.6) when all root traits were also considered along with seed traits.

Table 4.7. PCA results with three retained factors rotated with the varimax procedure showing relationship among seed traits only.

	Factor 1	Factor 2	Factor 3
Eigenvalue	3.01	2.40	1.31
Variance proportion	0.38	0.30	0.16
Seeds/floret	14	92*	-9
Florets/head	-17	59*	54*
Seeds/head	4	98*	14
Heads/plant	96*	-16	-15
Florets/plant	97*	8	5
Seeds/plant	80*	57*	-3
Peduncle length	-11	12	78*
Foliage dry weight	8	-7	80*

*** coefficients of eigenvectors greater than 50.**

c. **relationship between generations and families:** PCA was done by considering the average performances of each family for seeds per floret, florets per head, seeds per head, heads per plant, florets per plant, seeds per plant, peduncle length and foliage dry weight. The first three factors having eigenvalues greater than 1 and explaining 91% of the total variance, were retained (Table 4.8). Factor 1 showed strong positive linkage among numbers of heads, florets and seeds per plant. Associations were found among seeds per floret, florets per head and seeds per head, according to the factor 2. Peduncle length appeared linked with foliage dry weight and florets per head in factor 3.

Table 4.8. PCA results with 3 retained factors rotated with the varimax procedure based on average data of selected traits.

	Factor 1	Factor 2	Factor 3
Eigenvalue	3.4983	2.5905	1.2108
Variance proportion	0.4373	0.3238	0.1513
Seeds/floret	19	92*	-13
Florets/head	-34	65*	52
Seeds/head	-5	98*	17
Heads/plant	92*	-23	-28
Florets/plant	98*	-2	-9
Seeds/plant	87*	45	-17
Peduncle length	-26	16	85*
Foliage dry weight	-7	-6	91*

*** coefficients of eigenvectors greater than 50.**

The factors mentioned in Table 4.7 were used to draw a three-dimensional scatter plot to observe any pattern present between and within the two generations involved in this analysis (Figure 4.9). The families belonging to the S group were comparatively more clustered. There was clustering of BC_1F_2 at the lower end and of BC_2F_1 at the higher end of axis for factor 2. This pattern was prominent for families having their representation in both generations i.e., A, B and S.

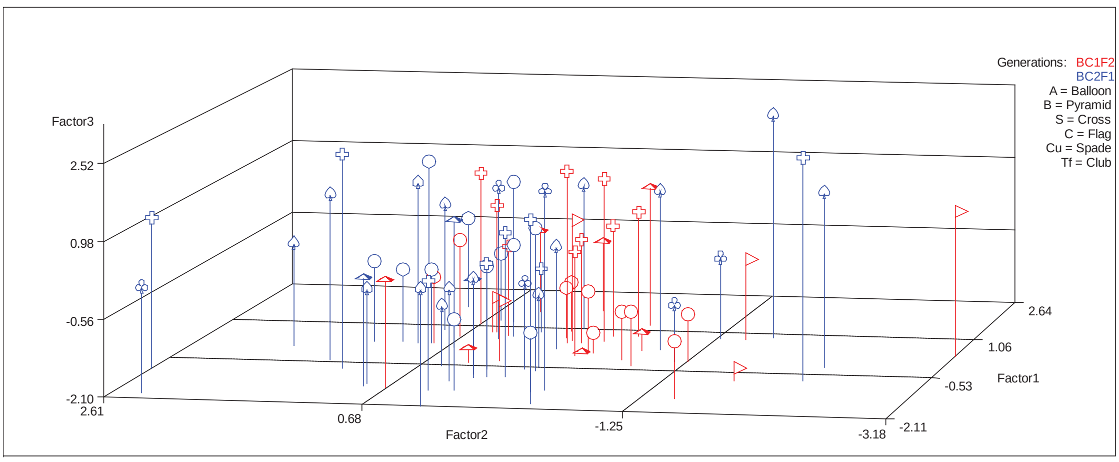


Figure 4.9. Three-dimensional scatter plot showing distribution pattern of generations and families based on first three principal components.

Averaged data of families were used to plot three important seed traits i.e., seeds per floret, florets per head and heads per plant (Figure 4.10). Clustering on a generation basis was clearly visible. Families belonging to the BC_1F_2 generation (in red) generally had numerous but small heads. In contrast, families representing BC_2F_1 (in blue) possessed comparatively fewer but bigger heads. A few families belonging to both generations appeared to have the combination of high seeds per floret and heads per plant. However, except for one family, they did not have large head size.

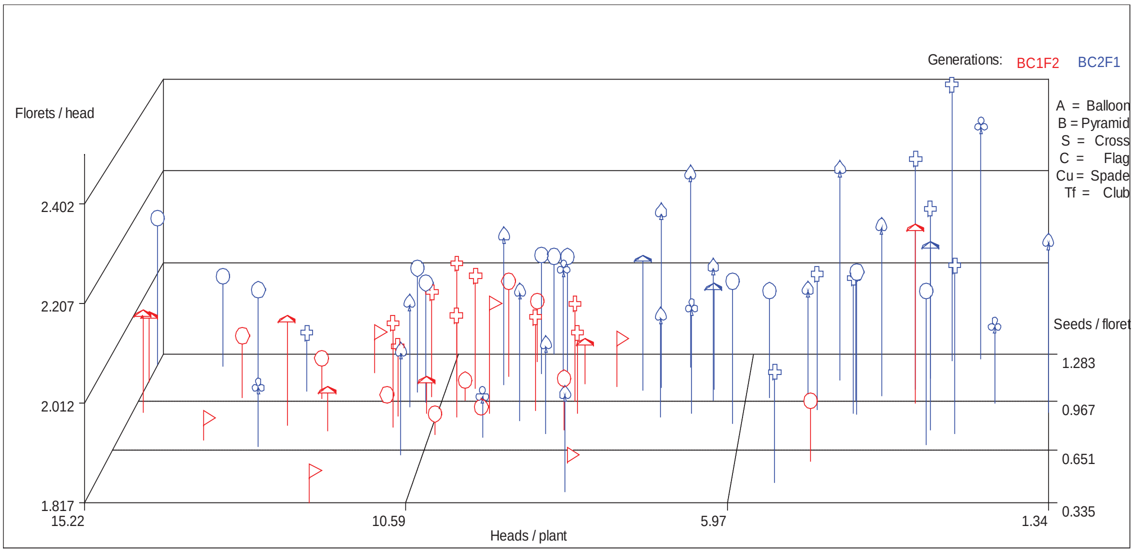


Figure 4.10. Three-dimensional scatter plot showing distribution pattern of generations and families based on three important seed production traits.

4.4.3.2 Path analysis

Path analysis was used to analyse interrelationships among variables and their direct or indirect effect on seed production. A model based on causal relationships between dependent and independent traits was used in the analysis. The analysis was done in the SAS 9.2 statistical package using the Covariance Analysis of Linear Structural Equations (CALIS) procedure (UCLA, 2012). Standardized estimates were taken as path coefficients and the square roots of the variance estimates were used as the residuals (errors). A considerable proportion of observations had random missing values in the data. This was because traits like pollen stainability, ovules per ovary, tap root diameter, etc, were studied in selected families only. Moreover, in some instances all 15 plants per family were unavailable due to lack of seeds to grow the plants or deaths of some plants during the experiment. In path coefficient analysis, the Full Information Maximum Likelihood (FIML) method was used. This method is recommended to fully utilize all available information from the data with high proportion of observations with random missing value (http://support.sas.com/documentation/cdl/en/statug/63347/HTML/default/viewer.htm#statug_calis_sect102.htm). The FIML method used all available information in the observations and plants with at least one non-missing value were included in the analysis. Relationships were explored among all traits and among selected traits.

a. Relationship among all traits: The analysis used 253 observations with complete data set and 601 observations with incomplete data set. Interactions among traits were analysed in relation to seed production (Figure 4.11). Heads per plant appeared to be slightly a more effective trait than seeds per head for seed production of the plant. Seeds per head were effectively influenced by number of seeds produced by a floret. Head size also contributed positively to seeds per head in two directions i.e., directly and via seeds per floret. Positive association between head size and seeds produced in a floret was observed. Vegetative growth of a plant moderately improved florets per head and peduncle length. It had negligible effect on head and root production. Tap root thickness had light relationship with root dry weight. Tap root thickness also exhibited a low positive influence on nodal root diameter and tap root length.

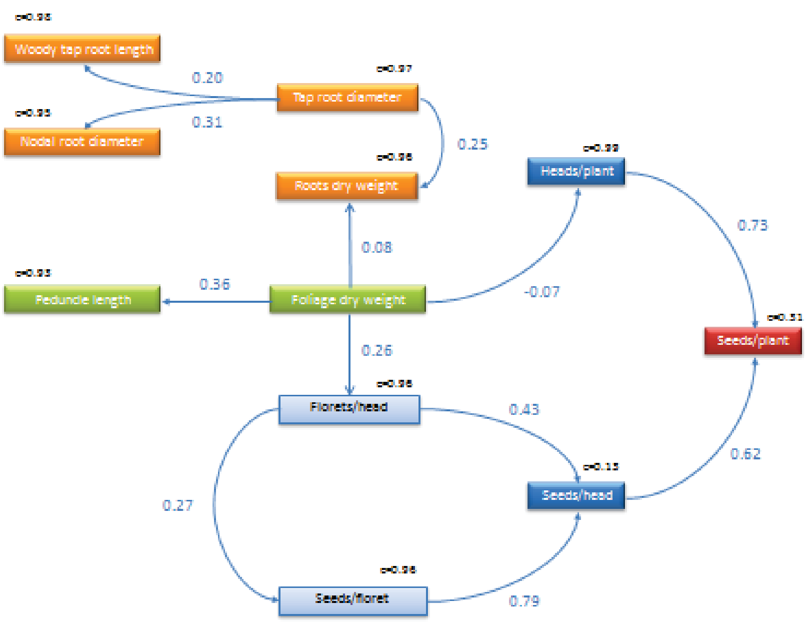


Figure 4.11. Path diagram with the coefficients (indicated on the arrows) and residuals (e), showing interrelationships among traits affecting seed production in white clover x *T. uniflorum* BC_2F_1 and BC_1F_2 hybrids.

b. **Relationship among seed production traits:** Root traits were removed from analysis to improve the observations with a complete set of data. The analysis was based on 724 observations with complete data set and 130 observations with incomplete data set. The results (Figure 4.12) remained same as produced by the data including root traits. The removal of root and vegetative traits further improved data structure (815 observations with complete and 39 observations with incomplete data set). It slightly improved the strength of effects of heads per plant and seeds per head on plant seed production. The overall structure of relationships among traits remained unaffected.

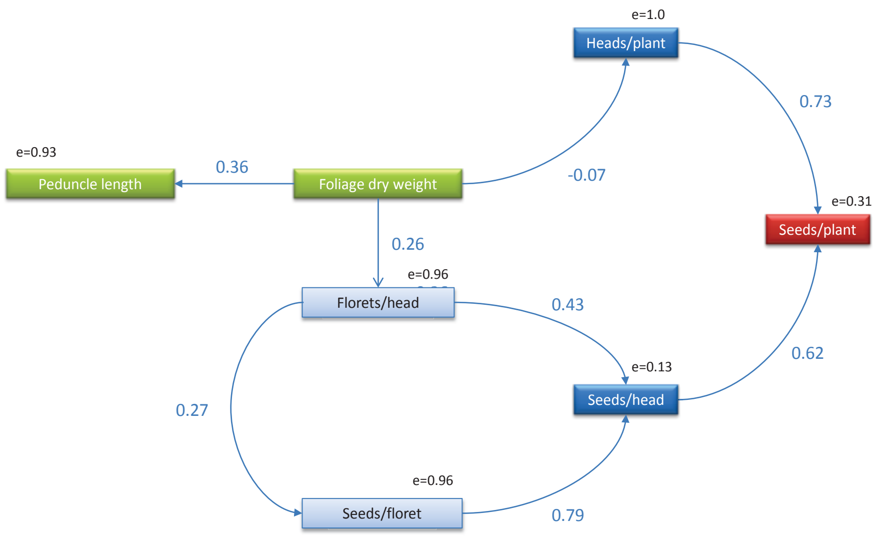


Figure 4.12. Path diagram with coefficients and residuals (e), showing interrelationships among selected traits affecting seed production in white clover x *T. uniflorum* hybrids.

4.4.4 Differences in parental effects

Progenies were evaluated to explore parental influences as white clover parent, BC_1F_1 parent, and male or female parent.

4.4.4.1 influence of white clover parent

Nine plans were designed to investigate the contributions of white clover backcrossed parents (Table 4.9). ANOVA results showed no clear pattern in expression of traits associated with change in white clover parent (Table 4.10). In about half of the traits (54%), change in white clover parent did not make a significant difference. For plan 5 this result appeared valid for all traits. Peduncle length and, heads, florets and seeds per plant appeared to be comparatively more influenced by white clover parent than per head traits.

Table 4.9. Details of plans 1–9, each with one BC_1F_1 plant backcrossed with different white clover genotypes.

Plan	Family	White clover (female parent)	BC_1F_1 (male parent)
1	36	CH_{13}	Cu_1
	37	AR_{28}	Cu_1
2	40	CH_5	Cu_2
	41	AR_{28}	Cu_2
	39	LA_8	Cu_2
3	43	CH_{13}	Cu_4
	44	AR_{28}	Cu_4
	81	LA_8	Cu_4
4	48	CH_{13}	S_{13}
	49	LA_4	S_{13}
5	51	CH_6	S_{14}
	54	LA_4	S_{14}
6	69	DU_{57}	A_1
	70	DU_{846}	A_1
	71	LA_1	A_1
	72	LA_8	A_1
	73	LA_4	A_1
	74	CH_5	A_1
	75	CH_2	A_1
	82	CH_4	A_1
7	69 + 70	DU	A_1
	71 + 72 + 73	LA	A_1
	74 + 75 + 82	CH	A_1
8	44	AR_{28}	Cu_4
	76	AR_{29}	Cu_4
	77	AR_{30}	Cu_4
	43	CH_{13}	Cu_4
	79	CH_6	Cu_4
	42	LA_3	Cu_4
	80	LA_2	Cu_4
	81	LA_8	Cu_4
9	44 + 76 + 77	AR	Cu_4
	43 + 79	CH	Cu_4
	42 + 80 + 81	LA	Cu_4

Table 4.10. P-values showing ANOVA results for plans used to observe effects of white clover parent on hybrids. Cells showing significant differences are highlighted ($P \leq 0.05$).

	Plans								
Source	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Seeds / floret	0.3053	0.0021	0.8079	0.8404	0.5652	<.0001	<.0001	0.012	0.4404
Florets / head	0.3303	0.0119	0.1738	0.0433	0.9992	0.0324	0.0012	0.1123	0.8455
Seeds / head	0.6788	0.0005	0.7499	0.4941	0.5622	0.0077	0.0003	0.0034	0.7454
Heads / plant	0.0951	0.2749	0.0004	0.634	0.5858	<.0001	<.0001	<.0001	0.001
Florets / plant	0.1692	0.4758	0.0021	0.3408	0.647	<.0001	<.0001	0.0004	0.0009
Seeds / plant	0.1096	0.0332	0.0011	0.4208	0.7347	<.0001	<.0001	0.019	0.0439
Peduncle length	0.0021	0.0109	0.6275	0.2951	0.0884	<.0001	0.0002	0.0366	0.0107
Foliage dry weight	0.0009	0.0258	0.0927	0.0541	0.4462	0.1836	0.7266	0.1028	0.1681
Root dry weight	.	.	0.0958	.	.	0.3842	0.297	0.0958	0.0958
Tap root diameter	.	.	0.7875	.	.	0.3748	0.2488	0.7875	0.7875
Woody tap root length	.	.	0.9045	.	.	0.7516	0.5524	0.9045	0.9045
Nodal root diameter	.	.	0.0013	.	.	0.0228	0.0083	0.0013	0.0013

Plans six and eight analysed the comparative effects of different plants of each of three white clover varieties crossed with the same BC_1F_1 male parent. In plan six the common BC_1F_1 plant was A_1. In plan eight progenies of crosses of different white clover genotypes with BC_1F_1 hybrid Cu_4 were evaluated. For most (63%) of the seed production traits, significant differences were observed among the white clover parents in these two plans. In-depth analysis showed that differences among the white clover parents were mostly due to differences within plants of a variety (Figure 4.13). For example, plant LA_3 was a significantly lower performer than the other plants of the same variety for seeds per floret, seeds per head and seeds per plant. White clover variety AR produced less variation, as compared to the variety LA. The difference was more at per plant level as compared to per floret and per head level.

Plan nine ignored the identities of the different genotypes of a white clover variety. It compared the average effects of white clover varieties on their progenies (Figure 4.14). The varieties LA, AR and CH did not show significantly different effects on their progenies with respect to seeds per floret, florets per head and seeds per head. However, progeny performances differed for heads per plant, florets per plant and seed per plant, with varieties CH and AR being the best and the worst, respectively. The progeny rankings became reversed for peduncle length. In general, variety CH positively influenced seed production of the progenies as compared to variety AR.

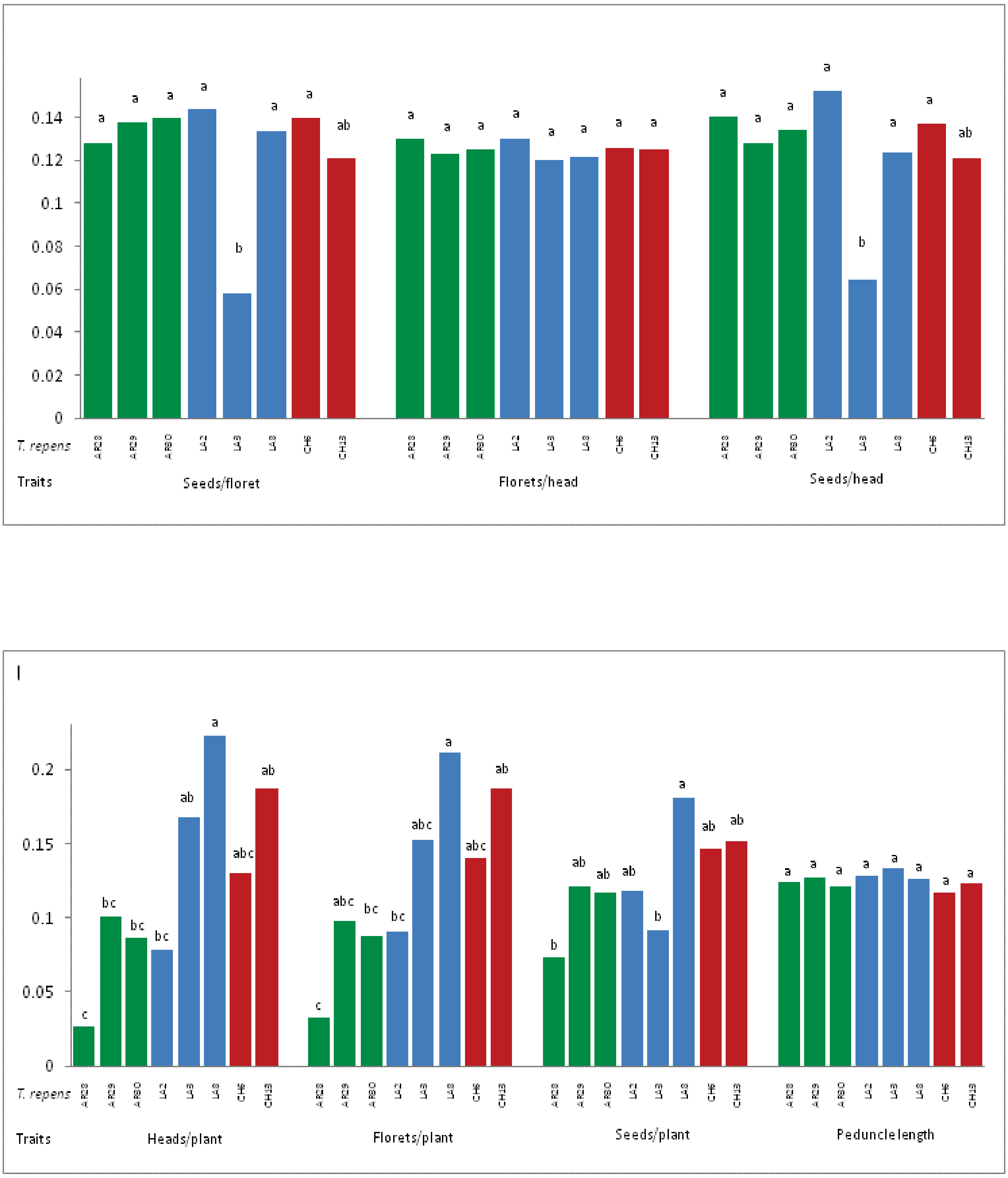


Figure 4.13. Comparative expression for seven different traits of progenies of a single BC_1F_1 (Cu_4) backcrossed with several plants of different white clover varieties for plan 8. Performances were expressed out of 1 (total of the family means for the traits) for all traits to plot different traits in a single graph. Different letters at the top of bars represent significant differences from each other at P≤ 0.05 (Tukey's test following ANOVA).

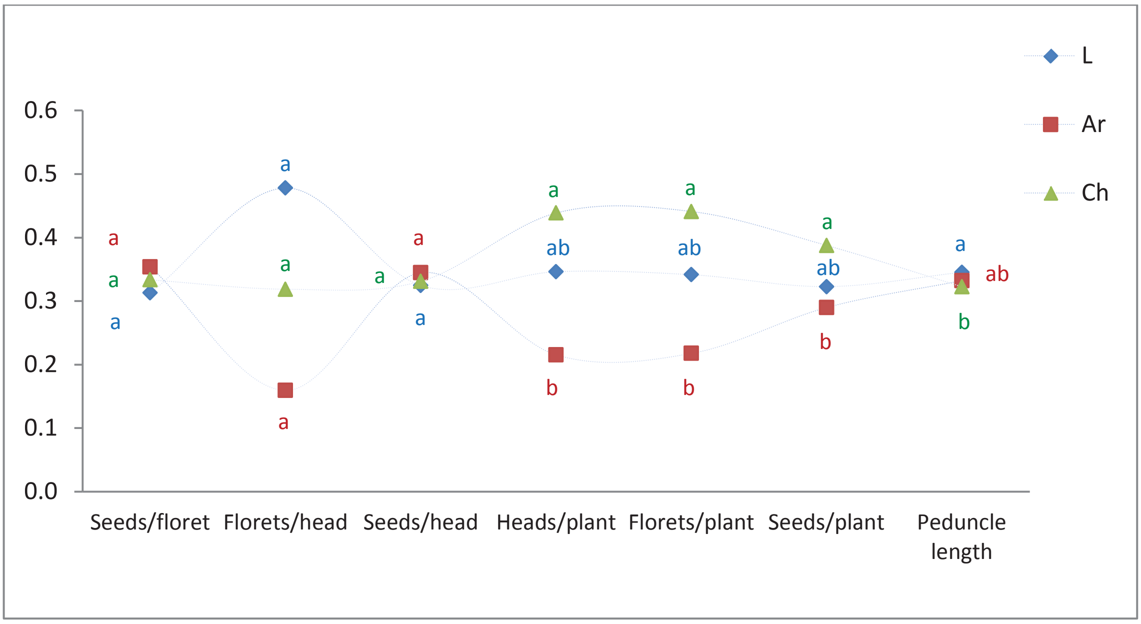


Figure 4.14. Comparative influence of different white clover parents on progenies in plan 9. Performances are derived out of 1 (total of the family means for the traits) for all traits to plot different traits in a single graph. Different letters near the data labels represent significant difference from each other at P≤ 0.05 (Tukey's test following ANOVA).

In plan seven, comparisons were made among white clover varieties LA, DU and CH. The relationships between LA and CH were the same as found in plan nine. The variety DU was in between CH and LA for seeds per floret, seeds per head and peduncle length. It was superior to both of the other varieties for heads, florets and seeds produced per plant, despite producing the smallest heads.

4.4.4.2 Influence of BC_1F_1 parent

Within and between family effects were analysed for the different BC_1F_1 parents used in intercrossing and backcrossing.

a. Intercrossing: Effects were evaluated for BC_1F_1 hybrids used as male as well as female parents. Plans 1 to 4 evaluated the effect on progenies of different female BC_1F_1 parents crossed with same male BC_1F_1 parent (Table 4.11). Plans 5 to 8 were designed to study the effect on progenies of different male BC_1F_1 parents crossed with same female BC_1F_1 parent.

Table 4.11. Details of plans used to evaluate effect of BC_1F_1 parents on progenies in intercrossing.

Plan	Family	Group	Pedigree
1	1	A	A_{10} X A_6
	3		A_9 X A_6
	4		A_{11} X A_6
	10		A_{15} X A_6
2	15	S	S_{12} X S_{10}
	16		S_8 X S_{10}
	17		S_1 X S_{10}
	18		S_{14} X S_{10}
3	22	B	B_4 X B_2
	23		B_3 X B_2
	24		B_8 X B_2
4	25	C	C_4 X C_8
	26		C_5 X C_8
5	9	A	A_6 X A_{10}
	6		A_6 X A_9
	7		A_6 X A_{11}
	8		A_6 X A_{15}
6	11	S	S_{10} X S_{12}
	14		S_{10} X S_8
	12		S_{10} X S_1
	13		S_{10} X S_{14}
7	20	B	B_2 X B_4
	19		B_2 X B_3
	21		B_2 X B_8
8	29	C	C_8 X C_4
	28		C_8 X C_5

ANOVA results showed no clear outcome for the effect of changing BC_1F_1 parent in intercrossing on progenies (Table 4.12). For example plan 1 showed significant differences in contributions made by different parents in progenies for most of the traits. In contrast, plan 2 expressed no differences for any trait among progenies having different BC_1F_1 parents. Many of the trait comparisons (64%) did not differ with change of BC_1F_1 parent.

Table 4.12. P-values showing ANOVA results for plans used to observe effects of BC_1F_1 parent on progenies in intercrossing. Cells showing significant differences are highlighted ($P \leq 0.05$).

	Plans							
Traits	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Seeds / floret	**<.0001**	0.2048	0.2104	0.1598	0.1697	0.1962	0.1291	**0.0004**
Florets / head	0.0904	0.1013	**0.001**	**<.0001**	0.0012	0.9024	0.2353	**0.0025**
Seeds / head	**0.0006**	0.127	**0.0072**	**0.0109**	0.0345	0.4331	0.5799	**<.0001**
Heads / plant	**0.0026**	0.5319	**0.0035**	**0.0014**	0.8471	0.5152	**0.034**	0.7327
Florets / plant	**0.023**	0.5554	**0.0053**	0.5191	0.6631	0.3692	**0.0474**	0.4812
Seeds / plant	**<.0001**	0.3341	0.1108	0.4994	0.1684	0.3345	0.1246	**0.0057**
Peduncle length	0.8755	0.2697	**0.0027**	0.0935	0.3068	0.1251	0.2701	**<.0001**
Foliage dry weight	**0.0273**	0.2352	**0.0524**	0.0861	0.1944	**0.0036**	0.0973	0.9989
Root dry weight	**0.0475**	0.1286	.	0.1825	**0.0048**	0.5819	0.5108	.
Tap root diameter	.	0.3822	.	0.0887	**0.0118**	0.4893	0.5991	.
Woody tap root length	.	0.7407	.	**0.0398**	0.2954	0.0936	0.6842	.
Nodal root diameter	**0.0258**	0.2545	.	0.9425	0.5885	**0.0331**	0.2203	.

b. **Backcrossing:** Progenies were observed to evaluate the effect of change in BC_1F_1 parent in backcrossing (Table 4.13).

Table 4.13. Details of plans used to evaluate effect of BC_1F_1 parents on progenies in backcrossing.

Plan	Family	white clover parent	BC_1F_1 parent
1	57	LA_5	Tf_1
	56	LA_5	Tf_4
	59	LA_5	Tf_5
2	62	PR_2	B_6
	61	PR_2	B_8
3	65	DU_{44}	A_6
	66	DU_{44}	A_{11}

Seed production ability by a floret remained unaffected by change in BC_1F_1 parent (Table 4.14). Root traits, although measured for plan 3 only, also appeared unresponsive to this change. The remaining traits showed different responses in different plans. A majority of the traits (61%) expressed insensitivity to the change of BC_1F_1 parent in backcrossing.

Table 4.14. ANOVA results, expressed by P-values, of plans used to observe effect of BC_1F_1 parent on progenies in backcrossing to white clover. Cells showing significant differences are highlighted ($P \leq 0.05$).

Traits	**Plans**		
	1	**2**	**3**
Seeds / floret	0.0749	0.3991	0.1086
Florets / head	0.0007	0.8527	0.1153
Seeds / head	0.0031	0.6039	0.0488
Heads / plant	0.0784	0.0015	0.3428
Florets / plant	0.1229	0.0006	0.0336
Seeds / plant	0.2862	0.0032	0.0304
Peduncle length	0.0013	0.5729	0.0029
Foliage dry weight	0.1697	0.8531	0.0012
Root dry weight	.	.	0.5188
Tap root diameter	.	.	0.139
Woody tap root length	.	.	0.9943
Nodal root diameter	.	.	0.115

4.4.4.3 Reciprocal crossing

The difference in influence of BC_1F_1 male and female parent on progenies was evaluated by using 13 different plans (Table 4.15). The first four plans consisted of BC_1F_1 genotypes of family group A. plans 5 to 8, 9 to 11 and, 12 and 13 were made up of family group S, B and C, respectively.

Table 4.15. Details of plans used to evaluate effect of reciprocal crossing on progenies.

Plan	Families	Group	Pairs compared
1	1 and 9	A	A_{10} x A_6 and A_6 x A_{10}
2	3 and 6		A_9 x A_6 and A_6 x A_9
3	4 and 7		A_{11} x A_6 and A_6 x A_{11}
4	10 and 8		A_{15} x A_6 and A_6 x A_{15}
5	11 and 15	S	S_{10} x S_{12} and S_{12} x S_{10}
6	12 and 17		S_{10} x S_1 and S_1 x S_{10}
7	13 and 18		S_{10} x S_{14} and S_{14} x S_{10}
8	16 and 14		S_8 x S_{10} and S_{10} x S_8
9	19 and 23	B	B_2 x B_3 and B_3 x B_2
10	22 and 20		B_4 x B_2 and B_2 x B_4
11	21 and 24		B_2 x B_8 and B_8 x B_2
12	25 and 29	C	C_4 x C_8 and C_8 x C_4
13	26 and 28		C_5 x C_8 and C_8 x C_5

In most of the cases (78%) no differences were evident in effects of reciprocal crossing (Table 4.16). However, 45% comparisons in family group C (plans 12 and 13) showed significant effects of reciprocal crossing.

Table 4.16. ANOVA results, expressed by P-values, of plans used to observe effect of reciprocal crossing on progenies. Cells showing significant differences are highlighted ($P \leq 0.05$).

	Plans												
Traits	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**
Seeds / floret	0.18	0.69	0.51	0.12	0.37	0.63	0.72	0.65	0.48	0.20	0.15	0.09	0.43
Florets / head	0.82	0.71	0.26	0.87	0.15	0.94	0.90	0.41	0.53	0.76	0.09	0.0002	0.14
Seeds / head	0.22	0.75	0.84	0.28	0.25	0.81	0.73	0.55	0.74	0.35	0.02	0.94	0.26
Heads / plant	0.88	0.50	1.00	0.02	0.77	0.61	0.89	0.74	0.67	0.42	0.01	0.06	0.004
Florets / plant	0.55	0.43	0.72	0.02	0.60	0.40	0.89	0.61	0.49	0.57	0.03	0.003	0.003
Seeds / plant	0.15	0.39	0.87	0.01	0.40	0.94	0.65	0.43	0.88	0.51	0.08	0.37	0.02
Peduncle length	0.25	0.88	0.64	0.55	0.34	0.06	0.12	0.32	0.02	0.45	0.15	0.97	0.001
Foliage dry weight	0.29	0.02	0.05	0.97	0.002	0.44	0.64	0.0003	0.32	0.35	0.61	0.01	0.50
Root dry weight	.	.	.	0.09	0.21	0.67	0.18	.	.	.	.	0.41	.
Tap root diameter	.	.	.	0.07	0.71	0.003	0.99	.	.	.	.	0.01	.
Woody tap root length	.	.	.	0.71	0.03	0.40	0.91	.	.	.	.	0.01	.
Nodal root diameter	.	.	.	0.25	0.01	0.14	0.26	.	.	.	.	0.79	.

4.4.5 Differences in family effects

The experiment consisted of families which belonged to two generations, BC_1F_2 and BC_2F_1. There were four BC_1F_1 families, A, B, C and S, were involved as parents in intercrossing to produce BC_1F_2 plants. Plants of each family were intercrossed to produce the BC_1F_2 generation for that family. The BC_1F_1 families used in backcrossing with white clover to produce BC_2F_1 were A, B, S, Cu and Tf. Both BC_1F_2 and BC_2F_1 were considered separately during data analysis. Plants were named after the family name of their BC_1F_1 parent. For example if the plants of BC_1F_1 family A were intercrossed to produce BC_1F_2 seeds, then plants produced from these seeds were called as family A. Selected families of BC_1F_2 and of BC_2F_1 (having the same recurrent white clover parent) were used to evaluate within and between generation differences (Table 4.17).

Table 4.17. Details of the families involved in comparing BC_1F_2 and BC_2F_1 generations of experiment-2.

Generation	BC_1F_2			BC_2F_1		
Group	**Family**	**Pedigree**		**Group**	**Family**	**Pedigree**
A	1	A_{10} X A_6		**A**	71	LA_1 X A_1
	3	A_9 X A_6			72	LA_8 X A_1
	4	A_{11} X A_6			73	LA_4 X A_1
	5	A_6 X A_1				
	6	A_6 X A_9				
	7	A_6 X A_{11}				
	8	A_6 X A_{15}				
	9	A_6 X A_{10}				
	10	A_{15} X A_6				
B	19	B_2 X B_3		**CU**	38	LA_8 X CU_3
	20	B_2 X B_4			39	LA_8 X CU_2
	21	B_2 X B_8			42	LA_3 X CU_4
	22	B_4 X B_2			80	LA_2 X CU_4
	23	B_3 X B_2			81	LA_8 X CU_4
	24	B_8 X B_2				
	34	B_2 X B_4				
C	25	C_4 X C_8		**TF**	2	LA_8 x Tf_6
	26	C_5 X C_8			56	LA_5 X TF_4
	27	C_8 X C_6			57	LA_5 X TF_1
	28	C_8 X C_5			59	LA_5 X TF_5
	29	C_8 X C_4			67	LA_5 X TF_3
	30	C_8 X C_7			68	LA_3 X TF_3
S	11	S_{10} X S_{12}		**S**	46	LA_4 X S_{10}
	12	S_{10} X S_1			49	LA_4 X S_{13}
	13	S_{10} X S_{14}			53	LA_1 X S_{14}
	14	S_{10} X S_8			54	LA_4 X S_{14}
	15	S_{12} X S_{10}				
	16	S_8 X S_{10}				
	17	S_1 X S_{10}				
	18	S_{14} X S_{10}				

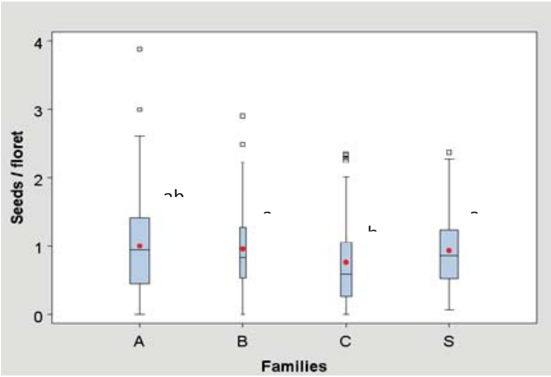
Box plots were constructed to compare the performances of families. Original data were used to form box plots and least significant differences (LSD) were determined by applying Tukey's test on transformed data. Box width varies with number of observations.

4.4.5.1 Comparison of BC_1F_2 families

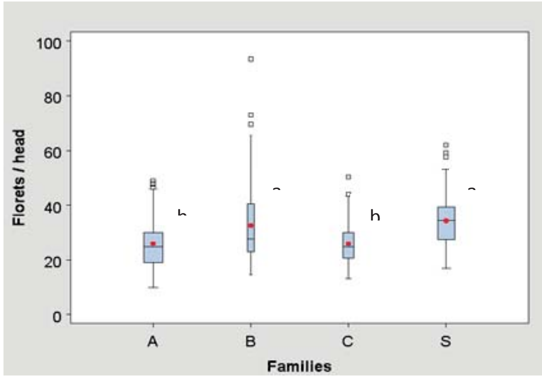
Families B and S produced more seeds per floret (Figure 4.15a) and florets per head than families C and A (Figure 4.15b). This pattern remained consistent for seeds produced by a head (Figure 4.15c). Although there were no significant differences among families in numbers of heads produced by a plant (Figure 4.15d), family A showed significantly lower floret production potential per plant than family B (Figure 4.15e). This trend remained consistent for overall seed produced by a plant (Figure 4.15f).

Plants of family S possessed longer peduncles than the other families (Figure 4.15g). These plants were also better performers for vegetative biomass production, with plants of family C having the second highest production (Figure 4.15h).

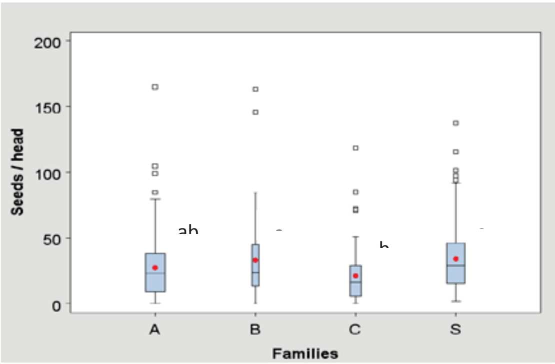
The families did not differ significantly from each other for root traits except that tap root diameter of family B was lower than the other families (Figures 4.15i – l).


Seeds / floret
0
1
2
3
4
A
B
C
S
Families

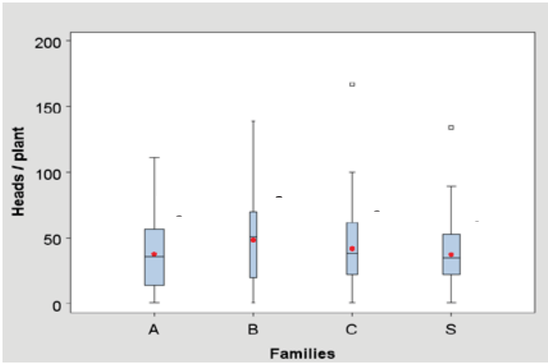
a


Florets / head
0
20
40
60
80
100
b
A
B
C
S
Families

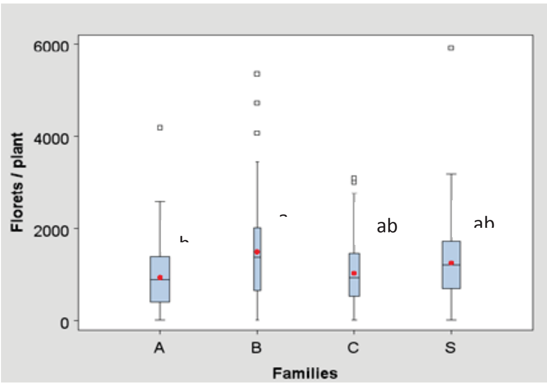
b


Seeds / head
0
50
100
150
200
A
B
C
S
Families

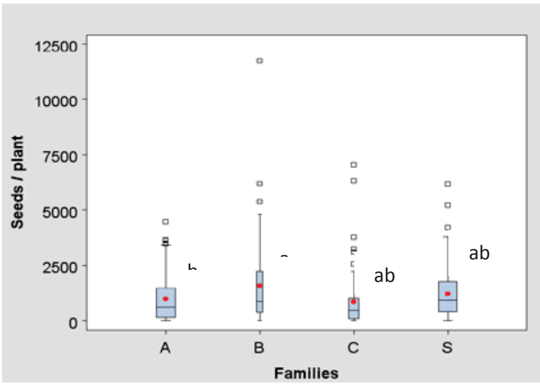
c


Heads / plant
0
50
100
150
200
A
B
C
S
Families

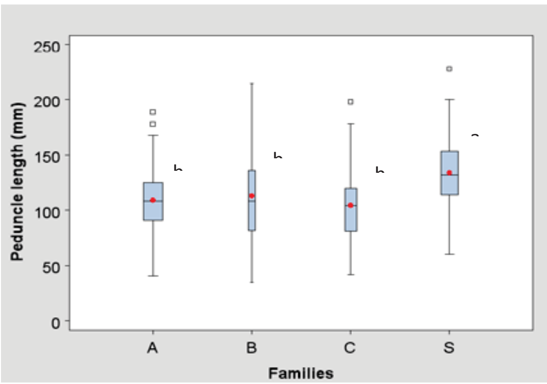
d


Florets / plant
0
2000
4000
6000
ab
A
B
C
S
Families

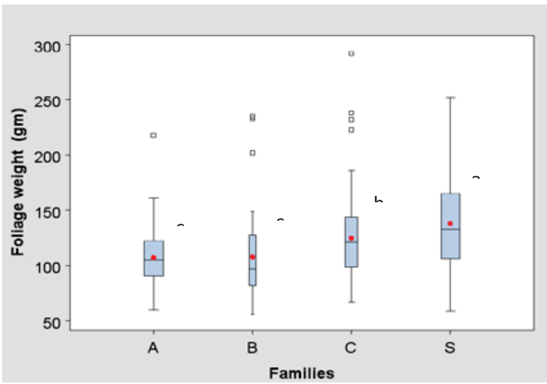
e


Seeds / plant
0
2500
5000
7500
10000
12500
ab
ab
A
B
C
S
Families

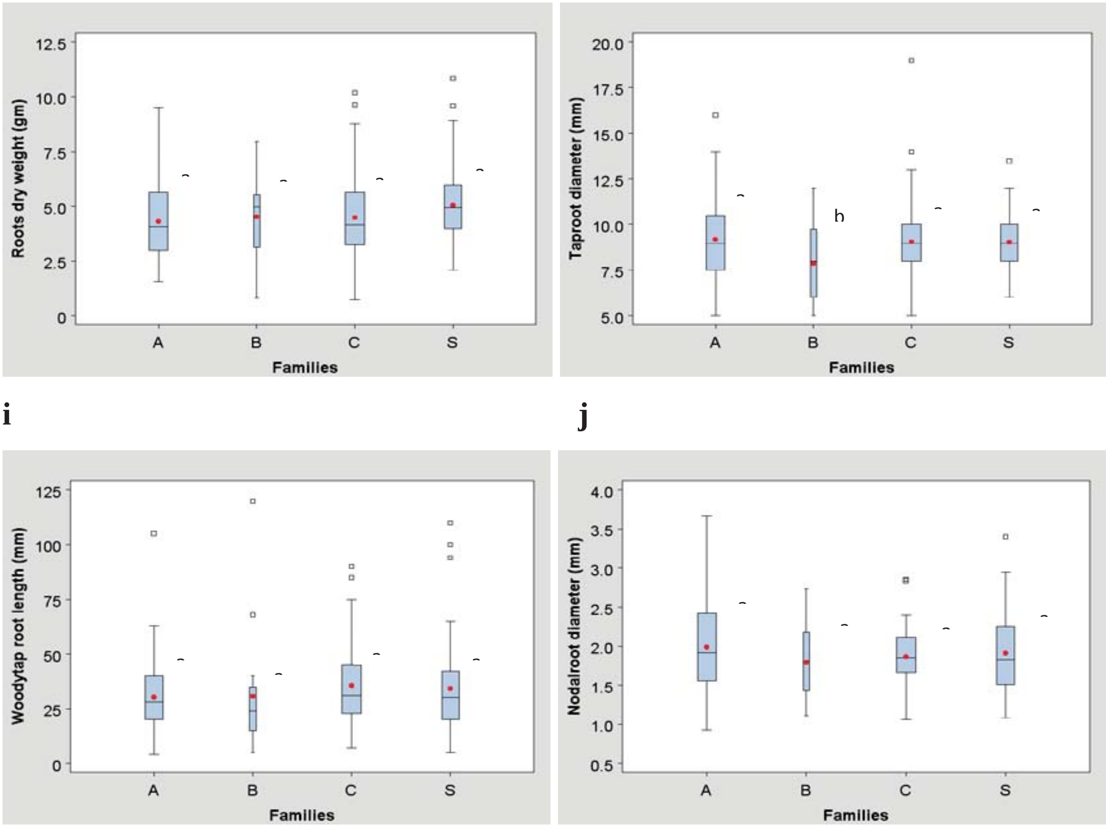
f


Peduncle length (mm)
0
50
100
150
200
250
A
B
C
S
Families

g


Foliage weight (gm)
50
100
150
200
250
300
A
B
C
S
Families

h



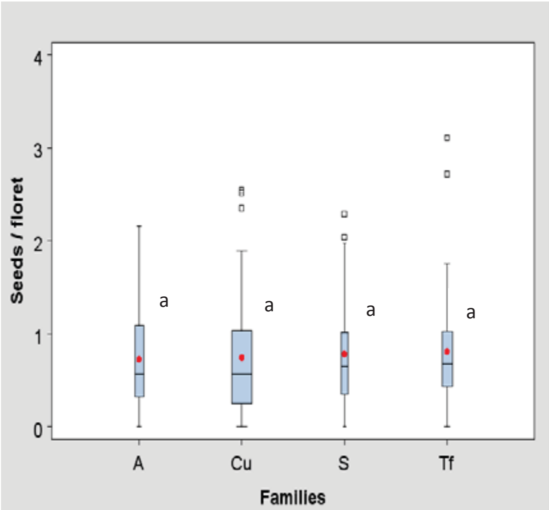
i j

k l

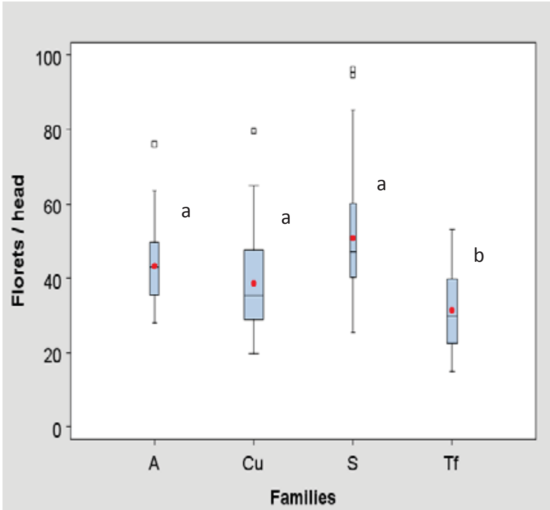
Figure 4.15. Comparison of families of BC_1F_2 generation for seeds/florets (a), florets/heads (b), seeds/head (c), heads/plant (d), florets /plant (e), seeds/plant (f), peduncle length (g), foliage dry weight (h), roots dry weight (i), taproot diameter (j), Woody tap root length (k) and nodal root diameter (l). The width of each box shows the number of observations. The lower and upper edges of the box represent first and third quartiles, respectively. The horizontal line drawn within the box marks the median. The red dot shows the sample mean. The whiskers are drawn to the most extreme points that lie within the fences. The upper fence is defined as the third quartile plus 1.5 times the interquartile range and the lower fence is the first quartile minus 1.5 times the interquartile range. Observations outside the fences (outliers) are marked as squares. Different letters at the top of bars represent significant differences at P≤ 0.05 (Tukey's test following ANOVA).

4.4.5.2 Comparison of BC_2F_1 families

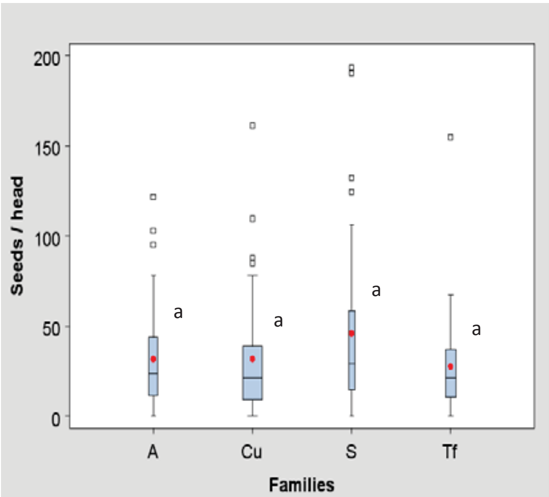
For generation BC_2F_1, comparisons were made among families A, Cu, S and Tf. All families showed similar potential for seeds present in a floret (Figure 4.16a). Plants of family S produced more florets per head than plants of family Tf (Figure 4.16b).


Seeds / floret
0
1
2
3
4
a
a
a
a
A
Cu
S
Tf
Families

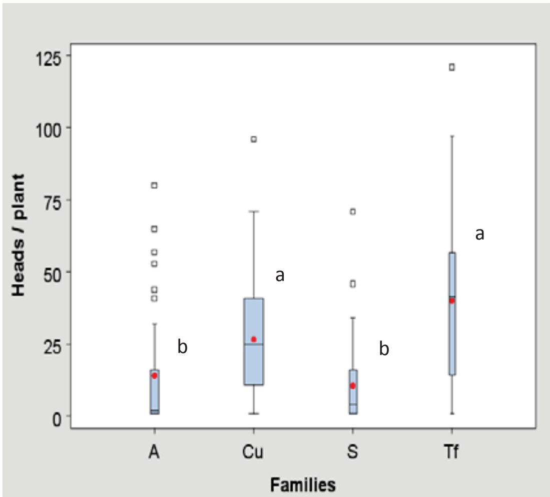
a


Florets / head
0
20
40
60
80
100
a
a
a
b
A
Cu
S
Tf
Families

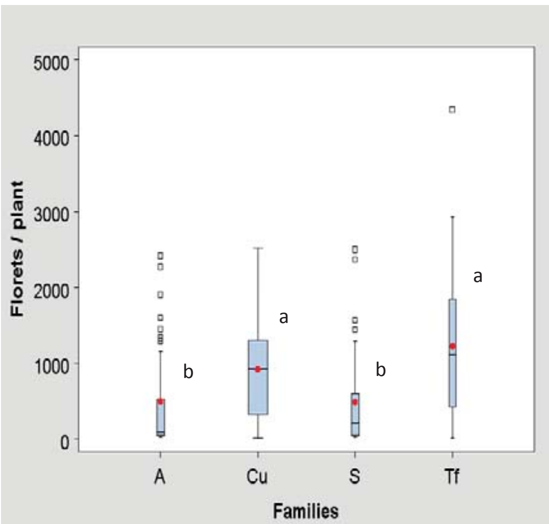
b


Seeds / head
0
50
100
150
200
a
a
a
a
A
Cu
S
Tf
Families

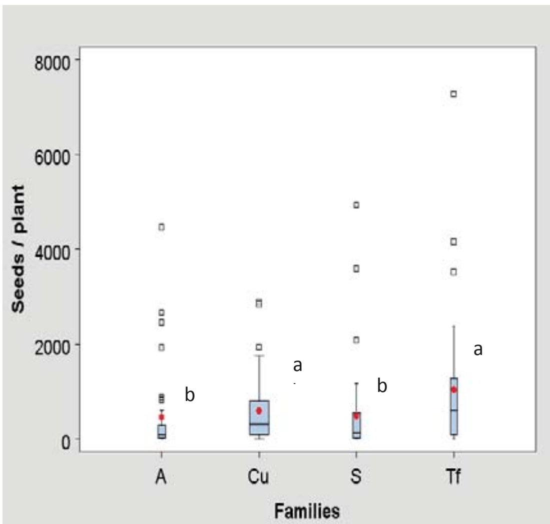
c


Heads / plant
0
25
50
75
100
125
b
a
b
a
A
Cu
S
Tf
Families

d


Florets / plant
0
1000
2000
3000
4000
5000
b
a
b
a
A
Cu
S
Tf
Families

e


Seeds / plant
0
2000
4000
6000
8000
b
a
b
a
A
Cu
S
Tf
Families

f

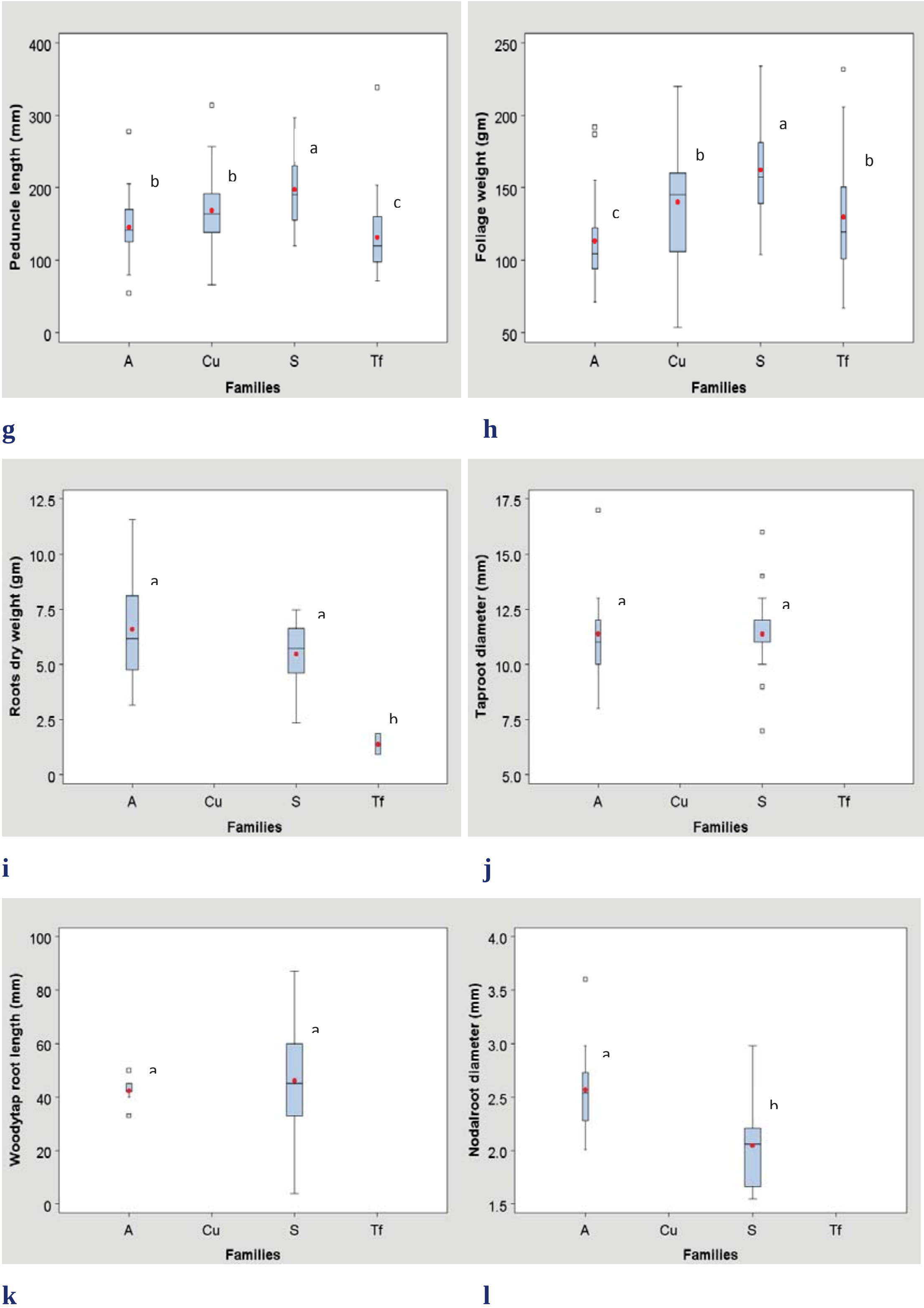


Figure 4.16. Comparison of families of BC_2F_1 generation for seeds/florets (a), florets/heads (b), seeds/head (c), heads/plant (d), florets /plant (e), seeds/plant (f), peduncle length (g), foliage dry weight (h), roots dry weight (i), taproot diameter (j), Woody tap root length (k) and nodal root diameter (l). The width of each box shows the number of observations. The descriptions of each figure are same as that of Figure 2.5.

Despite the presence of outliers at the upper limits, all families had similar means for seeds per head (Figure 4.16c). The plants of families Cu and Tf produced more heads per plant than the other two families (Figure 4.16d). The higher head production capacity of Cu and Tf also led their superiority in florets per plant (Figure 4.16e). The same trend continued and became more evident for seeds per plant (Figure 4.16f). Plants of both the A and S families produced fewer seeds than plants of the Tf family.

Family S produced the longest and family Tf produced the shortest peduncles (Figure 4.16g). Heads produced by family Cu had intermediate peduncles. The longer peduncles of family S appeared to be accompanied by better vegetative biomass production (Figure 4.16h). This trend was also showed by Cu family. The families A and Tf were low and statistically similer to each other for peduncle length and foliage dry weight.

The root dry weights of family Tf were significantly lower than families A and S (Figure 4.16i). Plants constituting families A and S showed similar expressions for tap root diameter (Figures 4.16j) and woody taproot length (Figure 4.16k). The diameters of nodal roots of family A were significantly larger than that of family S (Figure 4.16l).

4.4.6 Inheritance of the traits

A combination of index selection and florets per plant were used to select plants from experiment 1 to be used in experiment-2. Plants were divided into positive, medium and negative categories based on their good, medium and poor performance for seed production predicted by the selection procedures. The progenies of these categories were used to evaluate the inheritance of florets and seeds per plant. Two types of crosses i.e., intercrossed (Table 4.18) and backcrossed (Table 4.19), were used in this analysis. Within each family group genotypes of positive, medium and negative status were crossed with plants at similar status to enable comparisons among these genotypes of different status.

Table 4.18. Details of intercrosses used to evaluate the inheritance of numbers of florets and seeds per plant.

Plan	Class	Genotype	Pedigree	Group	Family
1	Positive	A_9	A_9 X A_6	A	3
		A_1	A_6 X A_9		6
			A_6 X A_1		5
	Medium	A_{10}	A_6 X A_{10}		9
		A_{11}	A_6 X A_{15}		8
		A_{15}	A_6 X A_{11}		7
			A_{10} X A_6		1
2	Positive	B_3	B_2 x B_3	B	19
		B_4	B_3 x B_2		23
			B_2 x B_4		20
			B_4 x B_2		22
	Medium	B_8	B_2 x B_8		21
			B_8 x B_2		24
3	Positive	C_4	C_8 x C_4	C	29
		C_5	C_4 x C_8		25
			C_8 x C_5		28
			C_5 x C_8		26
	Negative	C_6	C_8 x C_6		27
		C_7	C_8 x C_7		30
4	Positive	S_1	S_{10} x S_1	S	12
			S_1 x S_{10}		17
	Medium	S_8	S_{10} x S_8		14
			S_8 x S_{10}		16
	Negative	S_{14}	S_{10} x S_{14}		13
			S_{14} x S_{10}		18

Table 4.19. Details of backcrosses (BC_2F_1 generation) used to evaluate the inheritance of number of florets and seeds per plant.

Plan	Class	Genotype	Pedigree	Group	Family
5	Positive	A_6	DU_{44} X A_6	A	65
	Medium	A_{11}	DU_{44} X A_{11}		66
6	Medium	B_8	PR_2 x B_8	B	61
	Negative	B_6	PR_2 x B_6		62
7	Positive	Cu_1	CH_{13} x Cu_1	Cu	36
			AR_{28} x Cu_1		37
	Medium	Cu_4	CH_{13} x Cu_4		43
			AR_{28} x Cu_4		44
8	Positive	S_{10}	CH_{13} x S_{10}	S	45
			LA_4 x S_{10}		46
	Medium	S_{13}	CH_{13} x S_{13}		48
		S_{14}	LA_4 x S_{13}		49
			LA_4 x S_{14}		54
9	Positive	Tf_1	LA_5 x Tf_1	Tf	57
	Medium	Tf_3	LA_5 x Tf_3		67
		Tf_4	LA_5 x Tf_4		56
		Tf_5	LA_5 x Tf_5		59

The ANOVA results showed that in a majority (72%) of the plans, there was no difference among crosses of the different selection categories (Table 4.20). Even from the plans showing significant differences among crosses only one plan (plan 7), showed that the progenies in experiment-2 had retained the same status of seeds per plant as the parents in experiment 1. Although plans 5 and 6 showed significant difference among crosses of different categories for both florets and seeds per plant traits, Tukey's test revealed that the progenies of better performing plants of experiment 1 were poor in experiment 2, and vice versa.

Table 4.20. ANOVA results, expressed by P-values, of plans used to evaluate inheritance of florets and seeds per plant. The highlighted cells show significant difference (P ≤ 0.05).

	Intercrossed plans				Backcrossed plans				
Traits	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Florets / plant	0.7588	0.1134	0.0831	0.1302	**0.0336**	**0.0006**	0.9936	0.4747	0.8296
Seeds / plant	0.3792	0.0686	0.3	0.0665	**0.0304**	**0.0032**	**0.0527**	0.2421	0.8517

Progenies of plants having different peduncle lengths were evaluated for inheritance of this trait. In plans 1 to 4, plants having different peduncle lengths were intercrossed with a common female plant (Table 4.21). In plans 5 to 8, male parents having different peduncle lengths were backcrossed to a common white clover recurrent parent. The ANOVA results, presented by P-values in Table 4.21, showed that 50% of the plans expressed a significant difference ($P \leq 0.05$) in peduncle length. Tukey's test revealed that the order was correctly maintained for plans 3, 4 and 5 (in part).

Table 4.21. Details of plans used to observe inheritance of peduncle length. The highlighted cells show significant difference ($P \leq 0.05$).

Plan	Group	♀ Parent	Peduncle	♂ Parent	Peduncle	Family[1]	P-value	Family[2]
Intercrossed (BC_1F_2)								
1	A	A_6	90	A_1	68	5	0.1881	7
		A_6	90	A_{11}	108	7		9
		A_6	90	A_{10}	168	9		5
2	B	B_2	47	B_3	64	19	0.2701	21
		B_2	47	B_8	178	21		19
		B_2	47	B_4	215	20		20
3	C	C_8	121	C_4	60	29	**<.0001**	29
		C_8	121	C_6	132	27		27
		C_8	121	C_5	152	28		28
4	S	S_{10}	261	S_{14}	41	13	**0.0362**	13
		S_{10}	261	S_1	98	12		12
		S_{10}	261	S_{12}	192	11		11
Intercrossed (BC_2F_1)								
5	S	LA_4		S_{14}	41	54	**0.0378**	46
		LA_4		S_{13}	92	49		49
		LA_4		S_{10}	261	46		54
6	B	PR_2		B_6	65	62	**0.0512**	61
		PR_2		B_8	178	61		62
		PR_2		B_4	215	60		60
7	Cu	AR_{28}		Cu_2	115	41	0.7828	41
		AR_{28}		Cu_4	141	44		37
		AR_{28}		Cu_1	193	37		44
8	S	CH_{13}		S_{13}	92	48	0.5683	48
		CH_{13}		S_{10}	261	45		45

[1]families were arranged in ascending order of peduncle length of their male parent.

[2]families were arranged in ascending order of mean peduncle length as expressed by Tukey's test.

4.4.7 Differences in generation effects

Two generations i.e., BC_1F_2 and BC_2F_1 were compared for their mean performance for different traits. There were differences in measurement scales for different traits and so to compare two generations in one graph, mean values were standardized. For each trait, the mean values for both generations were added together and then each individual value was divided by that sum value. This provided the performance of each trait in the proportion of one, this allowing the possibility to compare two generations on the basis of different important traits. In such a comparison (Figure 4.17), the second intercrossed generation (BC_1F_2) showed significantly lower performance for per head traits, florets per head and seeds per head. However, this generation expressed higher potential for number of heads and florets per plant. The second backcrossed generation (BC_2F_1) showed inverse trends to that of the second intercrossed generation. The negative association between head size (florets per head) and numbers (heads per plant) apparently neutralized each other's effect in expression of seeds per plant. Since both generations expressed this negative association though in opposite order, their plants showed similar seed production.

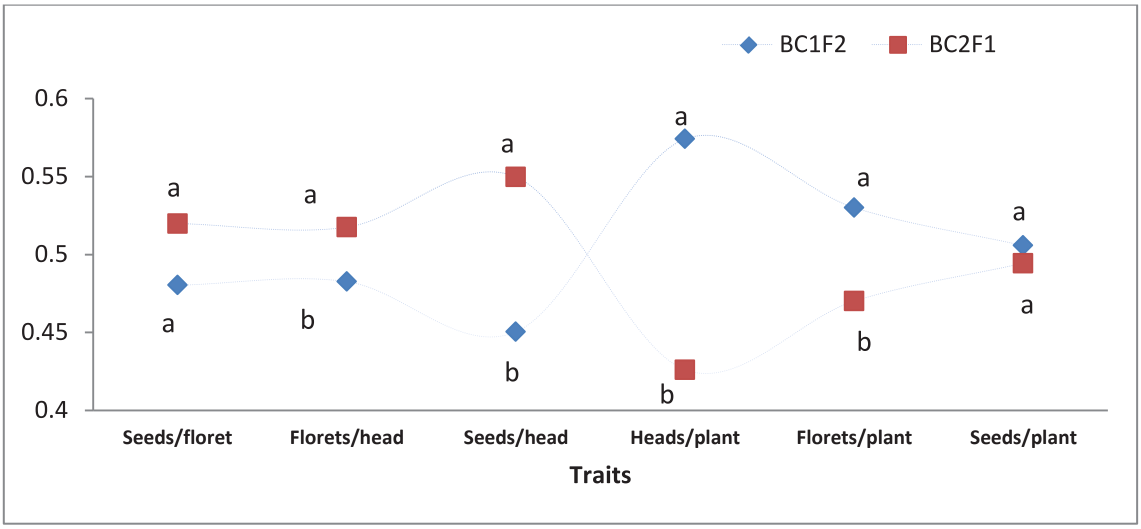


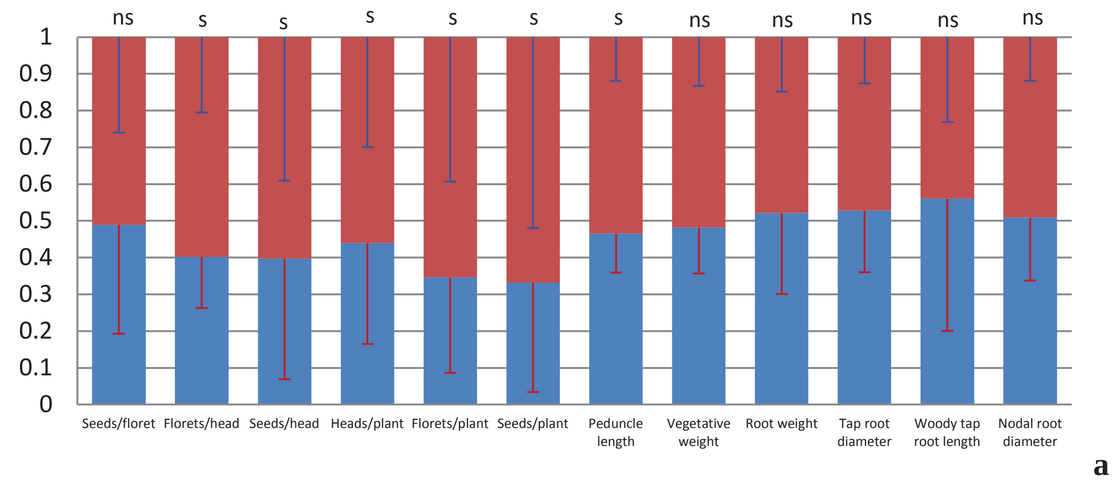
Figure 4.17. Comparison between BC_1F_2 and BC_2F_1 for seed traits. The average values of transformed data for each trait were standardized (expressed out of one, where one is the total of the generation means for the trait) to compare all traits in a single graph. Letters a and b near the data labels represent significant difference between them at $P \leq 0.05$ (Tukey's test following ANOVA on transformed data).

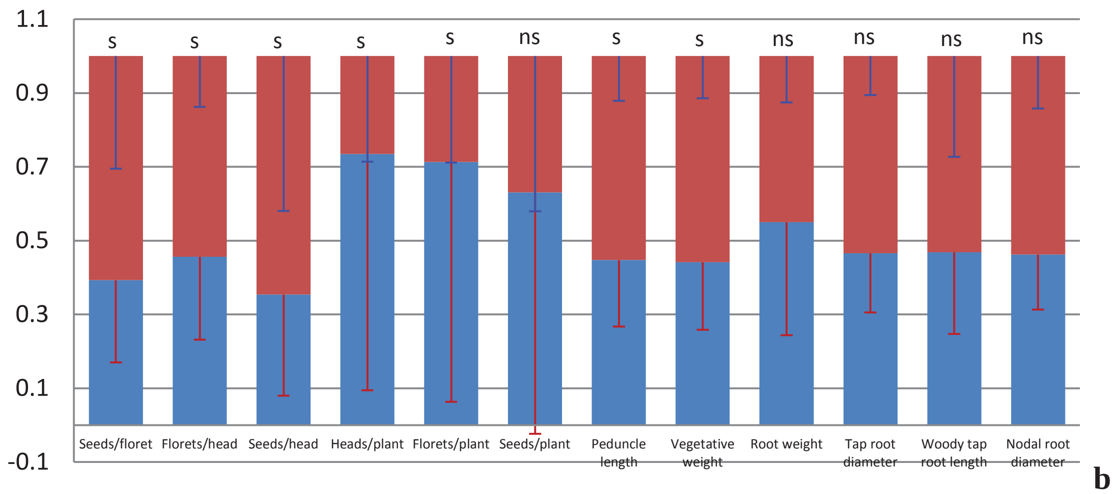
Similar families (having the same BC_1F_1 parents) were selected to make comparison between BC_1F_2 and BC_2F_1 (Table 4.22). Average values of original data for different traits were standardized as mentioned above, to compare all traits of both generations in a single graph.

Table 4.22. Selected families of BC_1F_2 and BC_2F_1 used for comparison.

Generation	**BC_1F_2**		**Vs**	**BC_2F_1**	
	Family	Pedigree		Family	Pedigree
Family					
A	1	A_{10} X A_6		63	DU_{45} X A_1
	3	A_9 X A_6		64	DU_{21} X A_{10}
	4	A_{11} X A_6		65	DU_{44} X A_6
	5	A_6 X A_1		66	DU_{44} X A_{11}
	6	A_6 X A_9		69	DU_{57} X A_1
	7	A_6 X A_{11}		70	DU_{846} X A_1
B	21	B_2 X B_8		60	PR_3 X B_4
	22	B_4 X B_2		61	PR_2 X B_8
	24	B_8 X B_2		62	PR_2 X B_6
	34	B_2 X B_4			
S	12	S_{10} X S_1		46	LA_4 X S_{10}
	13	S_{10} X S_{14}		53	LA_1 X S_{14}
	18	S_{14} X S_{10}		54	LA_4 X S_{14}

For family group A, BC_2F_1 showed better performance than BC_1F_2 for all seed traits (Figure 4.18a). This superiority was comparatively less prominent and statistically non-significant for seeds per floret. The differences between generations were not significant for vegetative weight and root traits. For families B (Figure 4.18b) and S (Figure 4.18c), BC_2F_1 was better than BC_1F_2 for seeds per floret, florets per head and seeds per head. This was reversed for heads per plant and florets per plant. These inverse relationships between number of heads per plant and, florets and seeds produced by a head resulted in similar seed production per plant by both generations. The peduncle length and vegetative biomass production appeared to be correlated with head size. Although root traits of the BC_2F_1 appeared to be better than that of the BC_1F_2, the differences were generally not significant. The only exception was the significantly thicker tap roots of BC_2F_1 plants in family S.





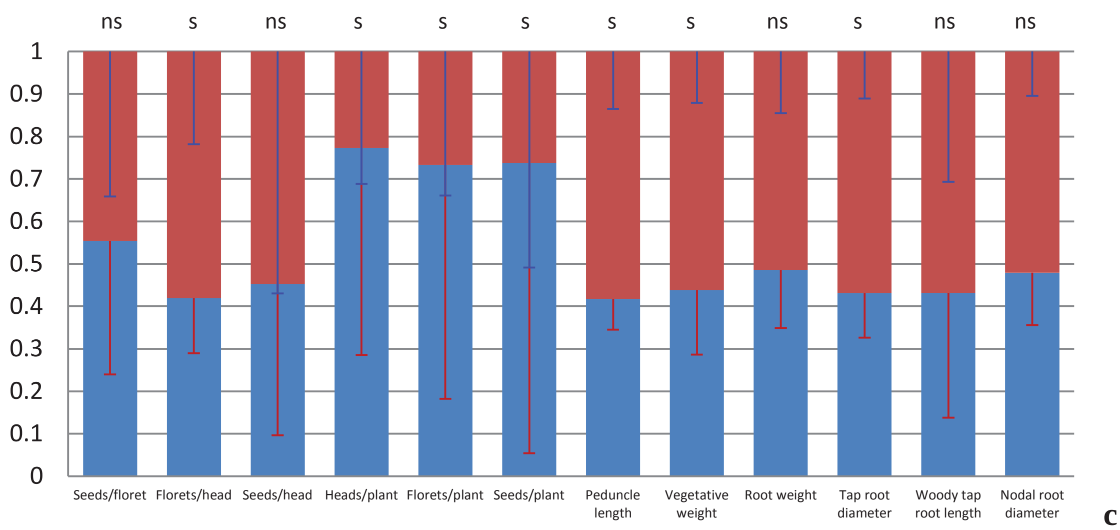


Figure 4.18. Comparison between BC_1F_2 (blue) and BC_2F_1 (red) for family A (a), B (b) and S (c). Standard deviations are shown by lines in the bars. Transformed data were used to determine the significance of difference between the generations. Significant (s) or non-significant (ns) differences.

4.5 SUMMARY

The experiment-2 analysed the BC_2F_1 and BC_1F_2 generations for seed production in an organized way. Backcrossing significantly improved head size, seeds per head, peduncle length, foliage production and tap root diameter, whereas intercrossing produced significantly more heads, florets and seeds per plant. There were positive associations among seeds per floret, head size, peduncle length, plant seed production, foliage production and woody tap root length. Head production ability did not appear to be associated with foliage production of the plant. Number of seeds per floret was a stronger contributor to seeds per head than head size. The generations and families within a generation significantly differed for most of the traits. Families B and S of the BC_1F_2 generation produced significantly more seeds per floret, larger heads and more seeds per head than families C and A. Family S of both the BC_2F_1 and BC_1F_2 generation had the longest peduncles and highest foliage production. There were strong positive associations among root traits. Families from both generations showed combinations of high seeds per floret and heads per plant. One of them also had better head size. There were no marked negative associations between root and seed production traits. Changes in white clover parental cultivars or hybrid parents did not result in considerably high proportion of significant differences among the progenies for seed production traits. Generally reciprocal crossing produced similar results. Changes of the parents affected head size the most, and heads per plant and seeds per floret the least among the seed production traits. The BC_1F_2 hybrids showed normal ovule production but only 11% of the ovules were successfully harvested as mature seeds. The hybrids showed considerable variation for harvestable proportion of potential seed yield. The index selection from experiment-1 and selection on the basis of florets per plant both proved to be ineffective.

It can be concluded that both backcrossing and intercrossing contributed to improvement in seed production. Variation could be created at both generation and family levels. Low realization of the potential seed yield was an important weakness of these hybrids. Parental influence in the expression of the traits remained unclear and required further investigation. The order of the traits for their contribution to plant seed production was 1. heads per plant, 2. seeds per floret and 3. head size (florets per head). Selection of the early hybrid generations should be based on individual primary traits and heritability estimates instead of cumulative criteria.

Chapter 5

TRAIT ASSOCIATIONS AND COMPARING MATING DESIGNS

5.1 INTRODUCTION

The preceding phases of this study revealed that both backcrossing and intercrossing were effective in improving seed production of the interspecific hybrids between white clover and *T. uniflorum*. Selected families of the advanced generations along with primary hybrids and parental species were subjected to a comprehensive analysis. A diverse range of backcrossed and intercrossed generations were compared to analyse the effects of different breeding strategies with particular focus on seed production traits. New aspects like inbreeding depression and previously identified important subjects like the difference between potential and actual seed yield, and unclear issues like parental influences on the hybrids, were assessed with more diverse plant material.

5.2 RESEARCH QUESTIONS

Following matters were assessed in this experiment.

i. Potential and actual seed yield.
ii. Association among the traits.
iii. Difference in parental effect.
iv. Relationship among families.
v. Inbreeding effects.
vi. Inheritance of the traits.
vii. Effect of different mating designs.

5.3 MATERIAL AND METHODS

5.3.1 Plant material

Seven hybrid generations, i.e. BC_1F_1, BC_2F_1, BC_3F_1, BC_2F_2-BI, BC_2F_2-IB, BC_1F_2 and BC_1F_3 and their parental species, i.e. white clover and *T. uniflorum* were used in this study (Table 5.1). These eight generations were represented by 81 families.

Table 5.1. Details of families used in experiment-3. In pedigree columns, letters A, B, C, Cu, S and Tf carrying numbers less than 15 represent BC_1F_1 plants having recurrent parent expressed by the letter. Rest of abbreviations represent white clover varieties. Abbreviation BC_2F_2-BI shows that during its development BC_1F_1 hybrids were backcrossed with white clover and during next season their progenies were intercrossed to produce BC_2F_2 generation and, the abbreviation BC_2F_2-IB shows that BC_1F_1 hybrids were first intercrossed and during next season their progenies were backcrossed with white clover to produce BC_2F_2 generation.

Families	Generation	Female parent(s)	Male parent(s)
6	BC_2F_2-IB	$Will_2$	C_5 x C_8
7	BC_2F_2-BI	A_{28} x Cu_2 (selfed)	
8	BC_1F_3	B_2 x B_3	S_{10} x S_1
9	BC_3F_1	$Will_2$	L_4 x S_{10}
10	BC_3F_1	$Will_2$	L_3 x Tf_3
11	BC_2F_2-IB	CH_{13}	S_{10} x S_{12}
12	BC_2F_2-IB	AR_{28}	S_{10} x S_{12}
13	BC_3F_1	AR_{28}	L3 x Tf_3
14	BC_2F_2-IB	CH_{13}	B_2 x B_3
15	BC_3F_1	$Will_2$	D_{44} x A_6
16	BC_1F_2	S_{14}	S_{10}
17	BC_1F_3	S_{10} x S_1	B_2 x B_3
18	BC_1F_2	Cu_2 (selfed)	
19	White clover Parent (selfed)	CH_5 (selfed)	
20	BC_2F_2-BI	L_4 x S_{10}	L_5 x Tf_4
21	BC_2F_2-IB	$Will_2$	S_{10} x S_{12}
22	BC_1F_2	B_2	B_3
23	BC_2F_2-BI	CH_5 x Cu_2 (selfed)	
24	BC_1F_2	B_3 (selfed)	
25	BC_2F_1	CH_5	CU_2
26	BC_1F_2	S_{14} (selfed)	
27	BC_1F_3	B_2 x B_3	A_{11} x A_6
28	BC_2F_2-IB	$Will_2$	A_{15} x A_6
29	BC_2F_2-IB	AR_{28}	B_2 x B_3
30	BC_2F_2-BI	D_{44} x A_{11}	D_{57} x A_1
31	BC_2F_2-BI	L_8 x A_1	L_4 x A_1
32	BC_3F_1	$Will_2$	D_{44} x A_{11}
33	BC_3F_1	CH_{13}	D_{44} x A_{11}
34	BC_2F_1	AR_{28}	Cu_2
35	BC_3F_1	CH_{13}	L_3 x Tf_3
36	BC_2F_2-IB	AR_{802}	B_2 x B_3
37	BC_3F_1	$Will_2$	L_5 x Tf_4
38	BC_1F_3	A_6 x A_{11}	S_{14} x S_{10}
39	BC_2F_2-IB	$Will_2$	A_{11} x A_6
40	BC_2F_2-IB	$Will_2$	B_2 x B_8
41	BC_2F_2-BI	CH_5 x Cu_2	D_{45} x A_1
42	BC_3F_1	AR_{28}	D_{44} x A_{11}

43	BC_2F_2-BI	D_{44} x A_{11}	L_3 x Cu_4
44	BC_3F_1	B_2 x B_3	C_8 x C_6
45	BC_2F_2-IB	$Will_2$	A_6 x A_{11}
46	BC_3F_1	AR_{802}	D_{44} x A_{11}
47	BC_3F_1	$Will_2$	A_{28} x Cu2
48	BC_2F_2-BI	CH_6 x Cu_4	L4 x S_{10}
49	BC_2F_2-BI	A_{28} x Cu_4	L_4 x A_1
50	BC_1F_3	A_6 x A_{10}	C_5 x C_8
51	BC_2F_2-BI	DU_{57} x A_1	CH_5 x Cu_2
52	BC_1F_3	S_{10} x S_{12}	A_6 x A_{10}
53	White clover Parent	Sustain	
54	BC_2F_2-BI	L_4 x A_1	L_8 x A_1
55	BC_2F_2-IB	$Will_2$	A_6 x A_1
56	BC_2F_2-IB	$Will_2$	S_{10} x S_1
57	BC_2F_2-IB	$Will_2$	C_8 x C_6
58	BC_2F_2-IB	DU_{44} x A_{11}	CH_5 x Cu_2
59	BC_1F_3	S_{10} x S_{12}	S1 x S_{10}
60	BC_2F_2-IB	AR_{802}	S_{10} x S_{12}
61	BC_3F_1	AR_{802}	L_3 x Tf_3
62	BC_1F_3	C_4 x C_8	S_{12} x S_{10}
63	*T. uniflorum* Parent	Az-4437	
64	BC_1F_3	B_2 x B_3 (selfed)	
65	BC_1F_3	B_2 x B_8	S_{14} x S_{10}
66	BC_2F_2-BI	DU_{44} x A_{11}	D_{44} x A_6
67	BC_3F_1	$Will_2$	D_{57} x A_1
68	BC_3F_1	$Will_2$	D_{21} x A_{10}
69	BC_3F_1	$Will_2$	L_5 x Tf_1
70	BC_2F_2-BI	L_4 x S_{10}	CH_{13} x Cu_1
71	BC_2F_2-BI	L_3 x Tf_3	L_5 x Tf_1
72	BC_2F_2-BI	AR_{28} x Cu_2	Ch_4 x A_1
73	BC_3F_1	$Will_2$	CH_5 x Cu_2
74	BC_2F_2-BI	L_3 x Tf_3	D_{21} x A_{10}
75	BC_1F_3	S_{10} x S_1	B_2 x B_8
76	BC_1F_3	S_{14} x S_{10} (selfed)	
77	White clover Parent	Aran	
78	BC_2F_2-IB	$Will_2$	A6 x A10
79	BC_1F_3	A_{11} x A_6	S14 x S10
80	BC_1F_3	S_{10} x S_{12}	C5 x C8
81	*T. uniflorum* Parent	Az4383-11-0P	
82	*T. uniflorum* Parent	Az4383-18-OP	
83	BC_1F_1	A bulk	
84	BC_1F_1	S bulk	
85	BC_1F_1	S_{18}	
86	BC_1F_3	B_2 x B_8	C_8 x C_6

5.3.2 Experimental plan

The experimental design used for this study was a RCBD with three replications. Each family had five plants per family per replication (81 x 5 x 3). Seeds were germinated on 24 June 2010 according to the methodology explained for experiment-2. Seedlings were allowed to grow in glasshouse. Then the trays carrying plantlets were placed outside for vernalization. During first week of September 2010, each plant was re-planted into a tapered cubical plastic pot (190 mm height × 28900 mm^2 top area × 21025 mm^2 bottom area and approximately 4.6 L in volume). The plants were arranged according to the experimental design. In each replication, all five pots carrying plants of a family were placed in a row in contact with each other (Plate 5.1). Moreover, six additional small pots (as used in experiment-2) filled with potting mix were placed adjacent on one side to accommodate extended plant growth (Figure 5.1). The area covered by each plot was 0.285 m^2 or 284790 mm^2 (330 mm x 963 mm). Three Families of parental generations i.e., *T. uniflorum*, white clover, and BC_1F_1, were grown to be used as reference.

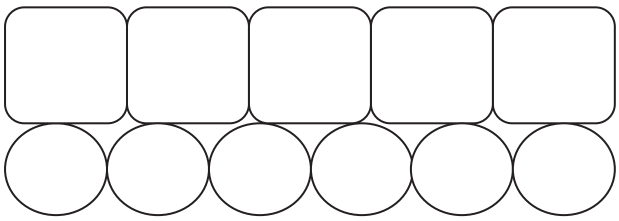


Figure 5.1. Schematic diagram showing the arrangement of pots for a family

Plate 5.1. Plants in flowering during December 2010 showing Layout of the experiment-3 "trait associations and comparing mating designs".

5.3.2.1 Potential and actual seed setting

A trial of 14 selected families was conducted to observe any differences between potential and actual seed setting. The methodology used in experiment-2 was followed.

5.3.2.2 Association among the traits

Different analytical techniques like PCA, path coefficient analysis, three dimensional graphs, etc, were used to explore the associations among traits involved in seed production.

5.3.2.3 Difference in parental effect

a. **Influence of white clover parent:** Intervarietal effects of white clover parents used in backcrossing with BC_2F_1 and BC_1F_2 generation for the recovery of seed production traits were studied. Two sets of each of the following crossing schemes show the pattern of crosses used to observe the differences. Subscripts A, B and C represent different varieties.

$$\left.\begin{array}{ll} WC_A & x \\ WC_B & x \\ WC_C & x \end{array}\right\} \quad BC_2F_1$$

$$\left.\begin{array}{ll} WC_A & x \\ WC_B & x \\ WC_C & x \end{array}\right\} \quad BC_1F_2$$

b. **Influence of BC_1F_2 parent:** Progenies of following mentioned 4 sets of upper and 2 sets of lower crossing patterns were employed to observe the contribution of the BC_1F_2 parent in backcrossing and intercrossing for recovery of seed production traits. Subscripts A, B, C and D represent different families of BC_1F_2. WC indicates a single white clover plant.

$$\left.\begin{array}{ll} & x \\ WC & x \\ & x \end{array}\right\} \quad \begin{array}{l} BC_1F_{2\text{-}A} \\ BC_1F_{2\text{-}B} \\ BC_1F_{2\text{-}C} \end{array}$$

$$\left.\begin{array}{ll} & x \\ BC_1F_{2\text{-}D} & x \\ & x \end{array}\right\} \quad \begin{array}{l} BC_1F_{2\text{-}A} \\ BC_1F_{2\text{-}B} \\ BC_1F_{2\text{-}C} \end{array}$$

c. **Reciprocal crosses:** Performance of progenies of reciprocal crosses was compared to note any maternal or paternal influence on the studied traits.

5.3.2.4 Relationships among families

Seed production characteristics of families from all generations were analysed by PCA. Relationship among the families based upon distance matrices were explored and represented in a tree diagram using Splits Tree4 program (Huson and Bryant, 2006).

5.3.2.5 Inbreeding effects

Four plans involving backcrossing and intercrossing were used to explore inbreeding depression at different levels. The following crossing schemes show two different patterns of crosses used to observe this effect. Subscripts A, B and C represent different families. Families in bold letters were used for evaluation.

PATTERN-1 (Back crossing before intercrossing)

$BC_1F_{1\text{-}A}$

(backcrossing) x x (selfing)

$WC_{\text{-}A}$ $BC_1F_{1\text{-}A}$

$\downarrow$ $\downarrow$

$\mathbf{BC_2F_{1\text{-}A}}$ $\mathbf{BC_1F_2}$

x (backcrossing) x (intercrossing) x (selfing)

$WC_{\text{-}B}$ $BC_2F_{1\text{-}B}$ $BC_2F_{1\text{-}A}$

$\downarrow$ $\downarrow$ $\downarrow$

$\mathbf{BC_3F_1}$ $\mathbf{BC_2F_2}$ $\mathbf{BC_2F_2}$

PATTERN-2 (Intercrossing before back crossing)

$BC_1F_{1\text{-}A}$

(intercrossing) x x (selfing)

$BC_1F_{1\text{-}B}$ $\rightarrow$ $\mathbf{BC_1F_{2\text{-}A}}$

$BC_1F_{1\text{-}A}$ $\rightarrow$ $\mathbf{BC_1F_2}$

(backcrossing) x x (intercrossing) x (selfing)

$WC_{\text{-}A,B,C}$ $\rightarrow$ $\mathbf{BC_2F_2}$

$BC_1F_{2\text{-}B,C,D}$ $\rightarrow$ $\mathbf{BC_1F_3}$

$BC_1F_{2\text{-}A}$ $\rightarrow$ $\mathbf{BC_1F_3}$

Comparisons were made at following levels:

i. All selfed families were compared with all crossed families irrespective of plans.
ii. All selfed families of a single plan were compared with all crossed families of that plan.
iii. The selfed and crossed progenies of only BC_1F_1 were compared.
iv. The selfed and crossed progenies of BC_2F_1 (in case of pattern 1) and of BC_1F_2 (in case of pattern 2) were compared.

5.3.2.6 Inheritance of the traits

Inheritance of the traits was studied in general and in terms of narrow sense heritability.

a. **General inheritance:** Representatives of all available generations produced from backcrossing and intercrossing i.e., BC_1F_3, BC_2F_2-BI, BC_2F_2-IB and

BC_3F_1, were sown by considering their high and low performance for important seed yield components. The structure of experiment-3 is shown in Table 5.2. Families identified as early, medium and late flowering in experiment-1 were also included in this study. For each category, the performances of families in the high category were compared with performances of families in the low category to check the inheritance of their status.

Table 5.2. Structure of experiment-3.

	HIGH		**LOW**	
Heads / plant	BC_1F_3	BC_2F_2-IB	BC_1F_3	BC_2F_2-IB
	BC_2F_2-BI	BC_3F_1	BC_2F_2-BI	BC_3F_1
Florets / plant	BC_1F_3	BC_2F_2-IB	BC_1F_3	BC_2F_2-IB
	BC_2F_2-BI	BC_3F_1	BC_2F_2-BI	BC_3F_1
Seeds / floret	BC_1F_3	BC_2F_2-IB	BC_1F_3	BC_2F_2-IB
	BC_2F_2-BI	BC_3F_1	BC_2F_2-BI	BC_3F_1
Florets / head	BC_1F_3	BC_2F_2-IB	BC_1F_3	BC_2F_2-IB
	BC_2F_2-BI	BC_3F_1	BC_2F_2-BI	BC_3F_1
Peduncle length	BC_1F_3	BC_2F_2-IB	BC_1F_3	BC_2F_2-IB
	BC_2F_2-BI	BC_3F_1	BC_2F_2-BI	BC_3F_1

b. **Narrow sense heritability:** Full-sibs and half-sibs were used to estimate narrow sense heritability (Figure 5.2). Ten groups were involved in this study. In each group, progenies of a single female parent crossed with different male parents were half-sibs, and progenies produced by a single cross were full-sibs. Each group had different male and female parents involved.

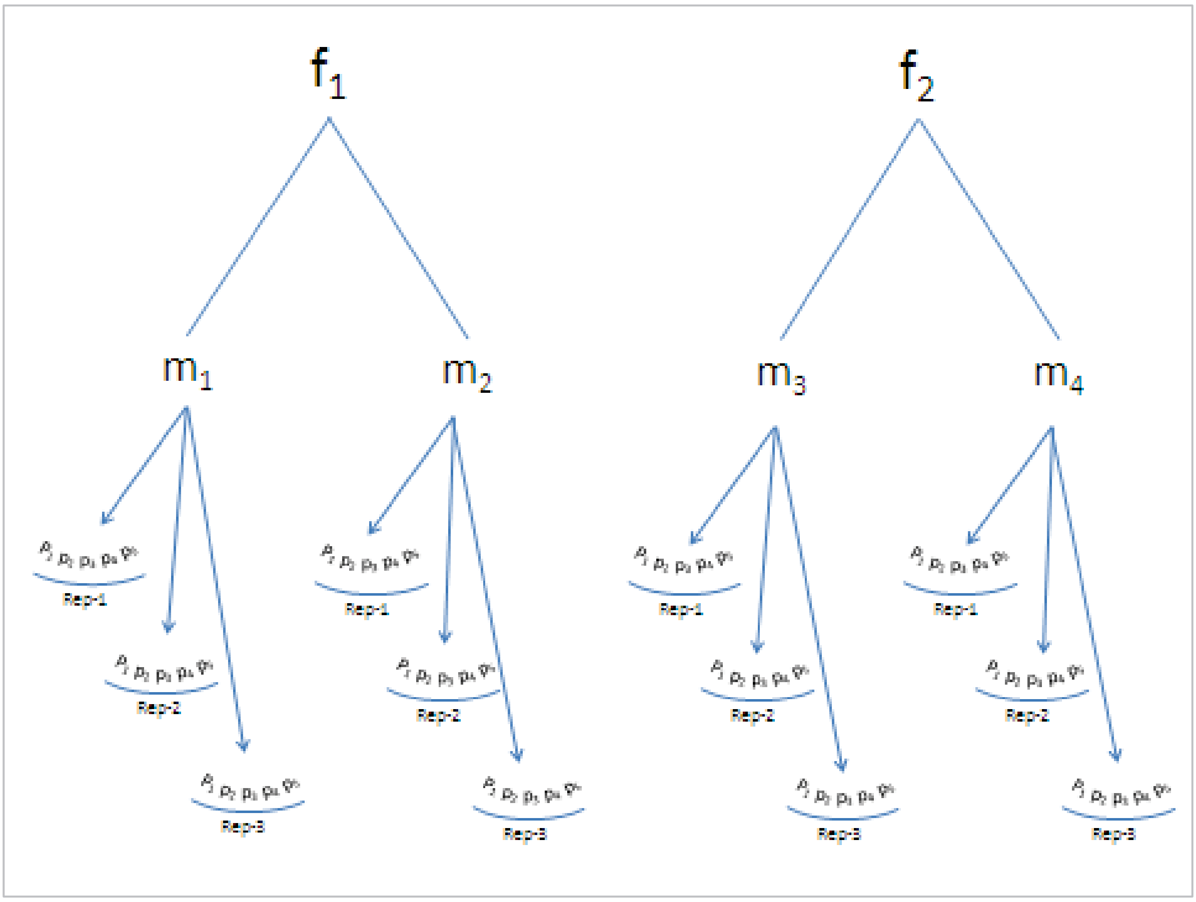


Figure 5.2. Schematic presentation of the pedigrees of full and half-sibs involved in the heritability study. Two groups are shown as an example. In each group, a common female was crossed with two different males. Each group involved a unique set of female and male parents. Female, male, progeny and replication are represented by abbreviations f, m, p and rep, respectively.

5.3.2.7 Effect of different mating designs

Different generations i.e., parents, BC_1F_1, BC_2F_1, BC_3F_1, BC_1F_2, BC_1F_3 and BC_2F_2 were used to evaluate the effect of different mating designs in the recovery of seed related traits in this interspecific hybrid breeding programme.

5.3.3 Measurements

Data were collected for the following parameters.

5.3.3.1 Reproductive traits

a. **Flower head emergence per plant:** Six counts were made to note head production from 12 November 2011 to 13 February 2012. The first five counts were taken at intervals of 10 days and heads showing petals were counted for each individual plant. This determined the starting date of flowering for all families. Last count was taken only for families used in inheritance of maturity study. In this count each family was divided into three portions i.e., the first plant, the middle portion (consisting of the 3 middle plants) and the last-plant. Heads present on the first and last plant were counted individually. For the middle portion, heads in an 18 x 18 cm quadrat were counted at 3 different places (Figure 5.3).

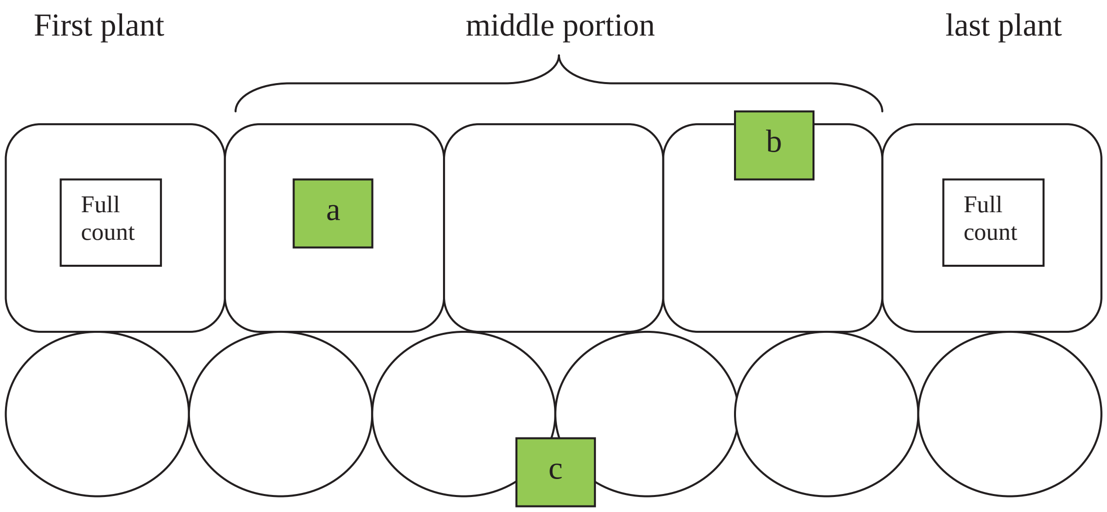


Figure 5.3. Diagram showing locations of head count at five positions of a family. Points a, b and c show quadrat positions for the middle portion.

b. **Pollen stainability:** Pollen viability of 15 selected families was checked by the method adopted in experiment-1.

c. **Selfing:** Three heads per plant were used to observe seed setting as a result of selfing. They were bagged and gently rolled daily between thumb and finger until all the florets got deflexed.

d. **Per head measurements:** Mature heads were removed from each plant during the second week of February 2012. First, bagged heads for selfing were evaluated. Then open-pollinated heads of a similar age were selected most preferably from middle top part and avoiding the peripheral hanging portion of the plant. Five normal and mature heads from each end plant were taken. For the middle portion, 15 heads were taken randomly with 5 heads from each plant. Data for seeds per floret, florets per heads and peduncle length were recorded and used to derive data for seeds per head, florets per plant and seeds per plant. Heads used for measuring selfing, pollen stainability, ovules count, peduncle length, florets per head, seeds per head and seeds per plant, were of the same age.

e. **Per plant measurements:** Data on a plant basis was derived by multiplying head production data of second last count taken on 25^{th} of December 2010, multiplied with per head data sampled at the end of experiment i.e., during second week of February 2011. Visual scoring was done to observe the maturity of flower heads on 11^{th} of February 2011 and 17^{th} of March 2011.

5.3.3.2 Vegetative traits

a. **Plant growth:** The growth of a family was measured by its foliage dry mass and covered area. Foliage mass was harvested from plants from 22^{nd} to 27^{th} November 2010. Plants were trimmed back at the height of 5cm from the top edge of the pot. The height of 5cm was suitable to remove only foliage, without harming or removing the stolons. In this way the buds formed in the stolons remained safe and available for producing heads in the subsequent season. A stick showing 5cm height was fixed in the first pot and was used as a reference to trim back all plants of that family. Stolons were permitted to grow into adjacent side pots of soil. The side stolons were not removed. However, their foliage, flowers and buds were trimmed to the 5cm height. The foliage mass was oven dried at 100^{0}C for 16 hours and its weight was used as “foliage dry weight” in data analysis. Covered area of each family was measured after the taking last counts for head production.

b. **Leaf area:** The areas of randomly selected healthy and fully grown leaves were measured with a leaf area meter (LI-COR Model LI-3000 Portable Area Meter). The middle lobes of five leaves per plant for the end plants and 15 leaves for the middle group were measured. Families used for evaluation of selfing performance were included in this sample. An additional 12 families were included, based on difference in their leaf sizes. In total 27 families were measured. Leaf area was represented by the single middle lobe of the leaf in cm^2.

c. **Stolon and nodal density:** After the last head count and sampling, plants were trimmed back. During the third week of February 2011, selected families were washed with a water blaster to clean their stolons and roots. Potting mixture and leaves were washed away by the water pressure. Plants were dried in the sun. Plants of a family were separated from each other without disturbing the plant shape (especially stolons). To note stolon and node density, a 216 x 217 mm card was used with four windows cut in it according to the shape and dimensions shown in Figure 5.4. Stolons expanding and crossed half way of the window towards the side 3 and 4 were counted in each window. Any stolon heading towards or reaching side 1 and 2 was ignored. This achieved a count in that window of all stolons originating from the centre of the plant. Any stolon entering into the window from the sides was taken as not originating from the centre of the plant and was ignored. Nodes were counted on all visible stolons in all windows. The data expressed density of stolons and nodes per unit area. The stolons of each plant were removed from roots and their weight was recorded after oven drying for 24 hours at 80^{o}C. This weight was used as "stolon dry weight" in data analysis.

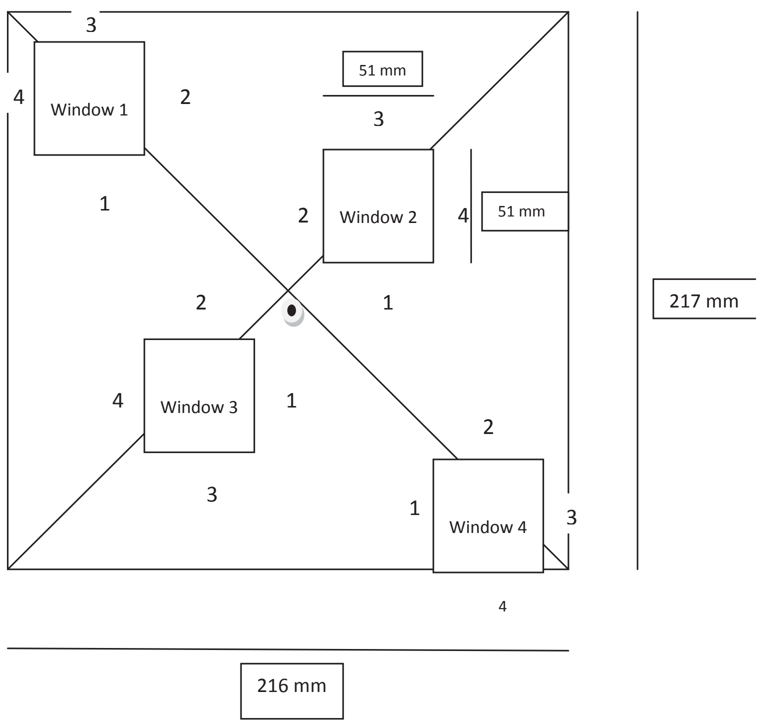


Figure 5.4. Dimensions of the card with four windows used to measure density of stolons and nodes.

5.3.3.1 Root traits

The following root traits were studied for the same families which were selected for stolon data.

a. **Root dry mass:** Roots mass was recorded after separating the roots from stolons and oven drying them at 80^{o}C for 24 hours.

b. **Tap root diameter:** Primary tap root diameter was measured at the base of the crown with an electronic Vernier Caliper (Mitutoyo digital Caliper-500.550.551).

c. **Woody tap root length:** Length of intact woody portion of the tap root was measured till its terminal branched end.

5.4 ANALYSES AND RESULTS

Data from 81 families representing eight different generations were analysed (Table 5.3). Except for peduncle length and 1000 seeds weight, all data were transformed using the Box-Cox transformation in GenStat v12. During transformation, *T. uniflorum* data were excluded to achieve an acceptable level of normality. Thus *T. uniflorum* was excluded from all analyses, other than where its inclusion specifically mentioned.

Table 5.3. Details of generations and families involved in the experiment.

Serial No.	Generation	No. of families	Code of families
1	BC_1F_1	3	83, 84, 85
2	BC_1F_2	2	16, 22
3	BC_1F_2 (selfed)	3	18, 24, 26
4	BC_1F_3	14	8, 17, 27, 38, 44, 50, 52, 59, 62, 65, 75, 79, 80, 86
5	BC_1F_3 (selfed)	2	64, 76
6	BC_2F_1	2	25, 34
7	BC_2F_2-BI	15	20, 30, 31, 41, 43, 48, 49, 51, 54, 58, 66, 70, 71, 72, 74
8	BC_2F_2-IB	16	6, 11, 12, 14, 21, 28, 29, 36, 39, 40, 45, 55, 56, 57, 60, 78
9	BC_2F_2 (selfed)	2	7, 23
10	BC_3F_1	16	9, 10, 13, 15, 32, 33, 35, 37, 42, 46, 47, 61, 67, 68, 69, 73
11	white clover	2	53, 77
12	white clover (selfed)	1	19
13	*T. uniflorum*	3	63, 81, 82

5.4.1 General features

The basic features of the original data have been summarized in Table 5.4. Being very different from the hybrids and white clover, data from *T. uniflorum* was not included in extracting these basic features.

5.4.1.1 Analysis of variance (ANOVA)

Transformed data were used to perform ANOVA based on a General Linear Model (GLM). Data from 70 families belonging to 8 generations (BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_1, BC_2F_2-BI, BC_2F_2-IB, BC_3F_1 and white clover) were analysed (Table 5.5). Significant variation was observed among generations for all traits except seed setting, as a result of selfing, tap root diameter and number of stolons per unit area. The families within a generation differed significantly for all traits except root to shoot ratio. Plants within a family were not statistically different from each other for any of the traits. The comparison between outside and middle plants of a family showed higher florets per head, heads per plant, florets per plant, seeds per plant and ovules per ovary produced by outside plants. Central plants produced longer peduncles. For the rest of the traits there was no significant effect of plant position. The replications had significant effects on 60% of the traits.

Table 5.4. Summary statistics of the untransformed data. Skewness and kurtosis are based upon the transformed data. Data of *T. uniflorum* is not included.

Traits	N	Mean	CV	SD	Min	Max	SE	Skewness	Kurtosis
Seeds/floret	1017	0.98	61.11	0.60	0	3.20	0.02	-0.10	-0.09
Florets/head	1017	40.69	33.93	13.81	8	88	0.43	0.02	0.07
Seeds/head	1017	42.08	76.15	32.05	0	231	1.00	0.07	-0.08
Heads/plant	1008	48.17	67.24	32.39	1	197	1.02	0.18	0.06
Florets/plant	1008	1885	69.84	1316	20	8738	41.46	0.21	-0.11
Seeds/plant	1008	1890	99.18	1875	0	13377	59.06	-0.07	-0.15
Pollen stainability%	153	78.6	6.68	5.35	67	94	0.43	0.39	-0.11
Ovules/ovary	158	5.26	14.89	0.78	3.38	7.25	0.06	0.10	-0.65
Selfing%	71	34.38	120.82	41.53	0	204	4.93	0.39	-0.15
Peduncle length (mm)	1017	192.20	30.97	59.53	38	368	1.87	0.06	-0.26
1000 seeds weight (gm)	557	0.53	21.12	0.11	0.24	0.85	0.00	-0.04	-0.06
Leaf area (cm^2)	193	3.59	33.18	1.19	1.02	6.38	0.09	0.12	-0.35
Foliage dry weight (gm)	1008	30.01	28.28	8.49	7.60	57.49	0.27	0.14	-0.26
Stolons dry weight (gm)	314	29.50	56.52	16.67	0.71	104.79	0.94	0.13	0.19
Roots dry weight (gm)	314	6.44	54.88	3.53	0.02	28.78	0.20	0.18	0.83
Tap root diameter (mm)	310	5.48	20.48	1.12	2.90	9.02	0.06	0.21	0.26
Woody tap root length (mm)	310	18.64	41.29	7.70	3	51	0.44	-0.02	-0.17
Stolons/104 cm^2	310	30.07	31.35	9.42	11	73	0.54	0.40	0.94
Nodes/stolon	310	5.47	25.21	1.38	3	12	0.08	0.32	0.00

Table 5.5. P-values showing ANOVA results of traits at different levels. Highlighted cells show no significant variation ($P > 0.05$).

Traits	Observations Used	Generation	Family	Plant	Plant position	Replication
	Levels	8	70	15	2	3
Seeds/floret	916	<.0001	<.0001	1.000	0.3576	<.0001
Florets/head	916	<.0001	<.0001	0.9966	0.0370	<.0001
Seeds/head	916	<.0001	<.0001	1.000	0.5590	<.0001
peduncle length	916	<.0001	<.0001	<.0001	<.0001	<.0001
Heads/plant	907	<.0001	<.0001	0.0382	0.0010	0.0044
Florets/plant	907	<.0001	<.0001	0..0560	<.0001	0.2515
Seeds/plant	907	<.0001	<.0001	0.5196	0.0010	0.0451
Foliage dry weight	907	<.0001	<.0001	0.9981	0.9297	<.0001
pollen stainability%	116	0.0032	0.0121	0.0731	0.9508	0.1510
ovules/ovary	129	<.0001	0.0083	0.0110	0.0134	0.5536
Selfing%	58	0.0934	0.0301	0.9562	-	0.2378
1000 seeds weight	506	<.0001	<.0001	1.000	0.3528	0.0003
Leaf area	160	<.0001	<.0001	1.000	-	0.1770
Stolons dry weight	281	<.0001	<.0001	0.8520	0.3832	0.8528
Roots dry weight	281	<.0001	0.0118	0.9241	0.4064	0.3515
Tap root diameter	282	0.1895	0.0003	0.8389	0.3779	0.0126
Woody tap root length	282	0.0051	0.0002	0.0740	0.4985	0.2543
Stolons/104 cm^2	282	0.1141	0.0020	0.0722	0.0826	<.0001
Nodes/stolon	282	0.0001	0.0001	0.9419	0.8476	0.0033
Root / shoot ratio	279	<.0001	0.1295	0.4314	0.7008	0.0004

5.4.2 Seed development in *T. uniflorum*

Ripe seeds could not be obtained from *T. uniflorum*. Pod swelling was observed as an indication of successful fertilization and embryo development. The embryos were taken out of the pod and development of plumule and radical was confirmed. The embryos within a pod were counted under a microscope. Data of these un-ripened but developed embryos were used to derive estimates for seeds per floret, head and plant for *T. uniflorum* (Plate 5.2*)*. This species showed the best seed production ability per floret, followed by white clover (Figure 5.30a).

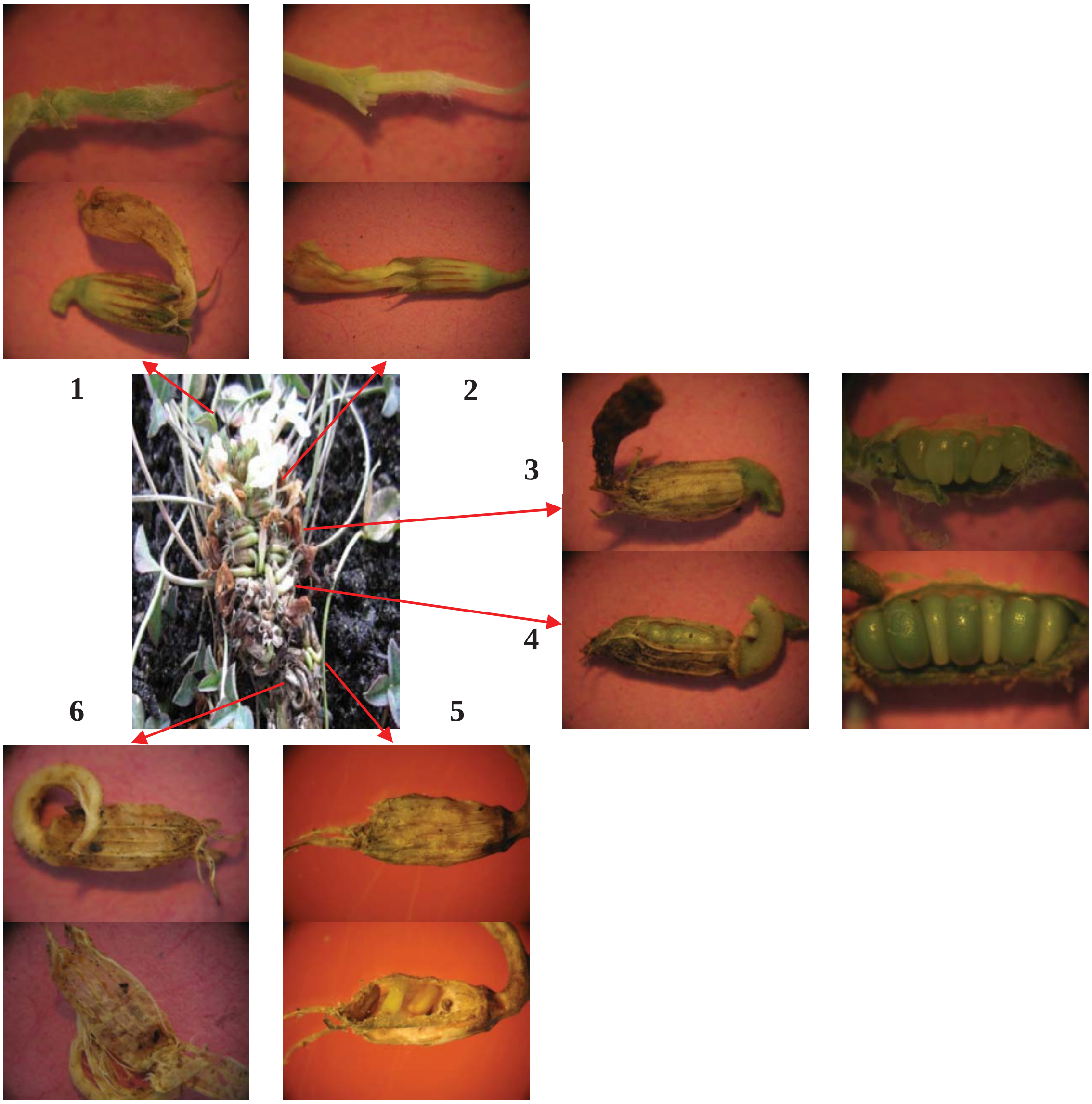


Plate 5.2. Different seed maturity stages of *T. uniflorum* under natural conditions. The left-middle picture is a stem with open flowers at the top and dry flowers at the bottom. The pictures in ascending order show observable developing seeds in the pods. Stage 5 is showing loss of pre-mature seeds due to rottening of the pod. The completely dried pod (stage 6) shows complete loss of the seeds.

During summer 2012-13, seed maturation in *T. uniflorum* was generally observed under field conditions. Variation for pod swelling among different accessions was observed. During the first week of December 2012, one to three stolons of the plants showing pod swelling were raised above the soil surface (Plate 5.3). A few stolons grown normally (very near to the ground surface) were marked on the same plant. Already ripened florets were removed from both types of stolon at the start of the observation. After about a month, all florets were removed from stolons on 6th of January 2013. A selected floret maturity range, i.e. from florets with green peduncle but dropped off withered petals to florets with brown but un-rotten peduncle and sepals, was analysed for seed maturity.

Plate 5.3. Naturally oriented (left) and uplifted (right) inflorescences of *T. uniflorum*.

The uplifted stolons were significantly higher than normal stolons for total embryos and seed production (Figure 5.5). The normal stolons retained a significantly higher proportion of unripened (green) seeds, whereas the drier nature of the uplifted stolons facilitated maturity of the seed and produced significantly more yellow and brown seeds. This later study showed the presence, although in low numbers, of mature seeds on normally grown stolons. This might be due to the fact that the plants were more than two years old and required repotting, which might have caused a slight uplifting of the stolons from their normal position, and this might have slightly favoured normal seed maturity.

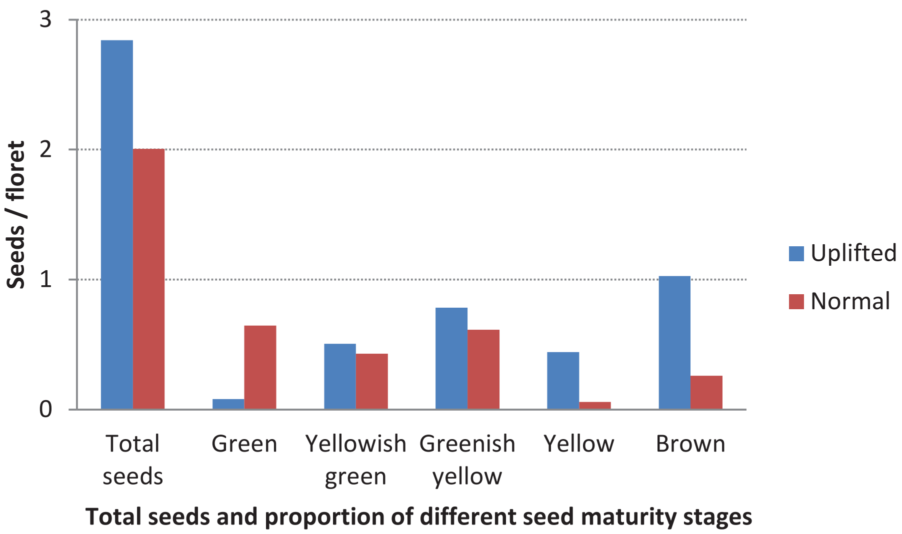


Figure 5.5. Comparison of seed maturation ability of normal and uplifted stolons of *T. uniflorum*. The colors on the X-axis indicate progressive seed maturity stages. The bars give the seeds per floret as the proportions of total seeds produced in a floret.

The observation during summer 2012-13 also showed that the sepal sheath became ruptured, prematurely exposing the developing pod to water (Plate 5.4). In white clover, the pod always remained protected from direct contact with water by the sepals. However, these observations were based on limited sample size (18 obsevations) and were restricted to the plants having swollen pods. Further detailed investigations are required to investigate fertilization and seed development and maturity in *T. uniflorum*.

A

B

Plate 5.4. Flower heads of white clover (A) and *T. uniflorum* (B) showing well protected pods in white clover and almost unprotected pods exposed to ground moisture in *T. uniflorum*.

5.4.3 Potential and actual seed setting

Ovules produced by an ovary were counted for a range of hybrid generations (Table 5.6). The average number of ovules produced in an ovary by the hybrids was 5.4 which were similar to that of white clover (5.0). Ovule production did not affect selfing efficiency (P = 0.04).

Table 5.6. Details of families involved in ovules per ovary study and results.

FAMIL	GENERATION	PEDIGREE		AVERAGE OVULES
		female	**male**	
83	BC_1F_1	A_{bulk} *		6.0
84	BC_1F_1	S_{bulk}		6.0
85	BC_1F_1	S_{18}		6.0
18	BC_1F_2 (selfed)	Cu_2		5.6
62	BC_1F_3	C_4 x C_8	S_{12} x S_{10}	5.6
79	BC_1F_3	A_{11} x A_6	S_{14} x S_{10}	4.8
64	BC_1F_3 (selfed)	B_2 x B_3		5.8
76	BC_1F_3 (selfed)	S_{14} x S_{10}		6.0
28	BC_2F_2-IB	$Will_2$	A_{15} x A_6	5.1
40	BC_2F_2-IB	$Will_2$	B_2 x B_8	5.3
9	BC_3F_1	$Will_2$	LA_4 x S_{10}	4.5
15	BC_3F_1	$Will_2$	DU_{44} x A_6	4.5
69	BC_3F_1	$Will_2$	LA_5 x Tf_1	5.0
	Average of hybrids			5.4
53	White clover	Cv. Sustain		5.3
77	White clover	Cv. Aran		4.8
	Average of white clover			5.0

*** mixture of different BC_1F_1 genotypes of the family.**

Numbers of ovules produced per ovary were multiplied by numbers of florets per plant to estimate the potential seed yield of a plant. This was compared with actual seed yield for 14 families belonging to different generations. The mean actual seed yield of the hybrids was very low (16%) relative to their potential seed yield. The relative mean actual yield of white clover was 31% of its potential seed yield. White clover expressed almost two fold the capability than that of the hybrids to realise the potential seed yield. Comparisons of families for their proportion of harvestable potential seed setting showed highly significant variation both within ($P = 0.0001$) and between ($P = < 0.0001$) families of different generations (Figure 5.6). Families of BC_1F_3 generations and white clover showed significantly higher capability for reaping the potential of seed production compared to families of other generations.

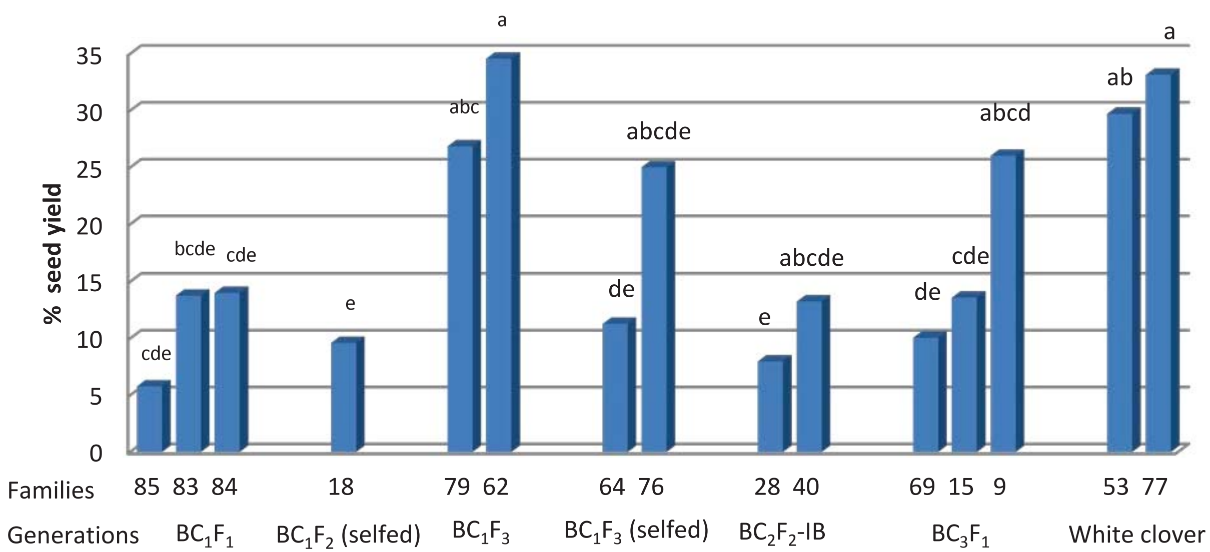


Figure 5.6. Comparisons of families of different generations for harvestable potential seed yield. Analysis is based on original data. Except BC_1F_2 (selfed), all generations (lower line of X-axis) are represented by more than one family (upper line on X-axis). Different letters at the top of bars represent significant difference from each other at P≤ 0.05 (Tukey's test following ANOVA).

5.4.4 Associations among the traits

Two analytical approaches, Principal Component Analysis (PCA) and path coefficient analysis, were used to explore associations among traits.

5.4.4.1 PCA

PCA was used as an exploratory tool to analyse the interactions among various plant traits which were expected to have an influence on seed production either directly or indirectly. In the SAS statistical package (SAS 9.2), the principal axis method was used in the factor procedure using one as the prior communality estimate. This was followed by a varimax (orthogonal) rotation. Analysis was done to explore relationships among all as well as selected traits.

a. **Relationships among all traits:** The analysis was based on complete sets of observations for 70 plants. The first six components (factors) had eigenvalues greater than one. Combined, they represented 77% of the total variance. Details of the PCA results (Table 5.7) reveal that factor one showed strong positive association among florets per head (head size), peduncle length, leaf area and foliage dry weight. Seeds per head were moderately linked with florets per head. However, number of ovules per ovary was negatively correlated with head size, peduncle length and foliage production. Factor two showed that high numbers of heads and florets per head had high contributions to plant seed production. Foliage production was moderately associated with these traits. Factor three had high scores for stolon and root traits. There appeared to be strong associations among stolon number, stolon dry weight and root dry weight. These traits were moderately positively linked with tap root diameter. In factor four, seed production capability per floret was strongly associated with number of seeds produced by a head. Although these two traits were positively associated with plant seed production, this association was not as strong as those shown by number of heads and florets per plant under factor two. Factor five showed that nodes per stolon were negatively associated with seed weight and pollen stainability. Factor six expressed positive association between tap root diameter and the length of the woody portion of the tap root.

Table 5.7. PCA results with six retained factors rotated with the varimax procedure showing relationship among all traits. The numbers for each variable are coefficients of the eigenvalues for each factor.

Variable	Factor1	Factor2	Factor3	Factor4	Factor5	Factor6
Eigenvalue	4.91	2.81	2.27	1.95	1.16	1.05
Variance proportion	0.27	0.16	0.13	0.11	0.06	0.06
Seeds/floret	-8	-1	17	93***	4	-8
Florets/head	80***	-3	9	34	28	5
Seeds/head	36*	-1	19	87***	16	0
Heads/plant	-29	90***	-1	-20	-6	12
Florets/plant	16	94***	8	-2	10	6
Seeds/plant	11	76***	11	57**	15	-3
Pollen stainability%	-10	10	7	-34	36*	-33
Ovules/ovary	-69**	-17	9	7	29	0
Peduncle length	82***	4	17	9	23	-6
1000 seeds weight	11	6	-1	9	84***	7
Leaf area	83***	-24	4	2	12	-8
Foliage dry weight	57**	49*	16	3	8	-10
Stolons dry weight	21	13	88***	12	1	-5
Roots dry weight	7	-3	83***	27	8	-9
Tap root diameter	22	5	52**	-28	-11	49*
Woody tap root length	-19	11	-5	-2	4	86***
Stolons number	-11	7	81***	4	17	6
Nodes/stolon	-29	-6	-33	-14	-62**	10

*, ** and *** highlight coefficient of eigenvectors greater than 35, 50 and 70, respectively.

b. **Relationships among seed production traits:** Data for all root and stolon traits were removed and characteristics directly related to seed production were analysed. The analysis was based on 1008 complete observations. The first three factors had eigenvalues greater than one and represented 87% of the total variance. These factors were retained for rotation and analysis. The results of this PCA were similar to those previously obtained using all traits with fewer (70) complete observations (Table 5.8). The associations expressed by factor 1, 2 and 3 of this analysis were similar to the associations shown by factor 3, 4 and 1, respectively, of the analysis with complete observations. The only major difference was that, in this analysis, factor 2 showed correlation of numbers of florets per head with number of seeds produced by a floret, head and plant.

Table 5.8. PCA results with three retained factors rotated with the varimax procedure showing relationship among selected traits.

Variable	Factor1	Factor2	Factor3
Eigenvalue	3.41	2.1	1.46
Variance proportion	0.43	0.26	0.18
Seeds/floret	3	93***	-14
Florets/head	2	57**	61**
Seeds/head	3	98***	15
Heads/plant	97***	-12	-15
Florets/plant	94***	14	14
Seeds/plant	72***	67**	2
Peduncle length	-3	-7	90***
Foliage dry weight	61**	2	59**

** and *** highlight coefficients of eigenvectors greater than 50 and 70, respectively.

5.4.4.2 Path analysis

Path coefficient analysis was conducted to investigate the interrelationships among the different traits involved directly or indirectly in seed production. The study was based on 12 generations each with different numbers of families and different amounts of data for each trait. Path analysis was applied as described in section 4.4.3.2 of experiment-2. The objective was to explore relationships among all traits, selected traits and seed production traits.

a. **Relationship among all traits:** The setup of the paths was based on biologically logical cause-and-effect assumptions. For example, seeds per head is determined by florets per head and seeds per floret. The analysis used 71 observations with complete and 946 observations with incomplete data set. Reproductive traits along with vegetative and root traits showed complex interactions in relation to seed production (Figure 5.7). Head production and seeds per head were taken as having direct effect on plant seed production. These both triats appeared important contributor in seed production per plant. Florets per head (head size) positively influenced seeds per head. It also showed a positive relationship with number of seeds produced by a floret. However, it had a negative association with number of ovules per ovary. Number of seeds per floret was major contributor to number of seeds per head. This trait appeared unaffected by the number of ovules in an ovary. Leaf area and foliage production showed positive association with head size. Foliage dry weight also had considerable positive effect on peduncle length. Leaf area moderately affected foliage production. The trait of nodes per stolon was the main determinant of heads per plant. Negative relationship between number of stolons and nodes present on a stolon was observed. High stolon numbers also had slight positive effect on heads and foliage production capacity. Number of stolons was moderately linked with stolon dry weight, which showed considerable effect on root weight. Tap root diameter had very weak positive and negative association with root weight and nodes per stolon, respectively. Thicker tap roots had woodier growth than thinner tap roots.

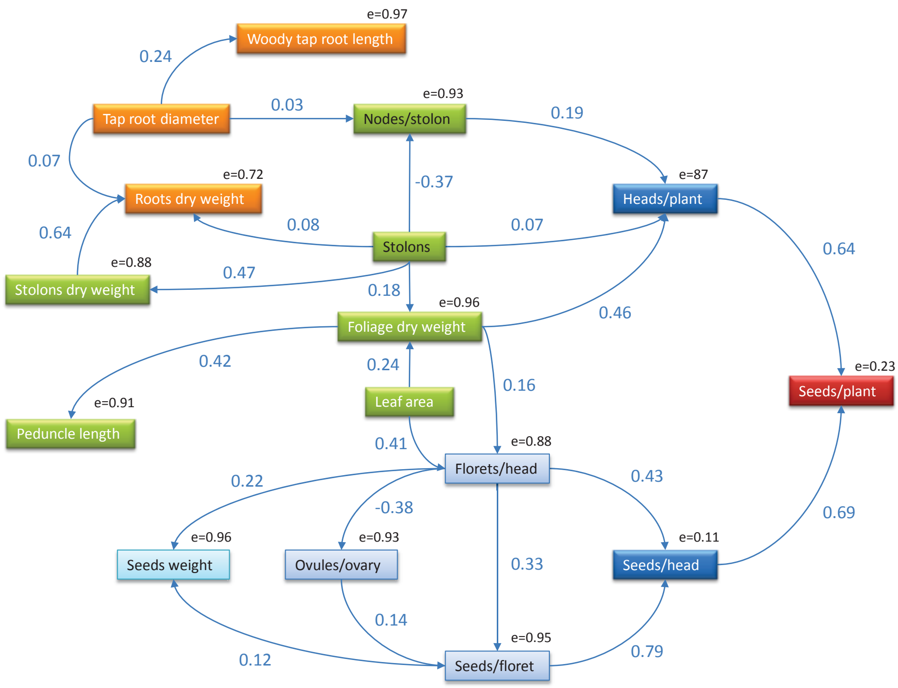


Figure 5.7. A path diagram with coefficients and residuals (e), showing interrelationships among traits affecting seed production in white clover x *T. uniflorum* hybrids.

Partitioning of effect showed that total effect of head size on seeds per head was considerably high (Table 5.9). The reduction of indirect positive effect of head size on seeds per head from total effect of seeds per floret on seeds per head leaved path coefficient of 0.51 as sole effect of seeds per floret on seeds per head. The direct positive association of stolons with head production got nullified with indirect negative association present between these two traits.

Table 5.9. Partition of direct and indirect effects.

Standardized Effects			
	Total	**Direct**	**Indirect**
Effects of Florets/head on Seeds/head	0.65	0.43	0.22
Effects of Florets/head on Seeds/floret	0.28	0.33	-0.05
Effects of Stolons on Heads/plant	0.08	0.07	0.01

b. **Relationship among selected traits:** All root and stolon traits were removed to observe relationships among leaf area, foliage production, peduncle length and seed production traits (Figure 5.8). The analysis used 85 observations with complete and 932 observations with incomplete data set. The relationships generally remained same as in case of path analysis based on data of all the traits. The exceptions were that seeds per head appeared a bit more contributing trait than heads per plant in seed production of the plant. The effect of leaf area on head size and foliage production increased, whereas association between foliage production and peduncle length considerably decreased. The removal of the traits effecting heads per plant and foliage production resulted in change of effects relating to these two traits. The relationship among seeds per head, florets per head, seeds per floret, ovules per ovary and seed weight remained consistent in both analyses.

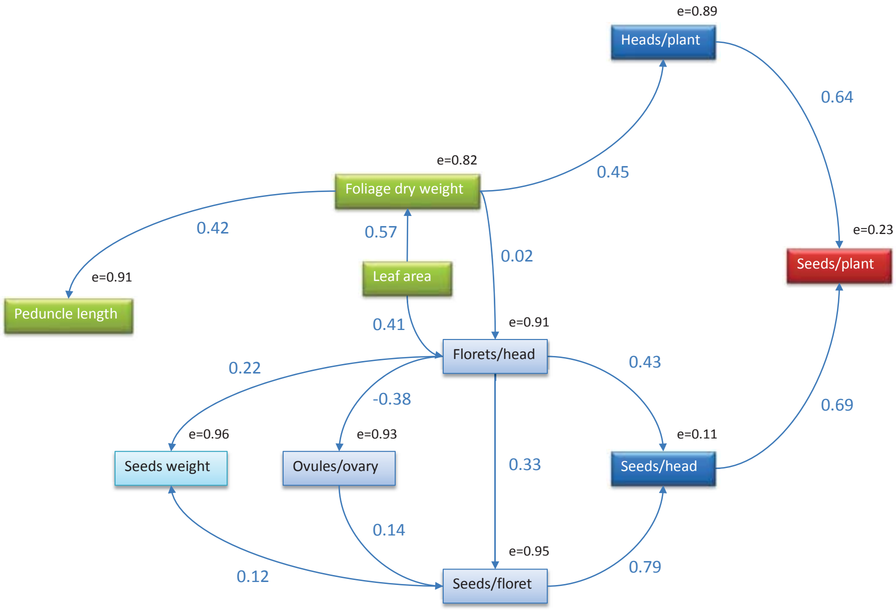


Figure 5.8. A path diagram with coefficients and residuals (e), showing interrelationships among selected traits affecting seed production in white clover x *T. uniflorum* hybrids.

c. **relationships among seed production traits:** Data of root and vegetative traits were removed to improve proportion of observations having complete data sets. The analysis used 1008 observations with complete and 9 observations with incomplete data set. Improvement in proportion of the observations with complete data set and removal of influence of foliage, leaf and peduncle traits only improved effects of heads per plant and seeds per head on plant seed production (Figure 5.9). The relationships among seed production traits appeared to be same as in Figure 5.8. The effects of seeds per floret and florets per head on seeds per head remained consistent in all three analyses.

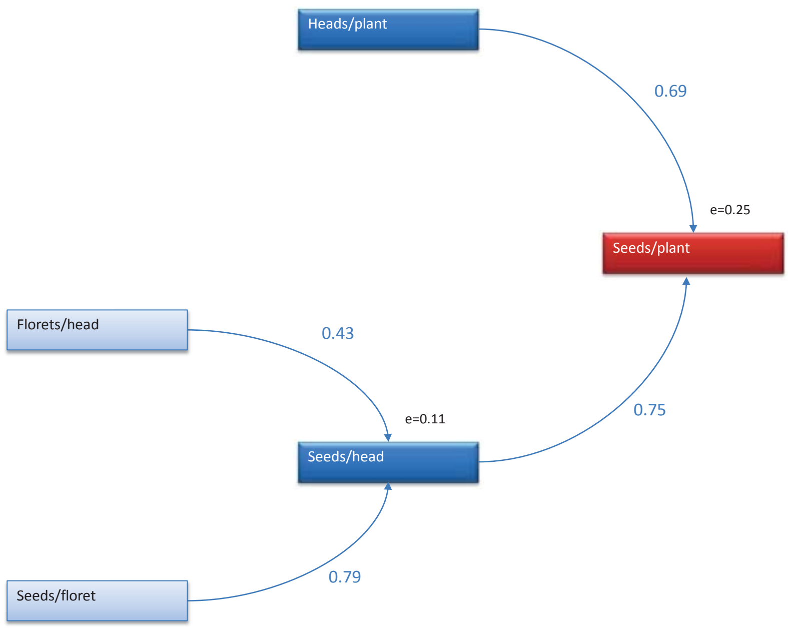


Figure 5.9. A path diagram with coefficients and residuals (e), showing interrelationships among seed production traits of white clover x *T. uniflorum* hybrids.

The path analysis of experiment-2 showed negligible association between foliage and head production (Figure 4.11), whereas a moderately positive association between these two traits in path analysis of experiment-3 (Figure 5.8). Experiment-2 was based on BC_1F_2 and BC_2F_1 generations, whereas path analysis of experiment-3 contained only 5.6% of the data of these two generations and 2.6% of the data of BC_1F_1 generation. The dominant proportion (87.8%) of the data used in experiment-3 was of more advanced hybrids than BC_1F_2 and BC_2F_1. Moreover, 4% of the data of this analysis was of white clover. The cumulative data proportion of the white clover and BC_3F_1 was 25.6% of the total data. Even in the development of BC_1F_3 hybrids, the selection was aimed to enhance seed production, which was the better trait from the white clover parent. This shows that the data used in experiment-3 was predominantly representing white clover like phenotypes. The comparison between parental species (Figures 5.32c and 5.30d) showed that *T. uniflorum* had lower foliage production and higher head production than white clover. The inverse trends of parents for these two traits might have weakened the relationships between these traits in the early hybrid generations i.e., BC_1F_2 and BC_2F_1. The advance hybrid generations having white clover type phenotype showed reasonable positive relationship between foliage and head production.

5.4.5 Differences in parental effects

5.4.5.1 Influence of white clover parents

To study the influence of a white clover parent on progenies in backcrossing, a common set of white clover varieties was crossed with each of two BC_2F_1 plants (plans 1 and 2) and BC_1F_2 plants (plans 3 and 4). The performance of their progenies was evaluated for a range of traits. In the majority (66%) of cases, the white clover parents gave significantly different results when crossed with the same hybrid plant (Table 5.10). However, the results were not completely consistent with respect to crosses and traits involved. Only plan 4 showed significant differences among crosses for all traits. The significant difference among progenies was on average 63% in case of BC_2F_1 hybrid parents involved, whereas it was 69% in case of involvement of BC_1F_2 hybrid parents. The parental composition affected the variation among progenies. Plan-1 and plan-2 involving BC_2F_1 hybrids expressed significant variation in 75% and 50% of the traits, respectively. Similarly, BC_1F_2 hybrids in plan-3 showed significant variation in progenies for 38% of the traits, whereas it was 100% in the plan-4. White clover parents differed in their efficiency in creating significant difference among progenies. The cultivars AR_{802} and $Will_2$ were less effective parents having 16% and 19% proportion in progeny comparisons with significant differences, respectively. This share of the white clover parents CH_{13} and AR_{28} was 32% and 33%, respectively.

Table 5.10. Plans used to observe effect of backcrossing with white clover on different traits. The highlighted cells show significant difference (P ≤ 0.05).

Generations	BC_2F_1				BC_1F_2			
Plans	**Plan-1**		**Plan-2**		**Plan-3**		**Plan-4**	
Parents	♀	♂	♀	♂	♀	♂	♀	♂
Crosses	AR_{28} CH_{13} AR_{802} $Will_2$	(D_{44} x A_{11})	AR_{28} CH_{13} AR_{802} $Will_2$	(LA_3 x Tf_3)	AR_{28} CH_{13} AR_{802}	(B_2 x B_3)	AR_{28} CH_{13} AR_{802} $Will_2$	(S_{10} x S_{12})
Observations	47		57		35		59	
Seeds/floret	<.0001		0.0107		0.2431		<.0001	
Florets/head	<.0001		0.2084		0.1276		0.0008	
Seeds/head	<.0001		0.0181		0.2843		<.0001	
Heads/plant	0.0413		0.1913		0.0035		0.0026	
Florets/plant	0.1078		0.6108		0.0017		0.0522	
Seeds/plant	0.0002		0.2573		0.0167		0.0173	
Peduncle length	<.0001		<.0001		0.1914		<.0001	
Foliage dry weight	0.3883		0.0003		0.3282		0.0599	

5.4.5.2 Influence of hybrid parents

The influence of different BC_1F_2 hybrids involved in intercrossing and backcrossing were investigated using two crossing plans for inter-crosses (BC_1F_2 x BC_1F_2) and one backcrossing plan. Hybrids were selected that showed apparently contrasting results for different traits during experiment-2 (Figure 5.10). The progenies of the crosses with a common parent were grown in experiment-3. These half sibs were evaluated for seed production traits to observe the effects of different parents.

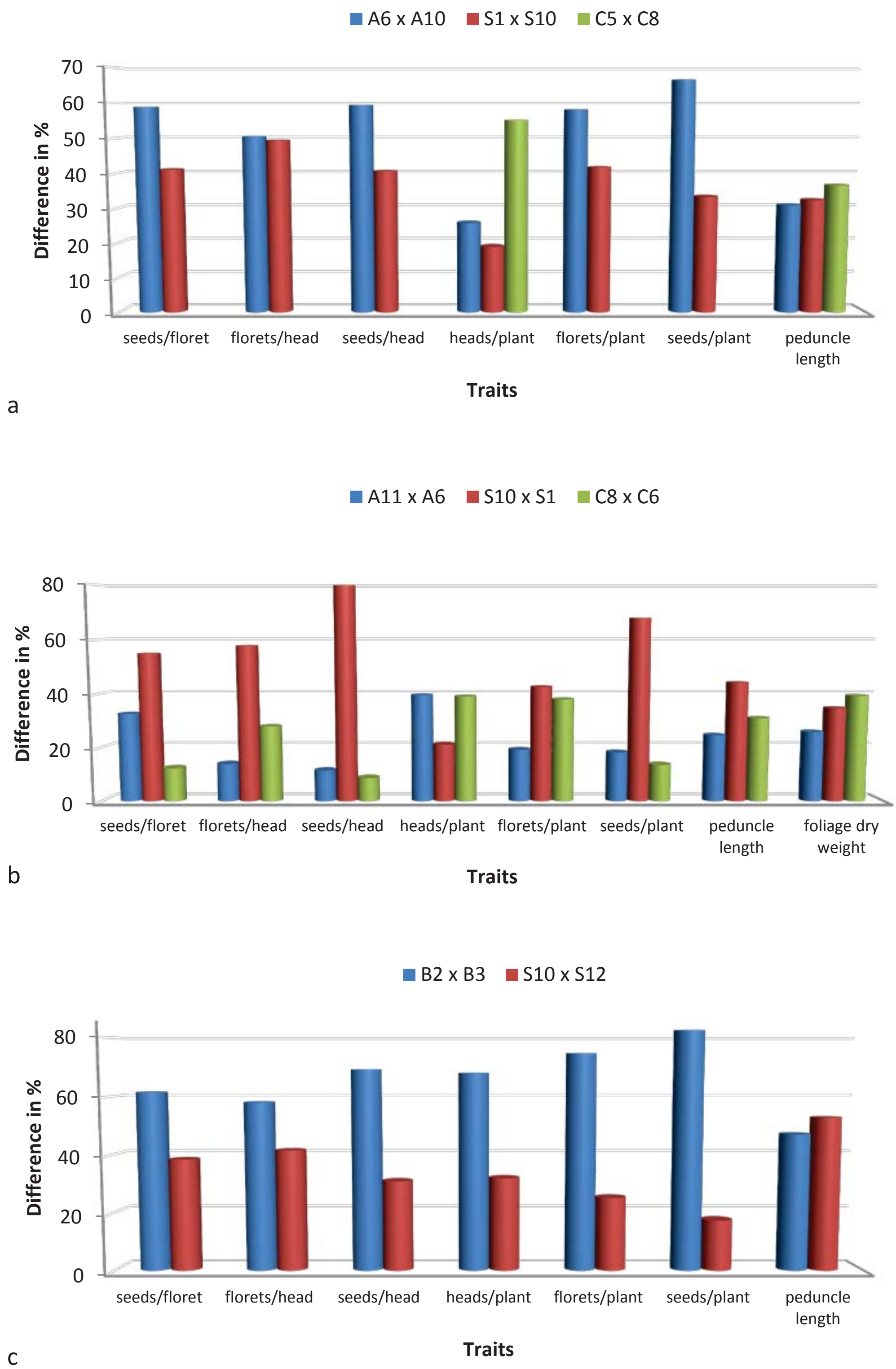


Figure 5.10. Comparative performances of different hybrids in experiment-2. These hybrids were intercrossed (a and b) and backcrossed (c) at the end of the experiment. Their progenies were used in experiment-3 to analyse influence of hybrids in crossing.

ANOVA of both intercrossing (Table 5.11) and backcrossing data (Table 5.12) showed that differences in hybrid parent did not create significant differences for most traits in the progeny. The significant variation appeared for 38% and 44% comparisons made in intercrossed and backcrossed progenies. Different BC_1F_2 hybrids crossed with a single BC_1F_2 hybrid could not maintain or enlarge the initial differences already present among the hybrids during experiment-2. The common female parent diluted the differences among the different male parents involved in each plan. The effect of parental composition was evident in this analysis. For example, Plan-1 of intercrossing scheme showed significant difference among progenies in 50% of the traits, whereas it was 25% in plan-2. Similarly, in backcrossing schemes, the significant difference among progenies was 13% for plan-1, while it was 63% for plan-4. The general analysis of results showed that seeds per floret, florets per head and seeds per head were clearly much effected (on average 61% case with significant difference among progenies) by change in hybrid parents than heads and florets per plant (0% case with significant difference among progenies). Seeds per plant and peduncle length showed similar effect as that on per head traits. Foliage production was less effected than all other traits except heads and florets per plant.

Table 5.11. Details and P values of ANOVA results of plans to analyse influence of hybrids in intercrossing. The highlighted cells showed no significant difference (P > 0.05).

Plans	Plan-1		Plan-2	
Parents	♀	♂	♀	♂
Crosses	(S_{10} x S_{12})	(A_6 x A_{10}) (C_5 x C_8) (S_1 x S_{10})	(B_2 x B_3)	(A_{11} x A_6) (C_8 x C_6) (S_{10} x S_1)
Observations	40		40	
	Pr > F-value			
Seeds/floret	0.0014		0.2092	
Florets/head	<.0001		<.0001	
Seeds/head	<.0001		0.8508	
Heads/plant	0.6204		0.7083	
Florets/plant	0.1687		0.7542	
Seeds/plant	0.0164		0.8102	
Peduncle length	0.8374		0.0001	
Foliage dry weight	0.333		0.375	

Table 5.12. Details and P values of ANOVA results for plans to analyse influence of hybrids in backcrossing. The female parent is the white clover and the male parents are hybrids. The highlighted cells showed no significant difference (P > 0.05).

Plan	Plan-1		Plan-2		Plan-3		Plan-4	
Parent	♀	♂	♀	♂	♀	♂	♀	♂
Cross	A_{28}	$(B_2 \times B_3)$ $(S_{10} \times S_{12})$	A_{802}	$(B_2 \times B_3)$ $(S_{10} \times S_{12})$	Ch_{13}	$(B_2 \times B_3)$ $(S_{10} \times S_{12})$	W	$(B_2 \times B_8)$ $(S_{10} \times S_{12})$
Observations	29		20		30		30	
	Pr > F-value							
Seeds/floret	0.1437		0.0175		0.0003		0.0021	
Florets/head	0.2707		0.0966		<.0001		0.3816	
Seeds/head	0.1376		0.0167		<.0001		0.0042	
Heads/plant	0.5892		0.2614		0.1619		0.6046	
Florets/plant	0.5194		0.972		0.8326		0.4556	
Seeds/plant	0.4084		0.1567		0.0352		0.0186	
Peduncle length	0.1205		0.0037		0.0049		0.0044	
Foliage dry weight	0.0017		0.6598		0.131		0.0177	

5.4.5.3 Reciprocal crosses

Progenies of reciprocal crosses were analysed to check presence of any maternal or paternal effects. Plan-1 analysed reciprocal intercrossing of BC_1F_2, whereas plan-2 used progenies of reciprocal crosses between BC_2F_1 plants. ANOVA results, in general, showed no such evidence (Table 5.13). The progenies of reciprocal crosses were similar for all traits except for florets per head of plan 1 and, seeds per floret and seeds per head of plan 2.

Table 5.13. Planning schemes and P-values of reciprocal crosses. The highlighted cells show significant difference (P < 0.05).

Plans	1		2	
Crosses	$\{(S_{10} \times S_1) \times (B_2 \times B_3)\}$ Vs $\{(B_2 \times B_3) \times (S_{10} \times S_1)\}$		$\{(LA_8 \times A_1) \times (LA_4 \times A_1)\}$ Vs $\{(LA_4 \times A_1) \times (LA_8 \times A_1)\}$	
	Observations	P-value	Observations	P-value
Seeds/floret	30	0.8095	15	0.0143
Florets/head	30	0.0013	15	0.1406
Seeds/head	30	0.4437	15	0.0116
Heads/plant	30	0.6562	15	0.9851
Florets/plant	30	0.1578	15	0.7683
Seeds/plant	30	0.3389	15	0.0551
Peduncle length	30	0.0989	15	0.5287
Foliage dry weight	30	0.0632	15	0.99

5.4.6 Relationships among the families

Families from each generation were compared based on transformed data of seeds per floret, florets per head (head size) and heads per plant (Figure 5.11). Families within each generation showed a considerable range of variation. For example, families of BC_3F_1 ranged from exceptionally low to high for number of heads per plant. Families of the BC_1F_1 generation showed low number of florets per head. The BC_1F_3 families produced medium sized heads and a wide range in seeds per floret and heads per plant. Families belonging to BC_2F_2-BI had medium to high head size and number of seeds per floret. They also had a wide range of numbers of heads per plant. Families of the BC_2F_2-IB were medium to high in head size and the range in heads per plant was less than for BC_2F_2-BI. Representative families of BC_3F_1 exhibited big heads with medium to high seeds per floret. Both families of white clover were good seed bearers per floret and displayed medium or high numbers of florets per head but were low for heads per plant.

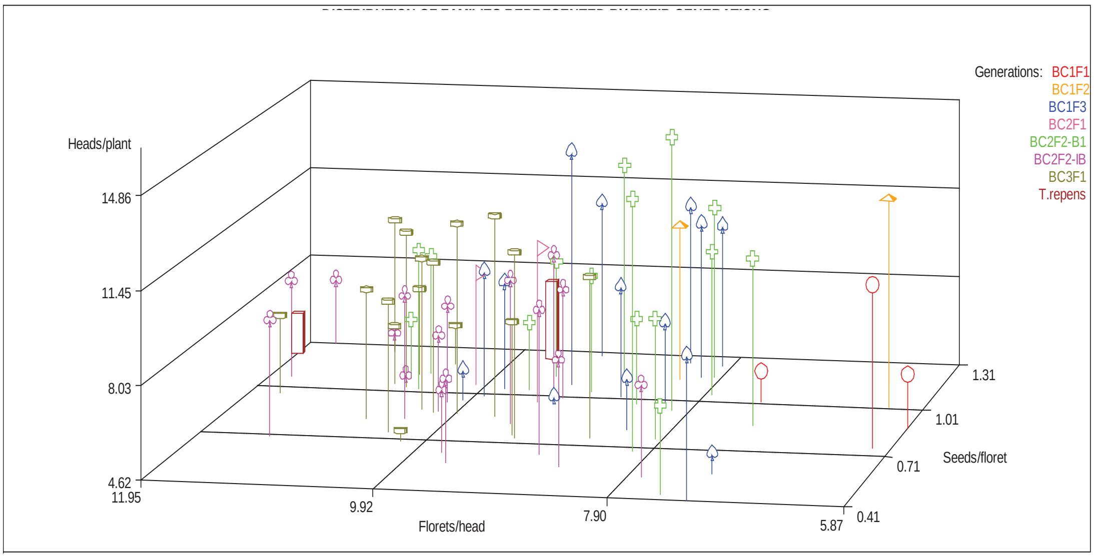


Figure 5.11. Three dimensional scatter diagram of families of different generations based on important seed traits.

Some families belonging to BC_2F_2-BI, BC_3F_1 and BC_1F_3 had above average count for all four important seed production traits i.e., seeds per floret, florets per head, heads per plant and seeds per plant (Table 5.14). They had more heads per plant and total seed production than both of the reference white clover cultivars. Aran produced bigger heads, whereas Sustain showed more heads making it a better seed producer per plant.

Table 5.14. Families having above average performance for important seed traits as compared to all families. Averages of cultivars Aran and Sustain are shown for comparison.

Family	Generation	Seeds/floret	Florets/head	Heads/plant	Seeds/plant
65	BC_1F_3	1.69	41	65	4669
33	BC_3F_1	1.69	55	52	4257
86	BC_1F_3	1.18	40	89	4236
13	BC_3F_1	1.11	52	59	3360
20	BC_2F_2-BI	1.10	51	53	2892
53	Sustain	1.63	44	39	2627
77	Aran	1.59	63	27	2341
Average of all families		1.06	40	50	1928

Considerable similarity was found between results of this family comparison and plotting of the first three factors (explaining 72% of the variance) extracted by PCA using data of seeds per floret, florets per head, seeds per head, heads per plant, florets per plant, seeds per plant, peduncle length and foliage dry weight. The resemblance of the results of these two analyses for families of selected generations (BC_1F_1, BC_1F_3, BC_3F_1 and white clover) is shown in figures 5.12 and 5.13.

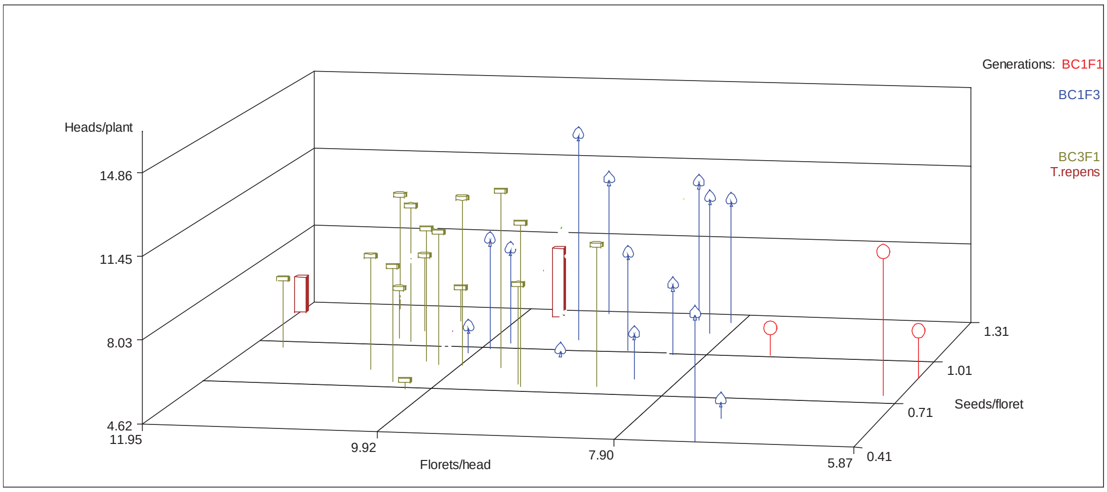


Figure 5.12. Comparison of families of BC_1F_1, BC_1F_3, BC_3F_1 and white clover, based on seed traits.

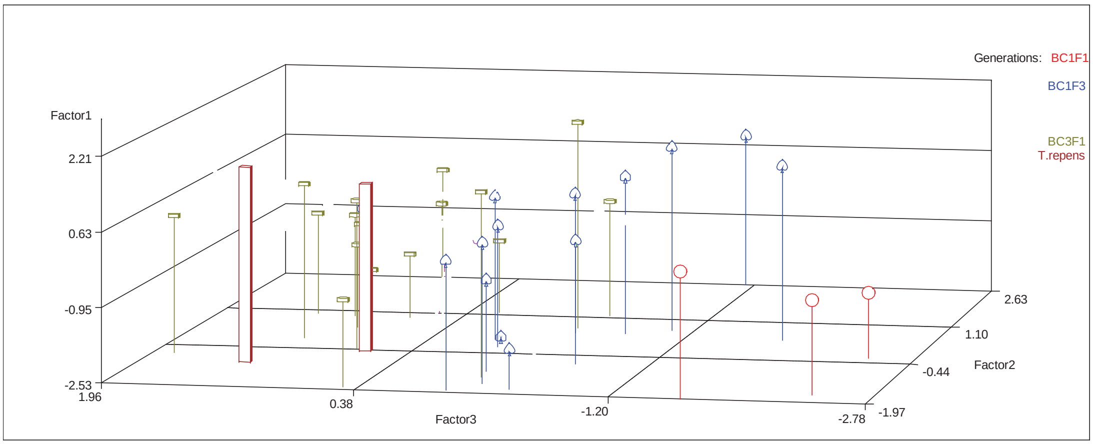


Figure 5.13. Comparison of families of BC_1F_1, BC_1F_3, BC_3F_1 and white clover based on principal components.

5.4.6.1 Association among families

Distance matrices were created in SAS by using original (untransformed) seed trait data (seeds per floret, florets per head, seeds per head, heads per plant, florets per plant and seeds per plant). Split tree analyses based on distance matrices showed resemblance of families irrespective of their generations (Figure 5.14). *T. uniflorum* entries were the most isolated and had a cluster of mostly BC_1F_1 families as their closest neighbours. They also had BC_1F_3 families like family 80 and 50 in its close vicinity. One family of BC_1F_3 (86), made up of only one plant, had exceptionally high head production. The branch having this family at its terminal end carried several other families of the BC_1F_3 generation. White clover (RET 53 and RET 77) families were very distant from *T. uniflorum* (UNI 63, UNI 81 and UNI 82) and clustered with families 33, 42, 68 and 9 of BC_3F_1, 36, 60, 29, 12 of BC_2F_2-IB and 70 and 48 of BC_2F_2-BI.

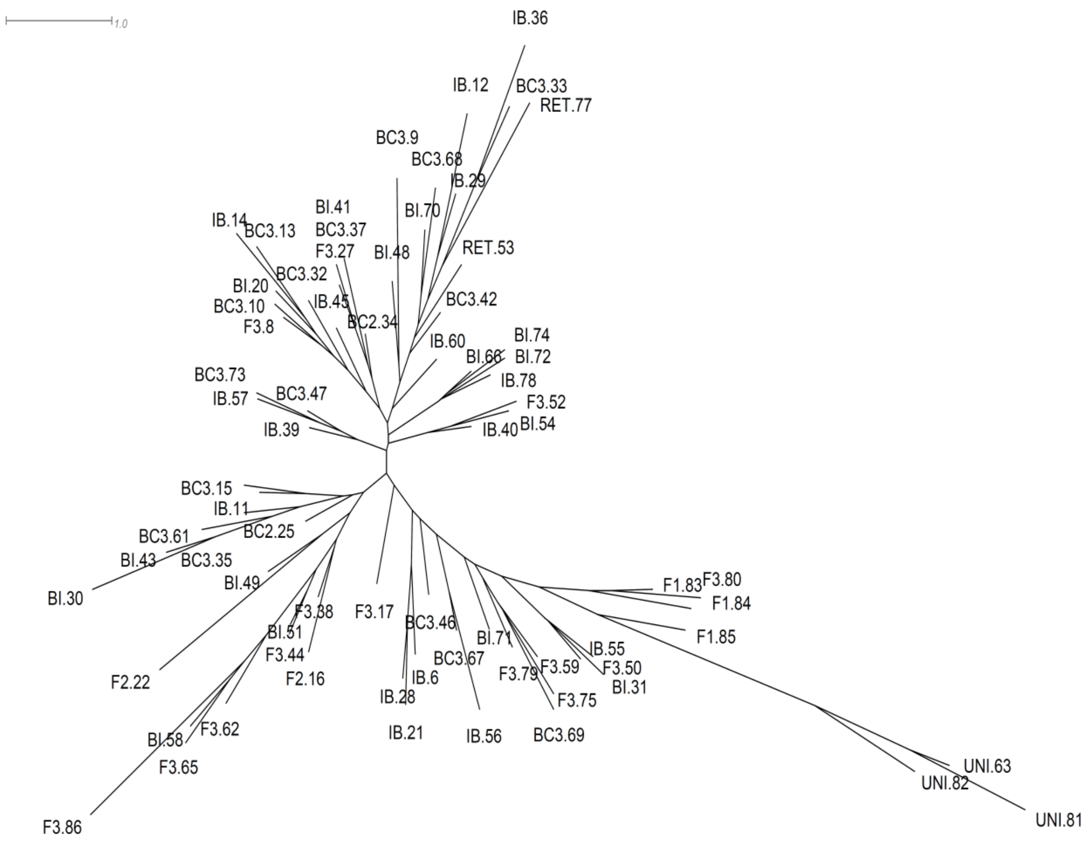


Figure 5.14. Tree diagram of original data produced with neighbour joining methods based on distance metrics. White clover cultivars are represented by abbreviations of RET 53 and RET 77. the accessions of *T. uniflorum* are shown by abbreviations UNI 63, UNI 81 and UNI 82).

A comparison between the outcomes of split tree (Figure 5.15) and plotting principal component 1 on 2 of PCA using transformed data (Figure 5.16) provided a consensus for the family interrelationships, i.e., the clustering pattern was similar in both analyses. Family clusters appearing as branches of the tree appeared in groups in the plot of principal components. The terminal families of the split tree appeared at the outer sphere of clusters of the PCA.

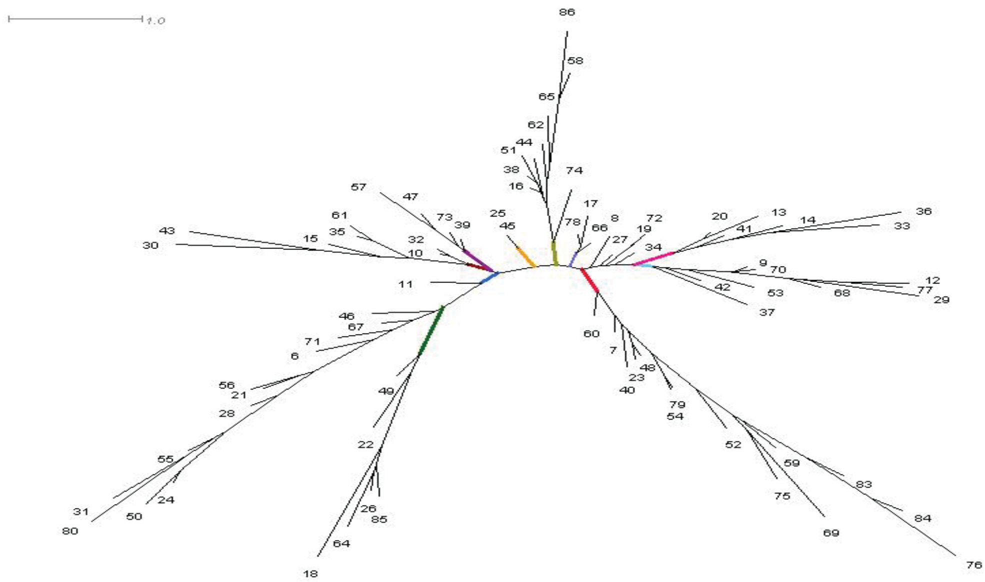


Figure 5.15. Tree diagram of transformed data produced by splits Tree4 with neighbour joining methods based on distance metrics created by SAS. Different colours at the start of each branch represent the group colour in Figure 3.13.

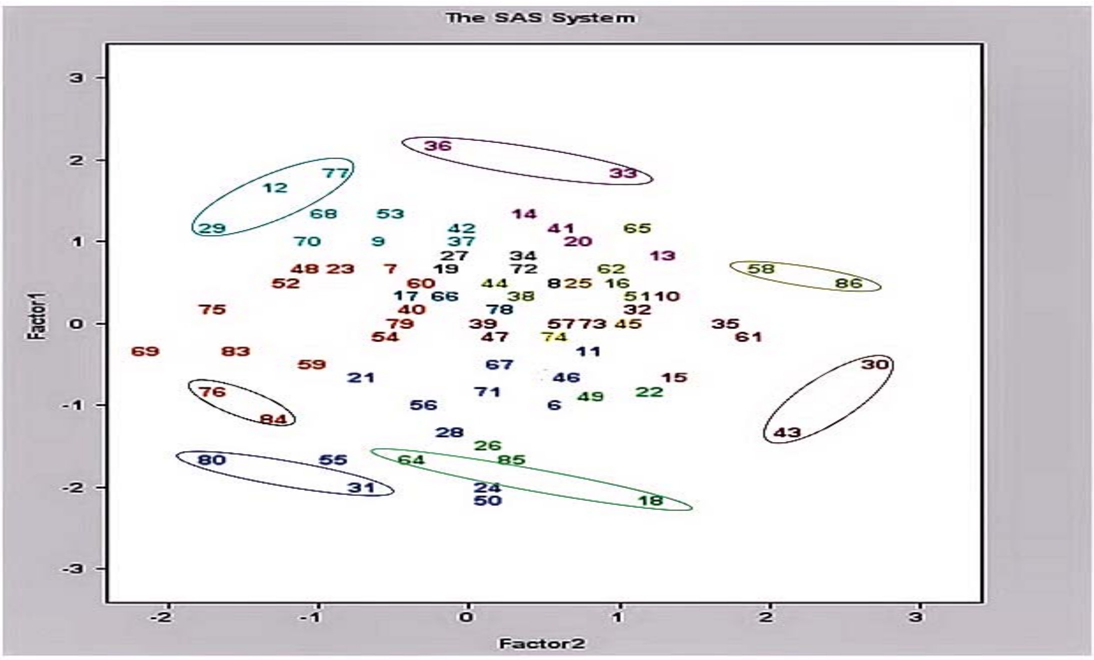


Figure 5.16. Scatter plot of families based on plotting factor 1 and factor 2 of the PCA. Different colours represent different branches of the split tree (Figure 5.15).

5.4.7 Inbreeding effects

Inbreeding effects were analysed by comparing selfed families to their relevant crossed families according to four plans i.e., a, b, c, and d (Table 5.15). These different plans were developed to analyse inbreeding effect in both second backcrossed and second intercrossed generations. The first generation in each plan was selfed BC_1F_1. The second generation in plans a and b was BC_2F_1 and in plans c and d it was BC_1F_2.

Table 5.15. Details of plans and their sub-levels involved in the inbreeding study.

Level	Comparison	Families involved in:			
		Plan-a	**Plan-b**	**Plan-c**	**Plan-d**
1	Selfed (x_1) Vs backcrossed (b_1) or intercrossed (i_1)	Selfed Cu_2 Vs A_{28} x Cu_2	Selfed Cu_2 Vs CH_5 x Cu_2	Selfed S_{14} Vs S_{14} x S_{10}	Selfed B_3 Vs B_2 x B_3
2	Selfed (x_2) Vs intercrossed (i_2)	Selfed A_{28} x Cu_2 Vs {(A_{28} x Cu_2) x (CH_4 x A_1)}	Selfed CH_5 x Cu_2 Vs {(DU_{57} x A_1) x (CH_5 x Cu_2)} + {(DU_{44} x A_1) x (CH_5 x Cu_2)}	Selfed S_{14} x S_{10} Vs {(A_{11} x A_6) x (S_{14} x S_{10})} + {(A_6 x A_{11}) x (S_{14} x S_{10})} + {(A_2 x A_8) x (S_{14} x S_{10})}	Selfed B_2 x B_3 Vs {(B_2 x B_3) x (S_{10} x S_1)} + {(B_2 x B_3) x (C_8 x C_6)} + {(B_2 x B_3) x (A_{11} x A_6)}
3	Selfed (x_2) Vs backcrossed (b_2)				Selfed B_2 x B_3 Vs {(CH_{13}) x (B_2 x B_3)} + {(A_{802}) x (B_2 x B_3)}
4	(x_1+x_2) Vs (b_1 + i_2)				
5	(x_1+x_2) Vs {(b_1 or i_1)+i_2+b_2}				
6	All x Vs All b + i				

Comparisons between selfed and crossed families were made at six levels. The first five comparison levels of plans a and b are explained in figure 5.17 and of plans c and d in figure 5.18. At level 6 an overall comparison between progenies of all selfed and crossed families was made of all plans.

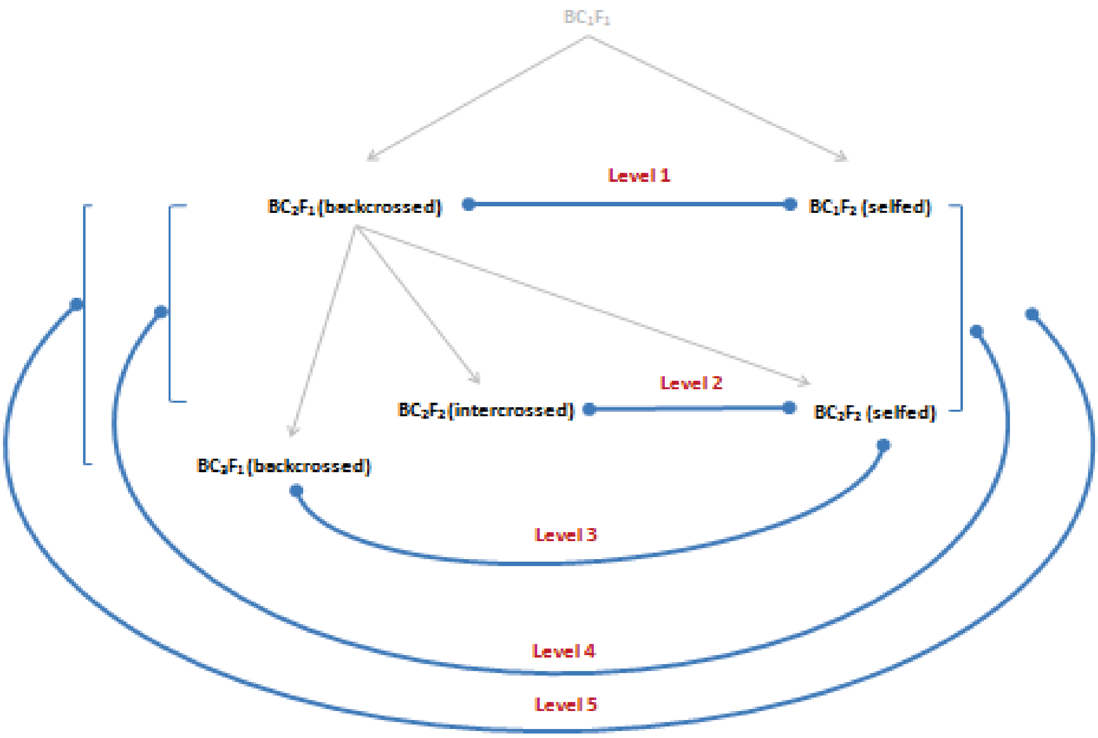


Figure 5.17. Different comparison levels for plans a and b of inbreeding effects.

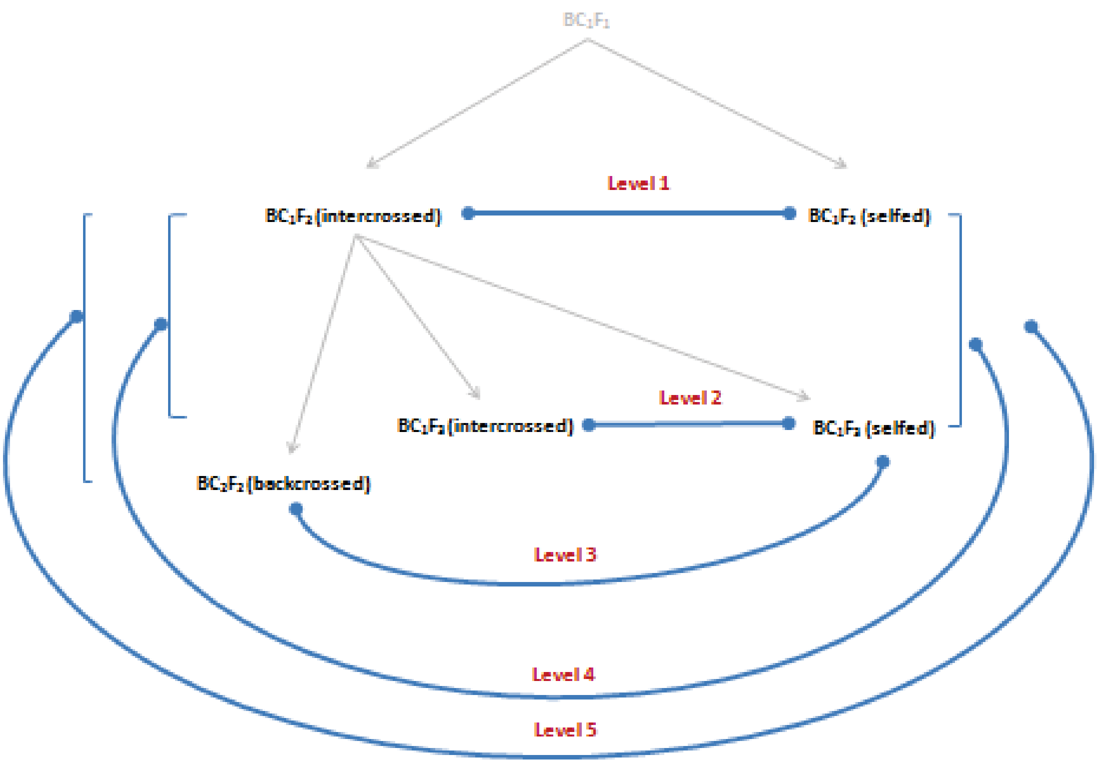


Figure 5.18. Different comparison levels for plans c and d of inbreeding effects.

ANOVA results revealed that 22% of the observed traits showed no significant difference between the selfed and crossed groups (Table 5.16). A considerable proportion of these (37%) were for heads per plant. Results showing a significant difference between selfed and crossed groups included very few (4 out of 106) abnormal outcomes, i.e. better performance of selfed as compared to crossed families. Three of these unexpected results were again for heads per plant. The order of traits for significant hybrid vigour was:

Peduncle length >	Foliage dry weight >	Seeds/floret Florets/head Seeds/head Seeds/plant	> Florets/plant >	Heads/plant

Selfing had the least impact on heads per plant but peduncle length was most affected. Foliage production also reduced with selfing.

Table 5.16. P-values for different traits of different plans used in inbreeding analysis (selfed vs crossed progenies). The highlighted cells showed no significant difference (P > 0.05).

PLAN	Level	Seeds /floret	Florets /head	Seeds /head	Heads /plant	Florets /plant	Seeds /plant	Peduncle length	Foliage dry weight
Plan-a	a1	<.0001	<.0001	<.0001	0.0001	0.2671	<.0001	<.0001	0.0263
	a2	0.1102	0.3987	0.3131	0.3198	0.4463	0.2884	0.0057	0.0667
	a3	0.023	0.138	0.0984	0.1186	0.0497	0.7406	0.0007	0.0059
	a4	<.0001	<.0001	<.0001	0.1713	0.1932	0.0051	<.0001	0.0007
	a5	0.0256	<.0001	0.0004	0.1491	0.0488	0.0171	<.0001	<.0001
Plan-b	b1	0.0009	<.0001	<.0001	0.0328	0.052	0.0007	<.0001	0.0003
	b3	0.0905	0.0008	0.9163	<.0001	0.0003	0.0001	0.0001	<.0001
	b5	<.0001	<.0001	<.0001	0.0039	0.0028	<.0001	<.0001	<.0001
Plan-c	c1	0.0068	<.0001	<.0001	0.7271	0.0297	0.0064	<.0001	0.0029
	c2	0.6597	<.0001	<.0001	0.0027	<.0001	<.0001	<.0001	<.0001
	c4	0.0108	<.0001	<.0001	0.0583	<.0001	<.0001	<.0001	<.0001
Plan-d	d1	0.0016	0.3111	0.0216	0.4228	0.6911	0.1125	0.0944	0.4135
	d2	0.0308	<.0001	0.0001	0.8744	0.0145	0.0029	<.0001	<.0001
	d3	0.0016	<.0001	<.0001	0.3447	0.0005	<.0001	0.0004	<.0001
	d4	0.0002	<.0001	<.0001	0.9865	0.0238	0.0002	<.0001	<.0001
	d5	<.0001	<.0001	<.0001	0.6928	0.0055	<.0001	<.0001	<.0001
All plans	6	0.0001	<.0001	<.0001	0.8243	0.0138	0.0001	<.0001	<.0001

5.4.7.1 Comparison of the generations

Original data from crossed and selfed families belonging to different generations were analysed to assess differences in performance between selfed and crossed combinations (Table 5.17). The average performance of crossed families was divided by average performance of the appropriate selfed families. This provided crossed:selfed ratio for each trait of every generation. For any trait value greater than one of this ratio showed better performance of hybrid, compared to its respective inbred.

Table 5.17. Generations and their pedigrees involved in the inbreeding depression study. Abbreviations with two capital letters represent white clover cultivars, whereas rest of the abbreviations indicate BC_1F_1 plants.

GENERATIO N	SELFED	CROSSED	FAMILY CODE
		female x male	selfed-crossed
BC_1F_2	S_{14} (selfed)	S_{14} x S_{10}	26-16
	B_3 (selfed)	B_2 x B_3	24-22
BC_2F_2	AR_{28} x Cu_2 (selfed)	(AR_{28} x Cu_2) x (CH_4 x A_1)	7 -72
	CH_5 x Cu_2 (selfed)	(DU_{57} x A_1) x (CH_5 x Cu_2)	23-51
BC_1F_3	S_{14} x S_{10} (selfed)	(A_6 x A_{11}) x (S_{14} x S_{10})	76-38
	B_2 x B_3 (selfed)	(B_2 x B_3) x (A_{11} x A_6)	64-27
Parent	CH_5 (selfed)	Sustain*	19- (average of 53 & 77)
		Aran*	

*** crossbred cultivars Grasslands Sustain and Aran.**

Comparisons of different generations for different traits showed that white clover expressed the least inbreeding depression and BC_1F_3 suffered the most severe vigor loss due to selfing (Figure 5.19). Intercrossing increased sensitivity to inbreeding, whereas movement towards white clover by backcrossing decreased the gap between crossed and selfed inbred. Only for florets per head in generation BC_2F_2-BI, selfed plants showed significantly better performance than crossed plants. Florets per plant appeared less influenced by the cross or inbred nature of the plants compared to other traits. Florets per head, peduncle length and foliage production were significantly improved by crossbreeding in all generations.

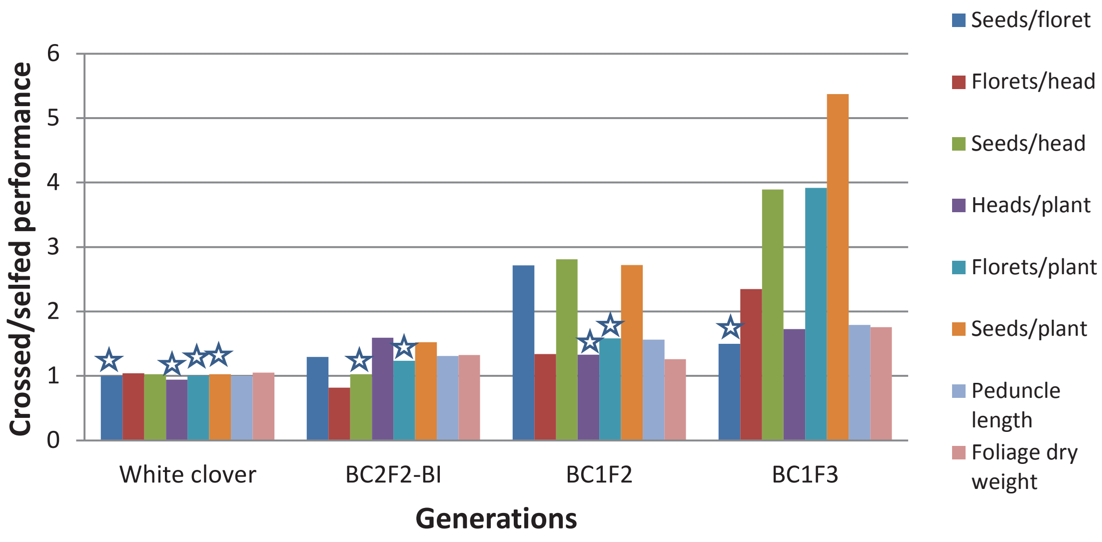


Figure 5.19. Comparison of generations for inbreeding depression. The Y-axis is based on performance of a parent in cross combination divided by performance of the same genotype in selfed form. Stars at each bar indicate no significant difference between cross and selfed combinations ($P < 0.05$). Transformed data of all traits, except peduncle length, were used for ANOVA.

5.4.8 Inheritance of the traits

Inheritance of the traits was evaluated in general and in terms of narrow sense heritability.

5.4.8.1 General inheritance

The general heritability of traits was judged by observing phenotypic expression of the available best and worst performers from experiment 2. Three intercrossed high and low performers of four generations were analysed for inheritance of selected traits (Table 5.18). For each trait, all high performers were also collectively compared with the group of all low performers.

As an example, the selection procedure is explained for seeds per floret of BC_1F_3 (Table 5.19). The plants of experiment-2 used in crossing for experiment-3 were arranged in descending order for seeds produced in a floret. A family of experiment-3 consisting of hybrids of high seeds producing plants of experiment-2 was assigned a status of “high” for seeds per floret in experiment-3. The three highest available families were selected.

Table 5.18. Details of the traits, families and generations involved in study of inheritance of traits. Families are represented by their respective code numbers.

Generation	Performance	Families of experiment-3 selected for different traits							
		Seeds / floret	Florets / head	Seeds / head	Heads / plant	Florets / plant	Seeds / plant	Peduncle length	Tap root diameter
BC_1F_3	High	8, 38, 79	8, 52, 65	8, 38, 79	27, 65, 86	8, 44, 75	8, 38, 65	65, 75, 80	
	Low	59, 62, 86	17, 62, 86	59, 62, 86	52, 59, 79	27, 59, 79	17, 52, 59	27, 44, 62	
BC_2F_2-IB	High	14, 29, 36	14, 29, 36	14, 29, 36	6, 36, 40	14, 29, 36	14, 29, 36	6, 21, 56	28
	Low	6, 28, 56	6, 28, 55	6, 28, 56	39, 57, 78	12, 28, 55	28, 56, 57	28, 55, 57	29
BC_2F_2-BI	High	20, 58, 70	20, 70, 72	20, 58, 70	30, 41, 51	41, 51, 58	41, 51, 58	20, 43, 72	
	Low	31, 54, 71	30, 43, 66	31, 54, 71	48, 49, 54	48, 49, 54	49, 54, 71	31, 54, 71	
BC_3F_1	High	37, 47, 68	9, 10, 68	9, 37, 47	15, 32, 67	32, 67, 68	33, 67, 68	9, 37, 47	9
	Low	10, 15, 61	15, 67, 69	10, 15, 69	9, 37, 47	9, 37, 69	15, 37, 69	10, 15, 69	15
All	High	8, 14, 20, 29, 36, 37, 38, 47, 58, 68, 70, 79	8, 9, 10, 14, 20, 29, 36, 36, 52, 65, 70, 72	8, 9, 14, 20, 29, 36, 37, 38, 47, 58, 70, 79	6, 15, 27, 30, 32, 36, 40, 41, 51, 65, 67, 86	8, 14, 29, 32, 36, 41, 44, 51, 58, 67, 68, 75	8, 14, 29, 33, 36, 38, 41, 51, 58, 65, 67, 68	6, 9, 20, 21, 37, 43, 47, 56, 65, 72, 75, 80	9, 28
	Low	6, 10, 15, 28, 31, 54, 56, 59, 61, 62, 71, 86	6, 15, 17, 28, 30, 43, 55, 62, 66, 67, 69, 86	6, 10, 15, 28, 31, 54, 56, 59, 62, 69, 71, 86	9, 37, 39, 47, 48, 49, 52, 54, 57, 59, 78, 79	9, 12, 27, 37, 39, 47, 48, 49, 54, 59, 69, 79	15, 17, 28, 37, 49, 52, 54, 56, 57, 59, 69, 71	10, 15, 27, 28, 31, 44, 54, 55, 57, 62, 69, 71	15, 29

Similarly, a family of experiment-3 consisting of hybrids of low seed producing plants from experiment-2 was assigned a status of “low” for seeds per floret in experiment-3. The three lowest families on-hand were selected.

Table 5.19. Selection procedure for high and low performers for seeds per floret trait of BC_1F_3.

EXPERIMENT-2	
Seeds/floret	Plant's identity
3.89	4.4.1
2.49	19.2.2
2.43	82.4.2
2.43	41.5.1
2.38	64.5.2
2.38	12.2.1
2.26	40.3.1
2.24	56.5.1
2.20	36.3.1
2.19	7.2.1
2.09	18.3.3
2.04	46.3.3
1.98	46.1.3
1.85	66.2.3
1.76	4.4.3
1.76	15.4.1
1.66	33.4.3
1.56	11.2.2
1.54	69.3.1
1.41	4.4.2
1.35	65.1.1
1.29	5.4.2
1.24	63.5.3
1.24	44.1.2
1.20	21.1.1
1.18	41.5.3
1.16	17.2.3
1.15	73.2.1
1.07	4.5.3
1.07	25.2.1
0.96	79.3.1
0.80	72.4.2
0.73	72.3.1
0.71	57.5.2
0.63	57.1.3
0.60	19.3.3
0.59	26.5.1
0.54	27.5.1
0.44	68.5.2
0.34	42.5.2
0.22	73.4.1
0.07	12.1.1
0.05	65.4.1
0.04	10.5.2

EXPERIMENT-3			
Female	Male	Family's identity	Selected as
19.2.2	12.2.1	8	High
7.2.1	18.3.3	38	High
4.4.1	18.3.3	79	High
11.2.2	33.4.3	52	
11.2.2	26.4.2	80	
12.2.1	19.3.3	17	
12.2.1	21.1.1	75	
19.2.2	4.4.2	27	
19.2.2	27.5.1	44	
33.4.3	26.4.2	50	
21.1.1	18.3.3	65	
11.2.2	17.2.3	59	Low
25.2.1	15.4.1	62	Low
21.1.1	27.5.1	86	Low

The results showed that 79% of the families maintained their status from experiment-2 to experiment 3 (Table 5.20). High performers remained high and low performers appeared to be low. Derived traits appeared to be less changed as compared to directly measured traits, particularly florets per plant. Backcrossed generations (BC_2F_2-IB and BC_3F_1) mostly showed unpredicted inheritance of derived traits, whereas BC_1F_3 generally showed abnormal inheritance for basic traits i.e., seeds per floret, seeds per head and peduncle length.

Table 5.20. ANOVA results of inheritance of seed production traits (comparisons of ‘high’ and ‘low’ families). Different generations are also compared for inheritance of each of these traits. The highlighted cells show significant difference ($P \leq 0.05$).

Traits	Generations	Observations	DF	Mean Square	F Value	Pr > F
Seeds / floret	**ALL**	309	1	6.03	66.6	<.0001
	BC_1F_3	70	1	0.01	0.11	0.7387
	BC_2F_2-IB	76	1	5.36	85.57	<.0001
	BC_2F_2-BI	75	1	2.47	28.56	<.0001
	BC_3F_1	88	1	0.50	6.35	0.0136
Florets / head	**ALL**	317	1	260.23	136.03	<.0001
	BC_1F_3	62	1	25.28	20.49	<.0001
	BC_2F_2-IB	76	1	166.73	122.52	<.0001
	BC_2F_2-BI	90	1	75.06	57.99	<.0001
	BC_3F_1	89	1	39.93	14.62	0.0002
Seeds / head	**ALL**	310	1	223.26	98.08	<.0001
	BC_1F_3	70	1	0.75	0.39	0.5354
	BC_2F_2-IB	76	1	199.55	103.88	<.0001
	BC_2F_2-BI	75	1	94.95	45.69	<.0001
	BC_3F_1	89	1	24.86	12.27	0.0007
Heads / plant	**ALL**	296	1	319.85	27.94	<.0001
	BC_1F_3	63	1	106.67	8.06	0.0061
	BC_2F_2-IB	64	1	7.64	1.07	0.3048
	BC_2F_2-BI	81	1	173.44	13.43	0.0004
	BC_3F_1	88	1	105.92	12.19	0.0008
Florets / plant	**ALL**	331	1	15602.92	16.04	<.0001
	BC_1F_3	83	1	468.65	0.46	0.4991
	BC_2F_2-IB	79	1	1346.16	1.28	0.2641
	BC_2F_2-BI	81	1	12694.17	24.48	<.0001
	BC_3F_1	88	1	4052.22	3.46	0.0661
Seeds / plant	**ALL**	306	1	475.39	61.41	<.0001
	BC_1F_3	75	1	138.77	21.57	<.0001
	BC_2F_2-IB	70	1	128.71	14.03	0.0004
	BC_2F_2-BI	81	1	227.88	39.22	<.0001
	BC_3F_1	80	1	19.61	2.4	0.1253
Peduncle length	**ALL**	313	1	92295.73	30.16	<.0001
	BC_1F_3	72	1	7565.09	2.84	0.0962
	BC_2F_2-IB	77	1	49421.95	20.55	<.0001
	BC_2F_2-BI	75	1	21784.80	12.05	0.0009
	BC_3F_1	89	1	18142.08	6.11	0.0154

5.4.8.2 Narrow sense heritability

The comparisons between full-sib and half-sib families were used to estimate narrow sense heritabilities (Fehr, 1987). Progenies of a single female crossed with more than one male were used as half-sibs (Table 5.21). Each single cross produced full-sib progenies to be used in this study. Progenies belonged to different hybrid generations. However, during selection of the families, preference was given to:

- Combinations with maximum numbers of the required observations.
- BC_2F_2 generation as it was an intermediate hybrid generation.
- Heterogeneous hybrids.

Table 5.21. Details of families used in heritability studies. There were 10 different comparisons involved. Each comparison had common female parent crossed with more than one male to produce half-sibs. For each cross there were on average 13.9 full-sibs (n). There were 10 females (N) and, on average, 2.3 males per female (M).

Comparisons	Family code	Female (common parent)	Male	Generation
1	52	S_{10} x S_{12}	A_6 x A_{10}	BC_1F_3
	59	S_{10} x S_{12}	S_1 x S_{10}	BC_1F_3
2	17	S_{10} x S_1	B_2 x B_3	BC_1F_3
	75	S_{10} x S_1	B_2 x B_8	BC_1F_3
3	8	B_2 x B_3	S_{10} x S_1	BC_1F_3
	27	B_2 x B_3	A_{11} x A_6	BC_1F_3
4	23	CH_5 x Cu_2	CH_5 x Cu_2	BC_2F_2-BI
	41	CH_5 x Cu_2	DU_{45} x A_1	BC_2F_2-BI
5	7	AR_{28} x Cu_2	AR_{28} x Cu_2	BC_2F_2-BI
	72	AR_{28} x Cu_2	CH_4 x A_1	BC_2F_2-BI
6	30	DU_{44} x A_{11}	DU_{57} x A_1	BC_2F_2-BI
	43	DU_{44} x A_{11}	LA_3 x Cu_4	BC_2F_2-BI
	66	DU_{44} x A_{11}	DU_{44} x A_6	BC_2F_2-BI
7	71	LA_3 x Tf_3	LA_5 x Tf_1	BC_2F_2-BI
	74	LA_3 x Tf_3	DU_{21} x A_{10}	BC_2F_2-BI
8	36	AR_{802}	B_2 x B_3	BC_2F_2-IB
	60	AR_{802}	S_{10} x S_{12}	BC_2F_2-IB
	61	AR_{802}	LA_3 x Tf_3	BC_3F_1
9	16	S_{14}	S_{10}	BC_1F_2
	26	S_{14}	S_{14}	BC_1F_2
10	39	$WILL_2$	A_{11} x A_6	BC_2F_2-IB
	55	$WILL_2$	A_6 x A_1	BC_2F_2-IB
	56	$WILL_2$	S_{10} x S_1	BC_2F_2-IB

ANOVA was done by using the GLM procedure to accommodate unbalanced data and missing values. Mean squares were used to partition the variation into components due to replication, female, males (female) and residual (Table 5.22). Phenotypic and additive genetic variances were derived and used to calculate narrow sense heritability.

Table 5.22. ANOVA design and estimation of phenotypic variation (σ^2_P), additive genetic variation (σ^2_A) and narrow sense heritability (h^2).

<table>
<tr><th>Source</th><th>DF</th><th>SS</th><th>Mean Square</th><th>Expected mean square</th><th>σ^2_p</th><th>σ^2_A</th><th>h^2</th></tr>
<tr><td>Replication</td><td>r-1</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
<tr><td>female</td><td>N-1 = df_f</td><td>SS_f</td><td>SS_f / df_f = MS_f</td><td>$\sigma^2_e + n\sigma^2_m + Mn\sigma^2_f$</td><td rowspan="3">$\sigma^2_e + \sigma^2_m + \sigma^2_f$</td><td rowspan="3">$\sigma^2_m$ x 4</td><td rowspan="3">σ^2_A / σ^2_p</td></tr>
<tr><td>Males (female)</td><td>N(M-1) = df_m</td><td>SS_m</td><td>SS_m / df_m = MS_m</td><td>$\sigma^2_e + n\sigma^2_m$</td></tr>
<tr><td>Error</td><td>MNn - MN = df_e</td><td>SS_e</td><td>SS_e / df_e = MS_e</td><td>σ^2_e</td></tr>
<tr><td>Total</td><td>MNn-1 = df_t</td><td></td><td></td><td></td><td></td><td></td><td></td></tr>
</table>

r, N, M and n represents number of replications, females, males/female and progenies per male, respectively.

σ^2_e, σ^2_m and σ^2_f represent variance component of error, male (female) and female parent, respectively.

The results showed a wide range of narrow sense heritabilities among different traits (Table 5.23). High heritability (0.93) was estimated for florets per head (florets per head). Number of heads per plant expressed moderate high inheritance. Both seeds per floret and seeds per head showed moderate heritability estimates. The derived traits of florets per plant and seeds per plant showed low moderate heritabilities. For foliage production and peduncle length, heritabilities appeared to be low in these hybrids.

The comparison between narrow sense heritabilities (Table 5.23) and heritability estimates calculated with the ‘high’/’low’ comparisons (Table 5.20) shows that florets per head expressed consistent selection effect, while florets per plant was mostly unresponsive to selection.

Table 5.23. Results of ANOVA and estimation of phenotypic variation (σ^2_p), additive genetic variation (σ^2_A) and narrow sense heritability (h^2).

Trait	Source	DF	SS	Mean Square	σ^2	σ^2_p	σ^2_A	h^2
Seeds/floret	**Replication**	2	3.34	1.67				
	Male	9	5.35	0.59	0.01	0.09	0.03	0.35
	Female (male)	13	4.23	0.33	0.02			
	Error	294	19.97	0.07	0.07			
Florets/head (head size)	**Replication**	2	14.54	7.27				
	Male	9	283.91	31.55	0.63	2.71	2.52	0.93
	Female (male)	13	149.03	11.46	0.73			
	Error	294	397.19	1.35	1.35			
Seeds/head	**Replication**	2	83.53	41.77				
	Male	9	169.47	18.83	0.25	2.50	1.01	0.40
	Female (male)	13	140.05	10.77	0.66			
	Error	294	466.24	1.59	1.59			
Peduncle length	**Replication**	2	61666.71	30833.36				
	Male	9	205778.98	22864.33	98.95	3098.00	395.79	0.13
	Female (male)	13	256202.70	19707.90	1298.32			
	Error	294	500013.68	1700.73	1700.73			
Heads/plant	**Replication**	2	30.47	15.24				
	Male	9	1029.47	114.39	2.10	15.32	8.39	0.55
	Female (male)	13	617.25	47.48	2.66			
	Error	292	3083.23	10.56	10.56			
Florets/plant	**Replication**	2	1758.86	879.43				
	Male	9	52886.80	5876.31	81.72	1122.21	326.87	0.29
	Female (male)	13	42503.30	3269.48	173.20			
	Error	292	253249.27	867.29	867.29			
Seeds/plant	**Replication**	2	136.94	68.47				
	Male	9	426.71	47.41	0.69	8.82	2.74	0.31
	Female (male)	13	332.17	25.55	1.35			
	Error	292	1978.97	6.78	6.78			
Foliage dry weight	**Replication**	2	107.24	53.62				
	Male	9	913.71	101.52	0.45	17.79	1.78	0.10
	Female (male)	13	1134.87	87.30	5.44			
	Error	292	3477.86	11.91	11.91			

5.4.8.3 Inheritance of flowering pattern

Flowering pattern is a very important factor influencing availability of mature heads for harvesting. Flower emergence, maturity and inheritance of flowering pattern were assessed for each family.

a. **Flower emergence:** Flower heads produced by each plant were counted five times at an interval of about ten days between the two consecutive counts. All the heads showing white petal color were counted each time. Figure 5.20 shows average head emergence in five different counts for each family. A distinct difference was evident in flowering pattern of *T. uniflorum* and white clover. *T. uniflorum* showed the earliest flower emergence among all families. The rate of flower emergence was relatively smooth. In contrast, cultivars of white clover started flower emergence much later than *T. uniflorum*. They showed a distinct flowering peak in the last week of December. Their flower production before this time was much lower than *T. uniflorum*. The hybrids showed wide variation in flower emergence. Many families showed the early flowering trend of *T. uniflorum*, however, none of them produced flowers earlier than *T. uniflorum*. The hybrids also showed wide variation in production of flower heads seen up to the last week of December coinciding with the longest day. In general, the early flowering families produced more heads, compared to late flowering families. However, as the head counts were not taken beyond the last week of December, it is uncertain which families had maintained their low flowering or shown peak flowering after that time.

b. **Flower maturity:** Plants were visually scored for head maturity status (indicated by brown coloration) at two different times, 11th of February, when heads sample were taken for per head data measurement and 17th of March before taking the second foliage harvest. The results showed that most of the families (55%) had most or all of their heads in a mature state during the first survey (Figure 5.21). Even the rest of the families at that time had at least 50% mature heads.

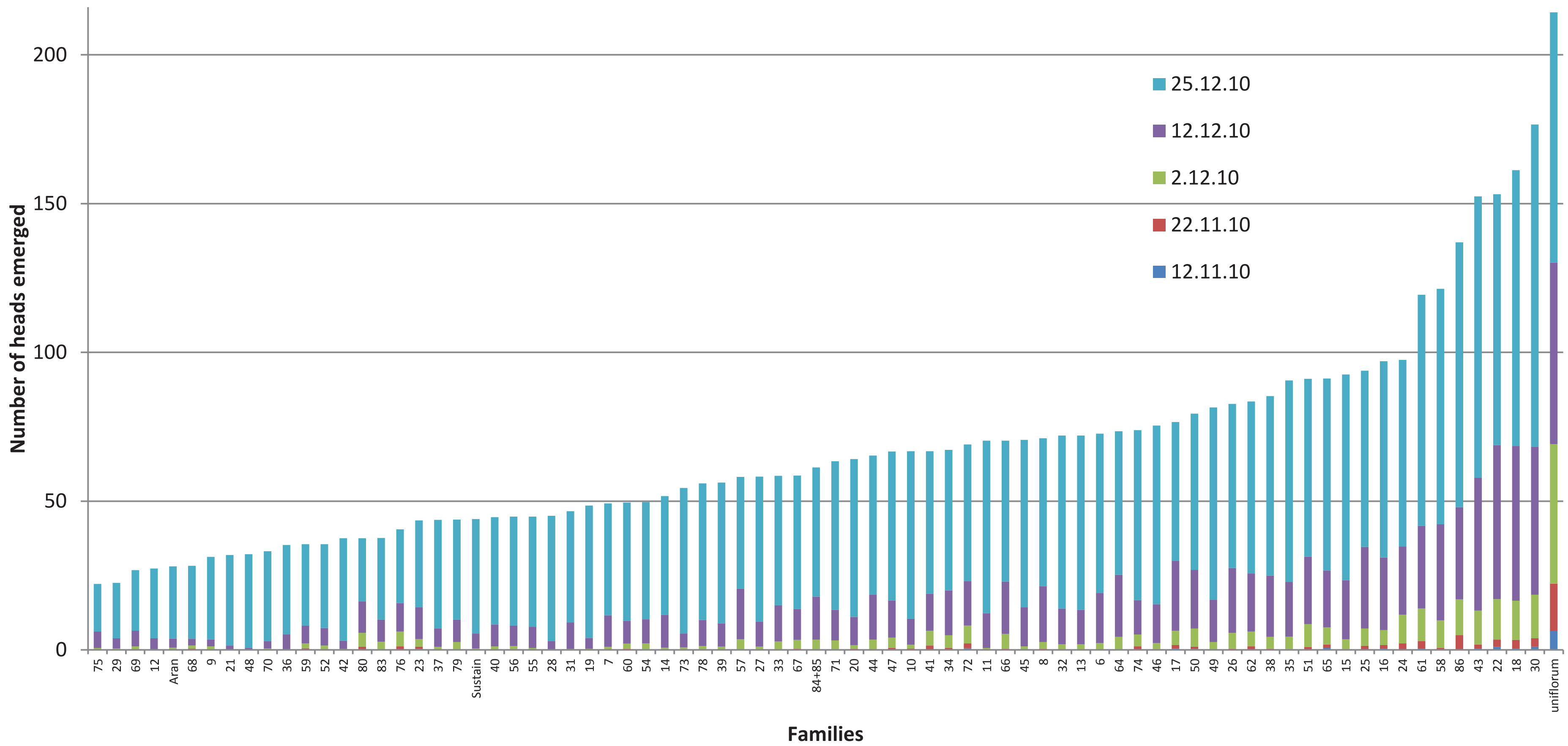


Figure 5.20. Average head emergence (number of heads showing white petals) for each family at different counting dates.

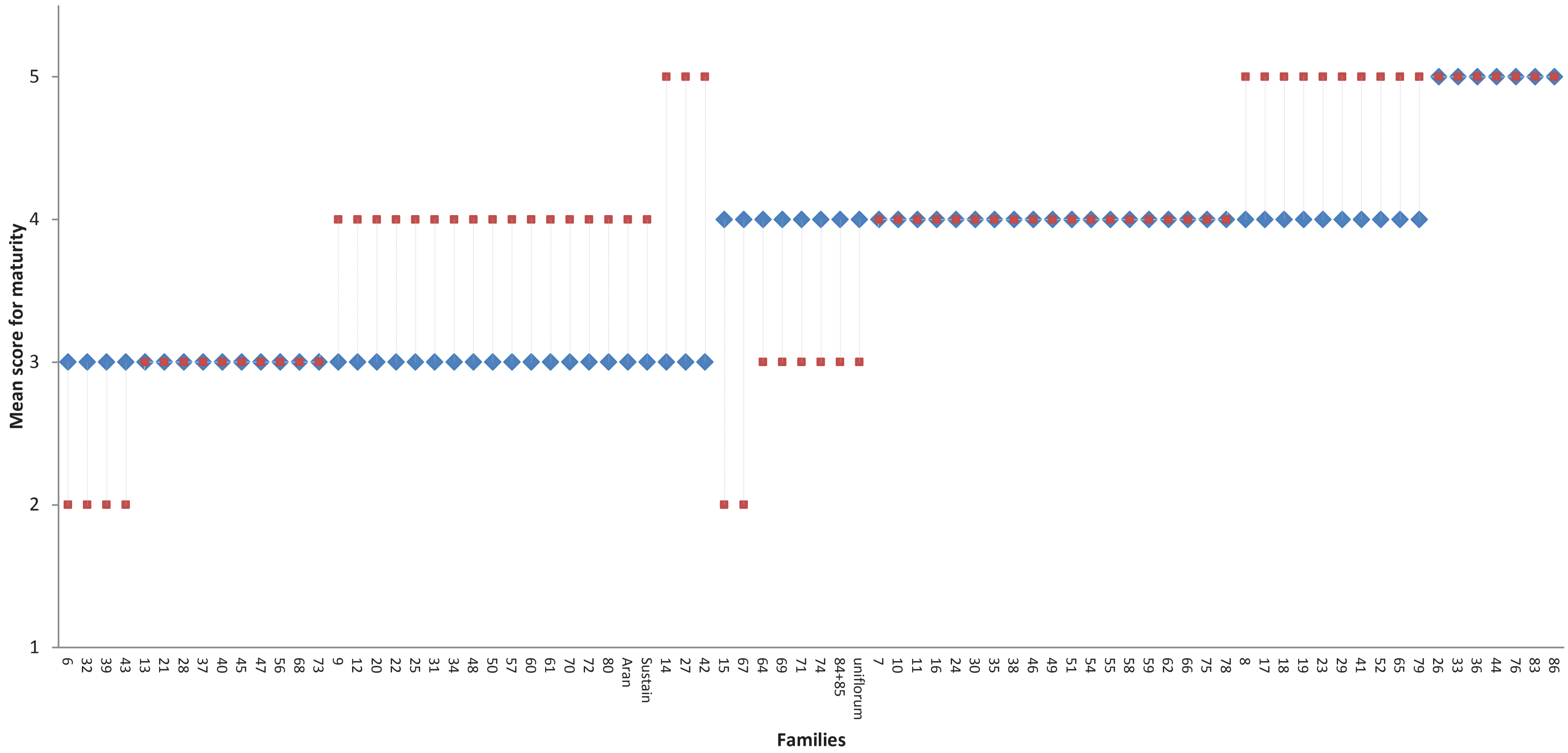


Figure 5.21. Mean family scores for maturity status at two different dates (11.2.11 ◆ and 17.3.11 ■). 1 = no head was brown, 2 = <50% heads were brown, 3 = 50% heads were brown, 4 = most heads were brown, 5 = all heads were brown.

All families were inclined towards maturity as no family had less than 50% brown heads during the second week of February. The nature of maturity patterns divided families into three categories. The first category maintained their maturity status in both counts and it consisted of majority (46%) of the families. Its one group including families 13, 21, 28, 37, 40, 45, 47, 56, 68 and 73 showed sustained flower production. The other group represented by the majority of the families having consistent status in both observation expressed existence of mostly all brown heads during both scoring dates. This could be due to reduction in flowering of the plants. The third group had mature heads during both counts. This might be due to their ability to keep ripened heads intact accompanied with unmeasured decrease in head number. The second prominent category consisting of 38% of the families showed a decline in flowering and ripening of the flower heads. The third category, comprising of 15% of the families, showed a second flush of flowering during the last count. This might be due to the intermittent flowering nature of the genotypes or to stimulation by rain during February. No pedigree effect could be found on flowering pattern.

c. **Inheritance:** Inheritance of flowering pattern was studied by comparing the following trends of selected progenies with their respective parents. Families involved in experiment-2 were sorted on the basis of flowering pattern. They were categorized as early, medium and late flowering on the basis of the start of flowering. Progenies of intercrossing of representative early, medium and late flowering families were analysed to observe inheritance of flowering pattern (Table 5.24 and appendix 2). Average head production at different dates was used to arrange the data for analysis.

Table 5.24. Families involved in study of inheritance of flowering pattern.

	START OF FLOWERING					
	Early		Medium		Late	
	Family	Generation	Family	Generation	Family	Generation
FAMILIES	8	BC_1F_3	50	BC_1F_3	20	BC_2F_2-BI
	30	BC_2F_2-BI	52	BC_1F_3	48	BC_2F_2-BI
	38	BC_1F_3	62	BC_1F_3		
	41	BC_2F_2-BI	72	BC_2F_2-BI		
	43	BC_2F_2-BI	74	BC_2F_2-BI		
	51	BC_2F_2-BI				
	66	BC_1F_3				
	86	BC_1F_3				

Results showed that generally progenies of early, medium and late flowering families flowered early, medium and late, respectively (Figures 5.22). Decline in their flower production followed the same order. Families of all three categories started flower emergence during November. Both the early and medium flowering groups reached peak flowering during late December. Flowering began declining in early families after mid-December, whereas medium families maintained peak flowering until mid-February. The late category showed a smooth rise in flowering from mid-December and peaked during mid-February. Although data was not recorded after this date, the late families probably continued to flower following the same pattern. Only two families, i.e. 66 of early category and 72 of medium category, expressed a deviation from the general trend of their categories. The general comparison of three categories showed that families had maintained their flowering pattern in the next generation (Figure 5.23A).The families of generation BC_2F_2-BI showed retention of the flowering pattern of their parents (Figures 5.23B), whereas a family representing the medium category of BC_1F_3 did not maintain its status during the last count and showed a decline in flowering similar that of the of early family (Figures 5.23C).

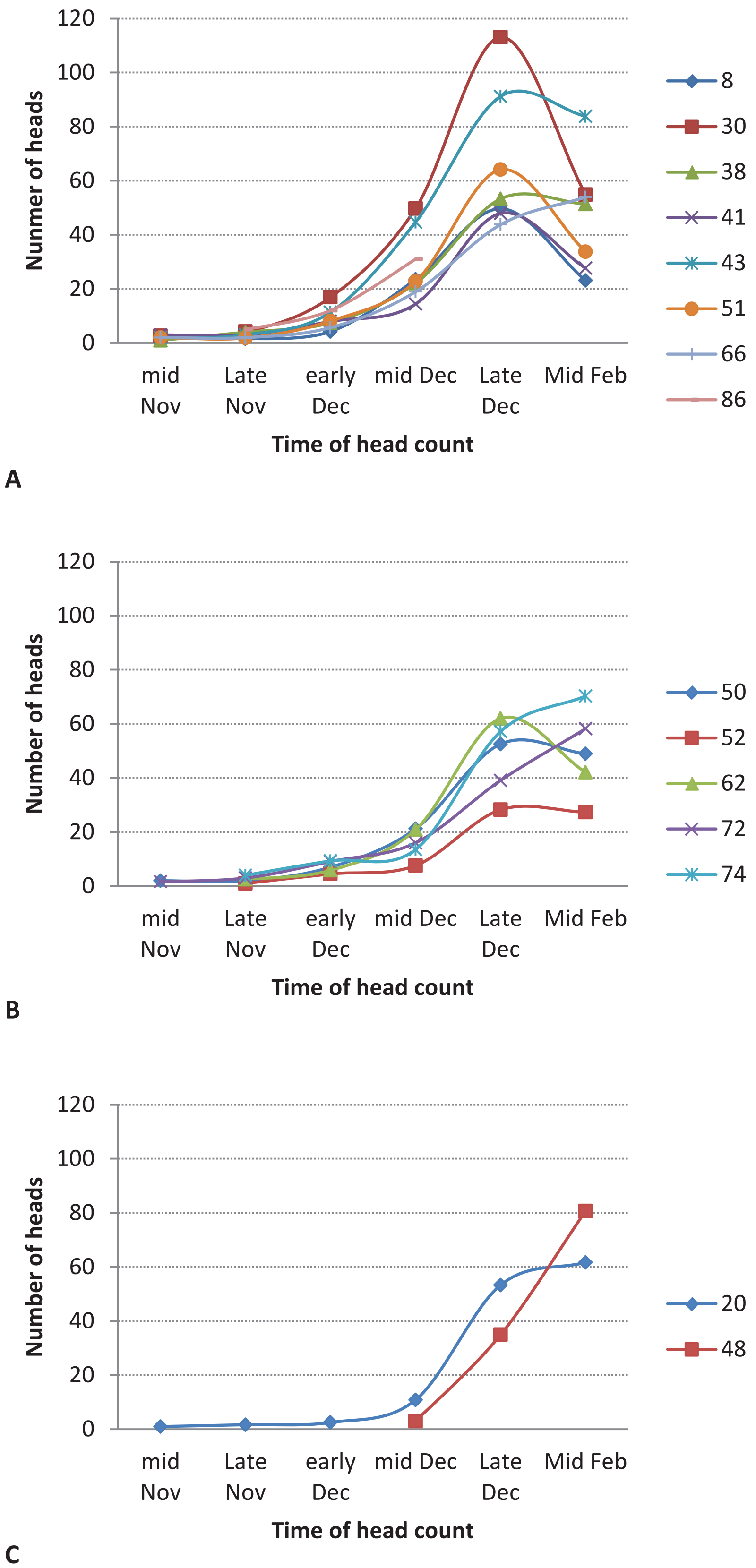


Figure 5.22. Flowering trends of early (A), medium (B) and late (C) families. Their early medium and late flowering status was decided on the basis of flowering trends of their parents in experiment-2.

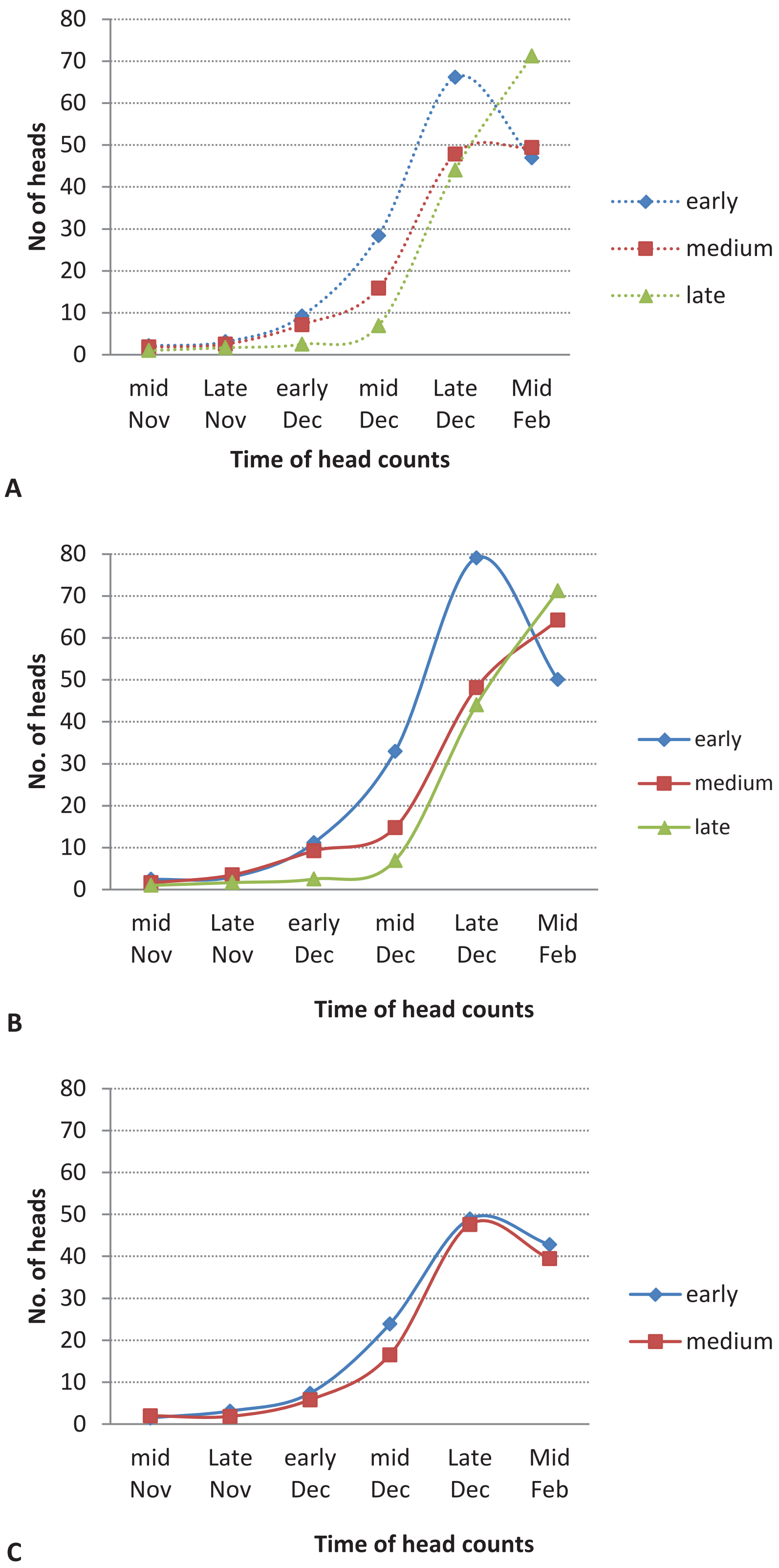


Figure 5.23. Average flowering trends of all (A), BC_2F_2-BI (B) and BC_1F_3 (C) families. Their early medium and late flowering status was decided on the basis of flowering trends of their parents in experiment-2.

5.4.9 Effects of different mating designs

5.4.9.1 General comparison

Eight generations i.e., parents (*T. uniflorum* and white clover), BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_2-IB, BC_2F_2-BI, BC_2F_1 and BC_3F_1 were compared for all traits. Each generation differed in the range of white clover cultivars used; this may have affected the comparison among generations. To investigate this issue, the following four groups of generations (Table 5.25) were selected:

1. Group A containing generations based only on A families.
2. Group AS containing generations based only on A and S families.
3. Group ASB containing generations based only on A, S and B families.
4. Group ALL containing generations based on all used families i.e., A, S, B, C, Cu and Tf.

Table 5.25. Family composition of generation groups. The groups having families presented in bold numbers had already been included in a previous group (group ALL for BC_1F_1, BC_1F_2 and white clover) or not had any of the A, S and B family involved (group *T. uniflorum* and group ALL of BC_2F_1). These groups were not considered in the comparative study of family effects on group performance.

Groups	**A**	**A,**	**A, S, B**	**ALL**
	Families			
Generations				
BC_1F_1	83	83,		**83, 84, 85**
BC_1F_2		16	16, 22	**16, 22**
BC_1F_3	38,	38,	8, 17,	8, 17, 27, 38, 44, 50, 52, 59, 62, 65,75, 79, 80, 86
BC_2F_1				**25, 34**
BC_2F_2-BI	30,			20, 30, 31, 41, 43, 48, 49, 51, 54, 58, 66, 70, 71, 72, 74
BC_2F_2-IB	39,	21,	21, 28,	6, 11, 12, 14, 21, 28, 29, 36, 39, 40, 45, 55, 56, 57, 60, 78
BC_3F_1	15,	9,	9, 15,	9, 10, 13, 15, 32, 33, 35, 37, 42, 46, 47, 61, 67, 68, 69, 73
white clover	77	53,		**53, 77**
T. uniflorum	**63, 81, 82**			

1. Group A had the following exceptional features:
    a. Recurrent parents involved in backcrossing were W, D and L.
    b. BC_1F_3 had hybrids of both A and S.
2. Group AS had following exceptional features:

a. Recurrent parents involved in backcrossing were W, D and L.

b. BC_1F_2 had only S.

c. BC_3F_1 were predominantly A.

3. Group ASB had the following exceptional features:

   a. Recurrent parents involved in backcrossing were W, D and L.

   b. BC_1F_2 had crosses of S and B. No cross of A was included.

   c. BC_3F_1 had predominantly crosses of A and no cross of B.

4. Group ALL had the following exceptional features:

   a. Recurrent parents involved in backcrossing were W, D, L, CH and AR.

   b. Consisted of different numbers of A, S, B, C, Cu and Tf families in different generations.

The data for the traits of each generation were compared for the above four groups having different families involved (excluding the groups indicated in bold letters in Table 5.25). As an example, Figure 5.24 shows such a generation comparison of mean numbers of heads per plant. There were no statistical differences among groups within any generation ($P > 0.05$).

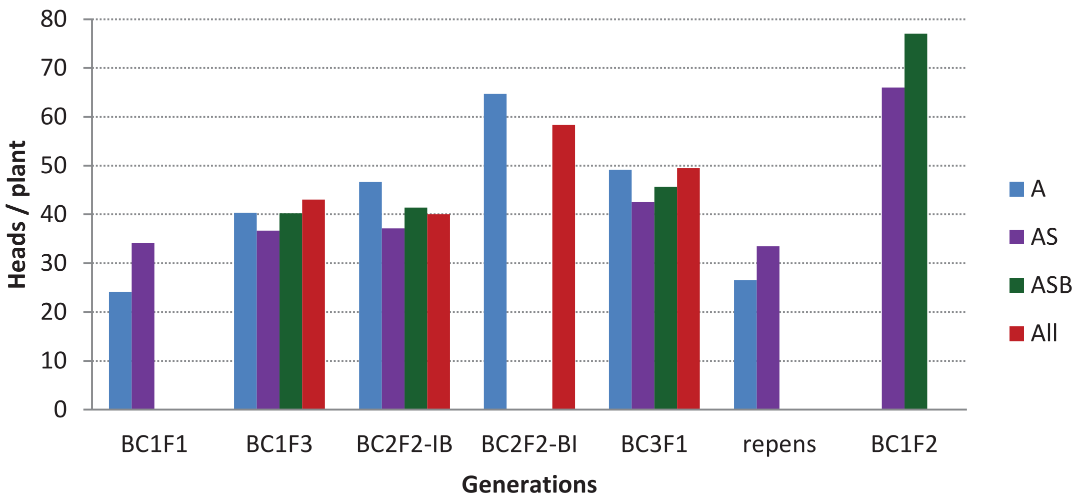


Figure 5.24. Comparison among four groups having different families' composition as indicated by their name. The group ALL contains families of A, S, B, C, Cu and Tf. The effect of different family composition was compared for heads per plant in seven different hybrid generations.

Possible comparisons (depending on availability of the data) among family groups of a generation were made for all traits. Eighty seven percent (98 out of 113) of the comparisons showed no statistical differences (Table 5.26). This showed that generation performance was not affected by the composition of families contributing to that generation. In other words, the generation effect was independent of the family effect.

Table 5.26. P-values of ANOVA for comparisons of groups having different family composition. The parental generation consisted of white clover cultivars. Shaded cells indicate presence of significant differences among groups (P ≤ 0.05).

GENERATION	BC_1F_1	BC_1F_2	BC_1F_3	BC_2F_2-BI	BC_2F_2-IB	BC_3F_1	Parent
GROUP	A, AS	AS,	A, AS,	A, ALL	A, AS,	A, AS,	A, AS
TRAIT							
Foliage dry weight	0.91	0.16	0.20	0.01	0.34	0.12	0.95
Florets/head	0.03	0.02	0.06	0.02	<.0001	0.18	0.06
Florets/plant	0.60	0.69	0.56	0.89	0.06	0.15	0.84
Heads/plant	0.36	0.71	0.64	0.45	0.07	0.33	0.51
Leaf area	0.10	.	0.67	0.13	0.68	0.00	0.10
Nodes/stolon	0.78	.	1.00	0.00	0.25	0.09	0.69
Ovules/ovary	0.87	.	0.39	.	.	0.37	0.18
Peduncle length	0.14	0.24	0.52	0.05	0.01	0.14	0.62
Pollen stainability	0.58	.	0.99	.	.	0.27	0.15
Root dry weight	0.90	.	0.89	0.36	1.00	0.84	0.77
Seeds/floret	0.31	0.56	0.36	0.07	<.0001	0.83	0.88
Seeds/head	0.12	0.14	0.24	0.02	<.0001	0.72	0.32
Seeds/plant	0.82	0.59	0.66	0.19	<.0001	0.31	0.86
1000 seeds weight	0.75	0.52	0.97	0.04	0.26	0.90	0.04
Selfing%	0.25	.	0.93	.	.	0.18	0.59
Stolon dry weight	0.65	.	0.71	0.01	0.55	0.47	0.29
Stolons/104 m^2	0.78	.	0.93	0.76	0.90	0.68	0.66
Taproot diameter	0.40	.	0.69	0.90	0.68	0.36	0.30
Woody tap root length	0.34	.	0.86	0.13	0.82	1.00	0.49

A simple comparison between the parents (white clover and *T. uniflorum*) for different important traits can be made by standardizing the data for these traits to make them presentable in a single graph. The following formula gave values of all traits around 1.

$$A = A_{org} \ / \ mean$$

where A = standardized value for parent A, A_{org} = original value of parent A, and mean = mean value of both parents

The parents clearly displayed contrasting expression for seed traits (Figure 5.25).

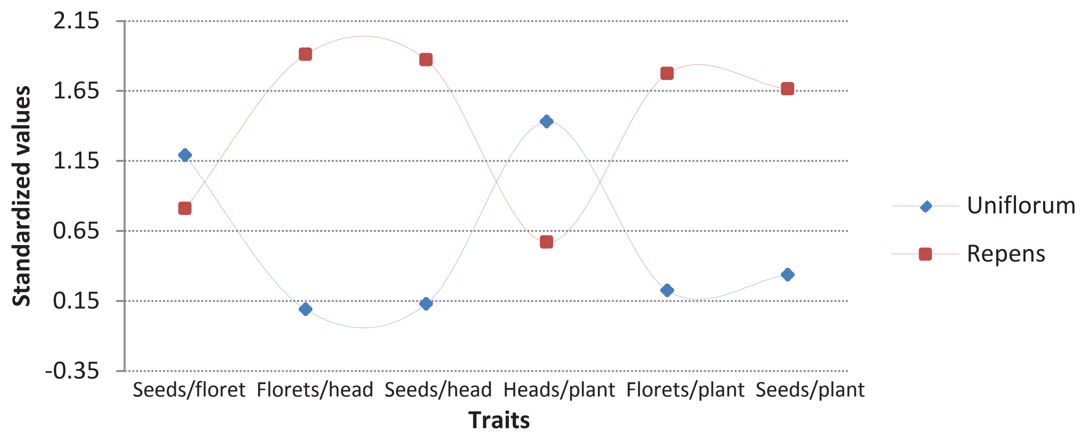


Figure 5.25. Comparison between white clover and *T. uniflorum* for seed production traits. Standardized values are used to make all traits comparable in one graph.

Figure 5.26 is a radar graph projection of the same standardized data (out of one) of the different seed traits for selected generations. This comparison among parent species and their third segregating (BC_1F_3) and backcrossed (BC_3F_1) generation showed that for most traits, hybrids were intermediate to their parents. One exception was that both hybrids were inferior to both parents for the seeds per floret trait. Another exception was the best performance of the BC_3F_1 generation for florets per plant. *T. uniflorum* was best for seeds per floret and heads per plant. The other parent white clover was the highest producer of florets per head, seeds per head and seeds per plant. Intercrossing (BC_1F_3) did not result in outstanding average performance for any trait.

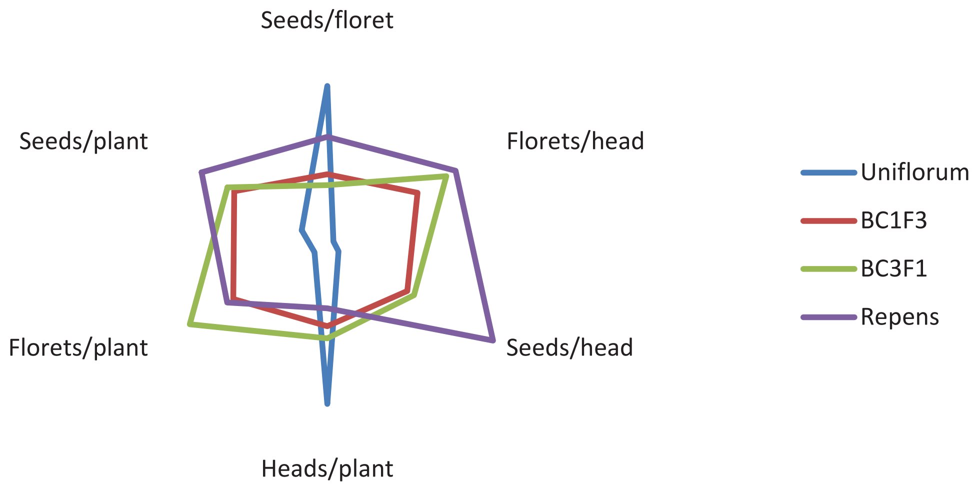


Figure 5.26. Comparison among Parents (white clover and *T. uniflorum*), BC_1F_3 and BC_3F_1.

Inclusion of all hybrid generations in this comparison showed that the hybrids were generally intermediate to the parent species for most seed traits (Figure 5.27). Exceptions were their inferior performance for seeds per floret and, except for BC_1F_1 and BC_1F_3, a superior floret production per plant, compared to the parents. Only the mean seed yield of BC_1F_2 plants was slightly higher in ranking than that of the high seed producing parent i.e., white clover.

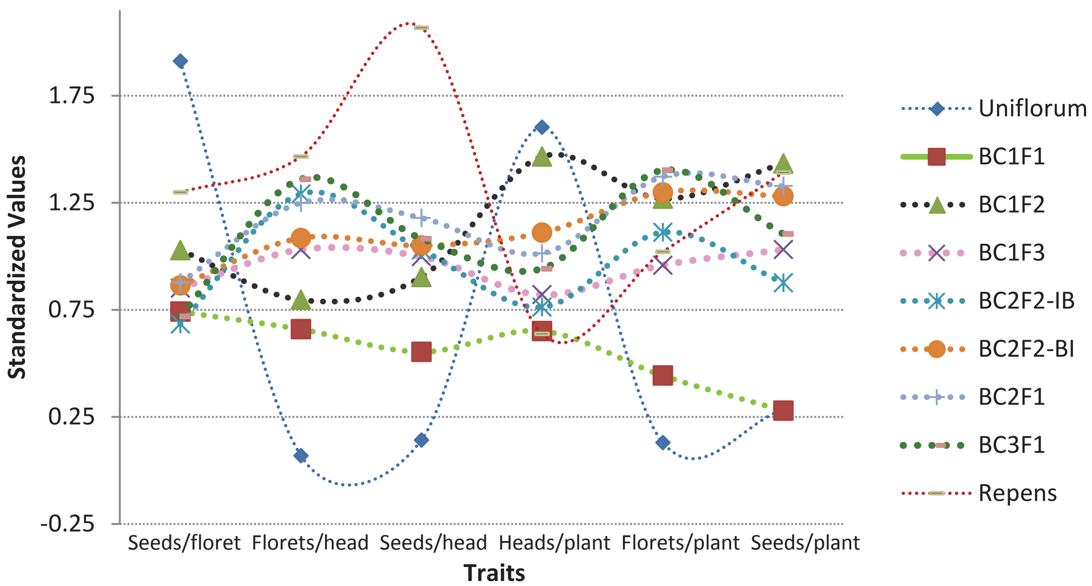


Figure 5.27. Comparison among white clover, *T. uniflorum* and their hybrid generations for seed production traits.

Transformed data were used for an ANOVA based on a General Linear Model (GLM). The differences among eight generations (BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_1, BC_2F_2-BI, BC_2F_2-IB, BC_3F_1 and white clover) were analysed for all traits. ANOVA results (Table 5.5) showed significant differences among generations for all traits except for tap root diameter. Because the transformed data did not include *T. uniflorum*, this species was unavailable for ANOVA and Tukey analysis.

A comparison of the coefficients of variation showed that the third backcrossed generation was less diverse than the third intercrossed generation in the phenotypic expression of seed traits (Figure 5.28). BC_1F_3 also appeared to be considerably more variable than white clover for peduncle length, seeds per floret, seeds per head, and seeds per plant. White clover genotypes were more variable than *T. uniflorum* plants for the majority of the traits.

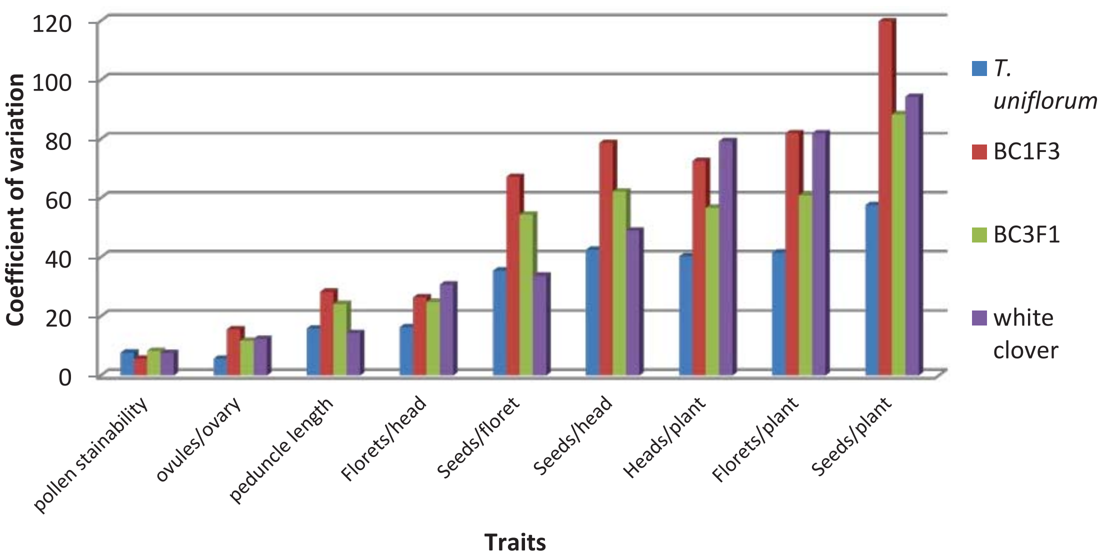


Figure 5.28. Comparison of coefficients of variation among different generations.

5.4.9.2 Cluster analysis

Cluster analysis was undertaken using SAS for those traits which had direct relevance to seed production and for which complete data were available for all generations. These traits were seeds per floret, florets per head, seeds per head, heads per plant, florets per plant, seeds per plant, peduncle length, 1000 seed weight and foliage dry weight. The dendrogram (Figure 5.29) showed *T. uniflorum* as the most isolated species. The BC_1F_1 was a link between *T. uniflorum* and white clover, while the later hybrid generations formed a distinct group with closer resemblance to white clover.

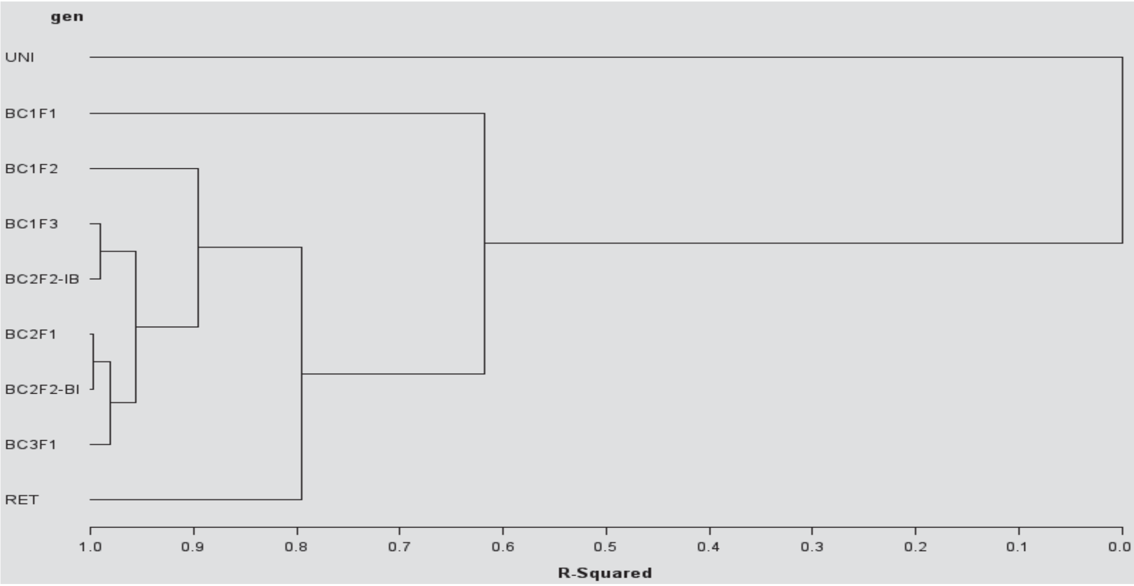


Figure 5.29. Dendrogram constructed by Ward's Minimum-Variance cluster analysis showing relationships among different generations. UNI= *T. uniflorum* RET = white clover

5.4.9.3 Box plot comparison

Box plots were built on the original data for the available generations of experiment-3. *T. uniflorum* showed the best seed production ability per floret, followed by white clover (Figure 5.30a). All of the hybrid generations showed inferior seed numbers per floret with the BC_2F_2-IB being the most inferior in this trait among all generations. However, each generation displayed a considerable range of variation. For example, the BC_2F_2-IB generation had some plants producing more seeds per floret than the average of the top performer, *T. uniflorum*.

The florets per head trait aligned hybrids between their parents, with *T. uniflorum* at the lowest and white clover at the highest end (Figure 5.30b). Both intercrossing and backcrossing of hybrids improved this trait. The BC_3F_1 displayed almost similar florets per head to that of white clover. Results showed that *T. uniflorum* was the worst and white clover was the best seed producer per head (Figure 5.30c). None of the hybrid generations produced average yields per head comparable to white clover. However, the upper range of most of the hybrid generations was higher than the average for white clover.

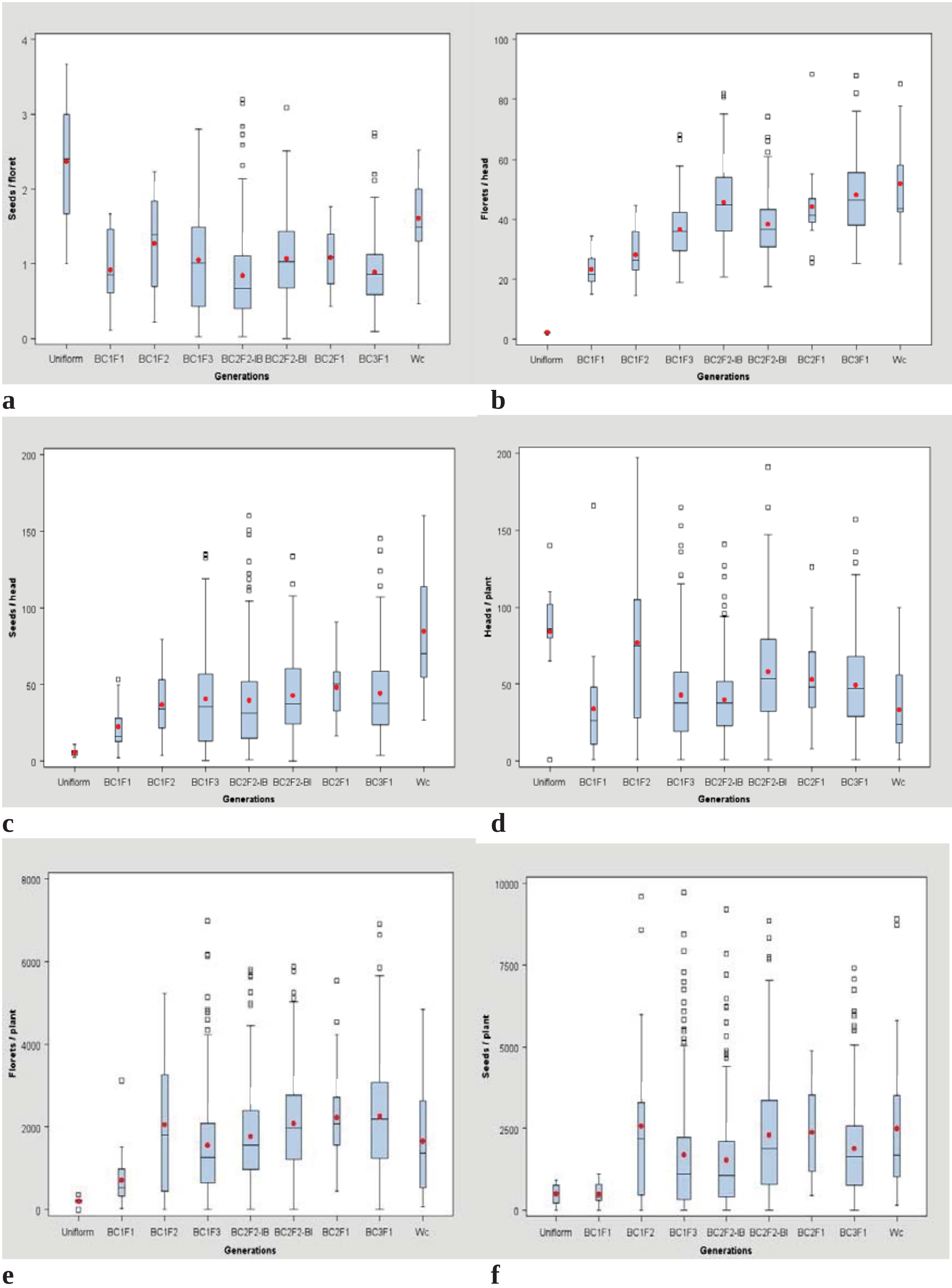


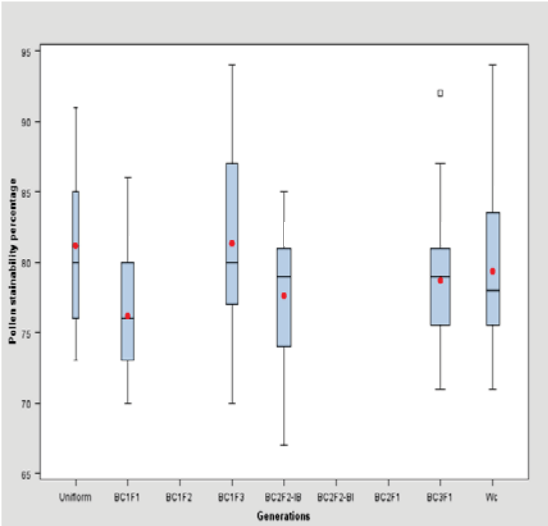
Figure 5.30. Comparison of families of generations for seeds/florets (a), florets/heads (b), seeds/head (c), heads/plant (d), florets/plant (e) and seeds/plant (f). The width of each box shows the number of observations. The upper and lower edges of the box represent first and third quartiles, respectively. The horizontal line drawn within the box marks the median. The red dot shows the sample mean. The whiskers are drawn to the most extreme points that lie within the fences. The upper fence is defined as the third quartile plus 1.5 times the interquartile range and the lower fence is the first quartile minus 1.5 times the interquartile range. Observations outside the fences (outliers) are marked as squares. Different letters at the top of bars represent significant differences at P≤ 0.05.

Number of seeds per head were approximately inversely proportional to heads per plant in parental species (Figure 5.30 c and d). *T. uniflorum* had the highest and white clover the lowest head numbers. The average head production of BC_1F_2 families was highest among the hybrids and was near to the average of *T. uniflorum* (Figure 5.30d). Except BC_1F_1, BC_1F_3 and BC_2F_2-IB, all hybrid generations exhibited better per plant head production ability than white clover. *T. uniflorum* had the fewest florets per plant and the BC_1F_1 was the lowest among the hybrid generations in this respect (Figure 5.30e). Both intercrossing and backcrossing improved this trait, with the BC_3F_1 and BC_2F_1 appearing better than white clover in having high average numbers of florets per plant. Specially backcrossed generations were significantly superior to white clover in this respect.

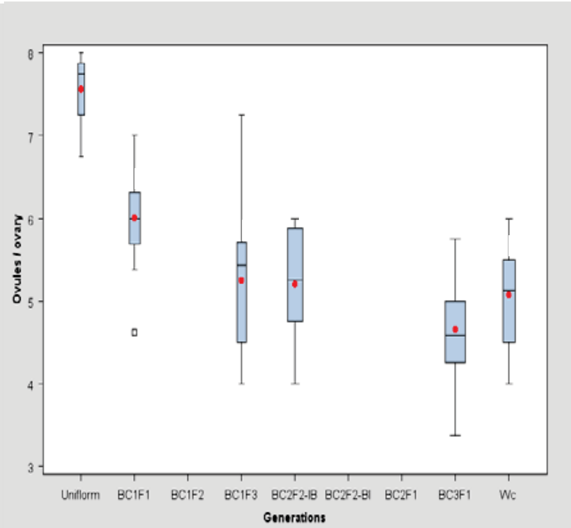
T. uniflorum and the BC_1F_1 were the lowest seed producers (Figure 5.30f). Although white clover had generally good seed production ability, the BC_1F_2, BC_2F_2-BI and BC_3F_1 generations produced similar seed numbers. Backcrossing or intercrossing beyond second generation did not increase seed numbers per plant. The upper limit of all hybrids consisted of plants with exceptionally high seed production.

Both, BC_1F_3 and BC_3F_1 generation showed pollen stainability percentages similar to those in white clover (Figure 5.31a). The BC_1F_1 and BC_2F_2-IB hybrids were significantly lower than BC_1F_3 in this respect. White clover and BC_1F_3 showed higher diversity for this trait than other generations. *T. uniflorum* contained the most ovules per ovary (Figure 5.31b), followed by the BC_1F_1 hybrids. Advancement in intercrossing caused reduction in ovule production and increase in variability for this trait. White clover was second last among all generations for this trait. Three generations of backcrossing had significantly decreased ovule production potential so that it was similar to, or slightly below, white clover.

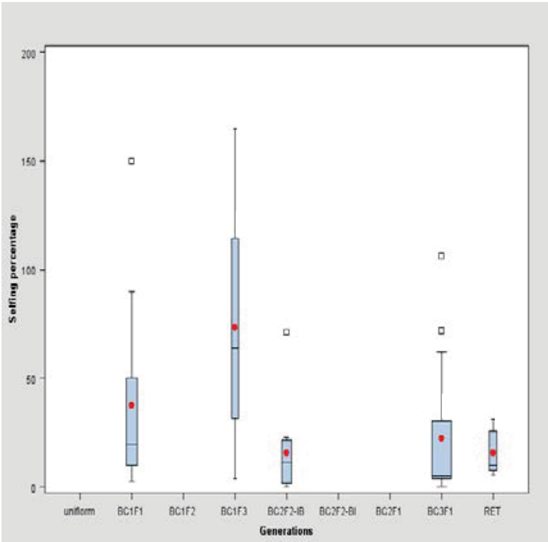
Study of a limited number of generations revealed higher mean selfing rates in BC_1F_3 than in all other generations except BC_1F_1 (Figure 5.31c). Backcrossing with white clover as the recurrent parent reduced number of seeds produced in a floret as a result of selfing. However, both the third intercrossed and backcrossed generation had much wider ranges compared to white clover. *T. uniflorum* parent had the heaviest seeds (Figure 5.31d). White clover produced the lightest seeds. Backcrossing improved seed weight more than intercrossing. All hybrid generations, except second backcrossed and intercrossed, had a higher range for this trait.



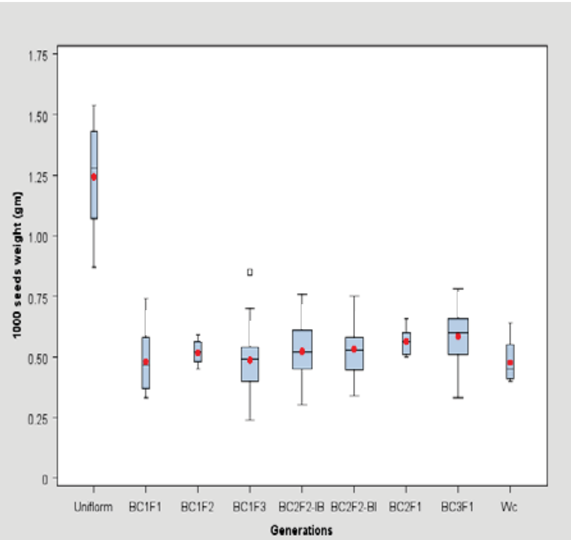
a



b



c



d

Figure 5.31. Comparison of generations for pollen stainability (a), ovules/ovary (b), selfing percentage (c), seed weight (d). Rest of the details of plots are same as described for Figure 5.30.

White clover produced the tallest peduncles (Figure 5.32a). The peduncle of *T. uniflorum* remained hidden under the stipule and was much shorter than the pedicel. As the pedicel was the mean to uphold the head, its length was used to compare with peduncle lengths of other generations. Not surprisingly, the *T. uniflorum* pedicels were very short as compared to the peduncles of all other generations. The BC_1F_1 was intermediate and peduncle lengths responded positively to the selection with advancement in generation by both intercrossing and backcrossing. The response rate was higher in backcrossing than in intercrossing. However, only the third generation of backcrossing restored the mean white clover peduncle length. Leaf area, as indicated by the area of the middle lobe of the leaf, also aligned *T. uniflorum* and white clover at the lowest and the highest ends, respectively (Figure 5.32b) and was improved through backcrossing and intercrossing. Only the third backcrossed generation restored the leaf area to that of white clover. Although the BC_2F_2-BI had significantly smaller leaves than white clover, it had some plants having high leaf area comparable with the larger leaves of white clover. The leaf area of the BC_1F_3 was significantly smaller than white clover and the BC_3F_1.

Foliage production was minimum for *T. uniflorum* and maximum in white clover, while intermediate in (Figure 5.32c). The BC_1F_1 generation was inferior most among hybrid generations in this respect. Both intercrossing and backcrossing facilitated progressive improvement. The range of diversity within generations appeared to be linked with sample size. Plant growth expressed by area of ground cover showed marked differences between the tiny plants of *T. uniflorum* and the well spread growth of white clover (Figure 5.32d). All hybrids recovered this trait efficiently. However, BC_1F_1 and BC_1F_2 showed significantly lower growth compared to the other hybrids and white clover. All other hybrids expressed similar growth to that of white clover. A few families of BC_1F_3, BC_2F_2-IB, BC_2F_2-BI and BC_3F_1 appeared as outliers inclined towards the *T. uniflorum* parent.

Stolons dry weight was the highest in white clover and the least in *T. uniflorum* (Figure 5.32e). Both intercrossing and backcrossing improved this trait. The data of second backcrossed and intercrossed generations were not available for this comparison. However, the third cycle of both ways of crossing did not enhance this trait to the white clover level. Both BC_2F_2 generations i.e., BC_2F_2-IB and BC_2F_2-BI, were able to show similar potential to the white clover. Both forms of BC_2F_2 and white clover expressed high diversity for this trait.

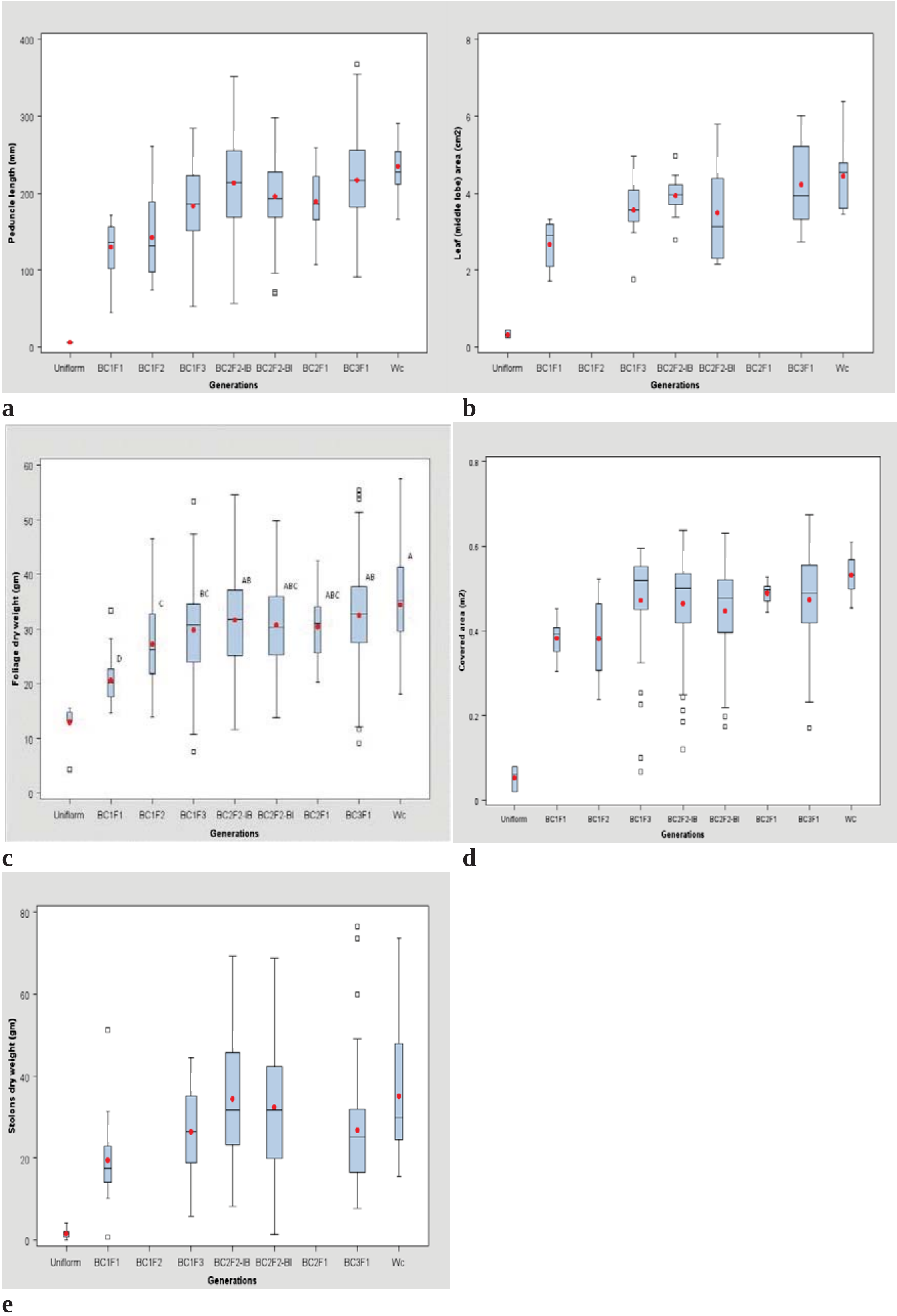


Figure 5.32. Comparison of generations for peduncle length (a), leaf area (b), foliage dry weight (c), plant covered area (d) and stolon dry weight. Rest of the details of plots are same as described for Figure 5.30.

T. uniflorum and BC_1F_1 had high root to shoot ratios as compared to the other hybrid generations and white clover (Figure 5.33a). In general, both intercrossing and backcrossing reduced this ratio. The third backcrossed generation showed a more drastic reduction than the third intercrossed generation. The BC_2F_2 produced by first backcrossing and then intercrossing (BC_2F_2-BI) also showed increased reduction in this ratio compared to BC_2F_2 produced by first intercrossing and then backcrossing (BC_2F_2-IB). The range in all hybrids for this trait was high. *T. uniflorum* produced the fewest stolons per plant (Figure 5.33b) among all generations. All other generations were similar to each other and to white clover in this aspect. A contrary trend was observed for numbers of nodes on a stolon (Figure 5.33c). *T. uniflorum* contained the most nodes per stolon, followed by the BC_1F_1. Remaining generations were not different from white clover for this trait. *T. uniflorum* exhibited the lowest, while white clover showed the highest root dry weight (Figure 5.33d). The BC_1F_1 generations also showed high root weights and further crossing could not improve this trait. Backcrossing negatively influenced this trait. A distinguishing characteristic of *T. uniflorum* was its tap root which had a wide diameter and a long woody portion (Figure 5.33e and 5.33f). By contrast, the hybrid generations did not differ from white clover in having narrower and less woody length of taproot than *T. uniflorum*. The BC_2F_2-IB had significantly longer woody tap root portion than BC_2F_2-BI.

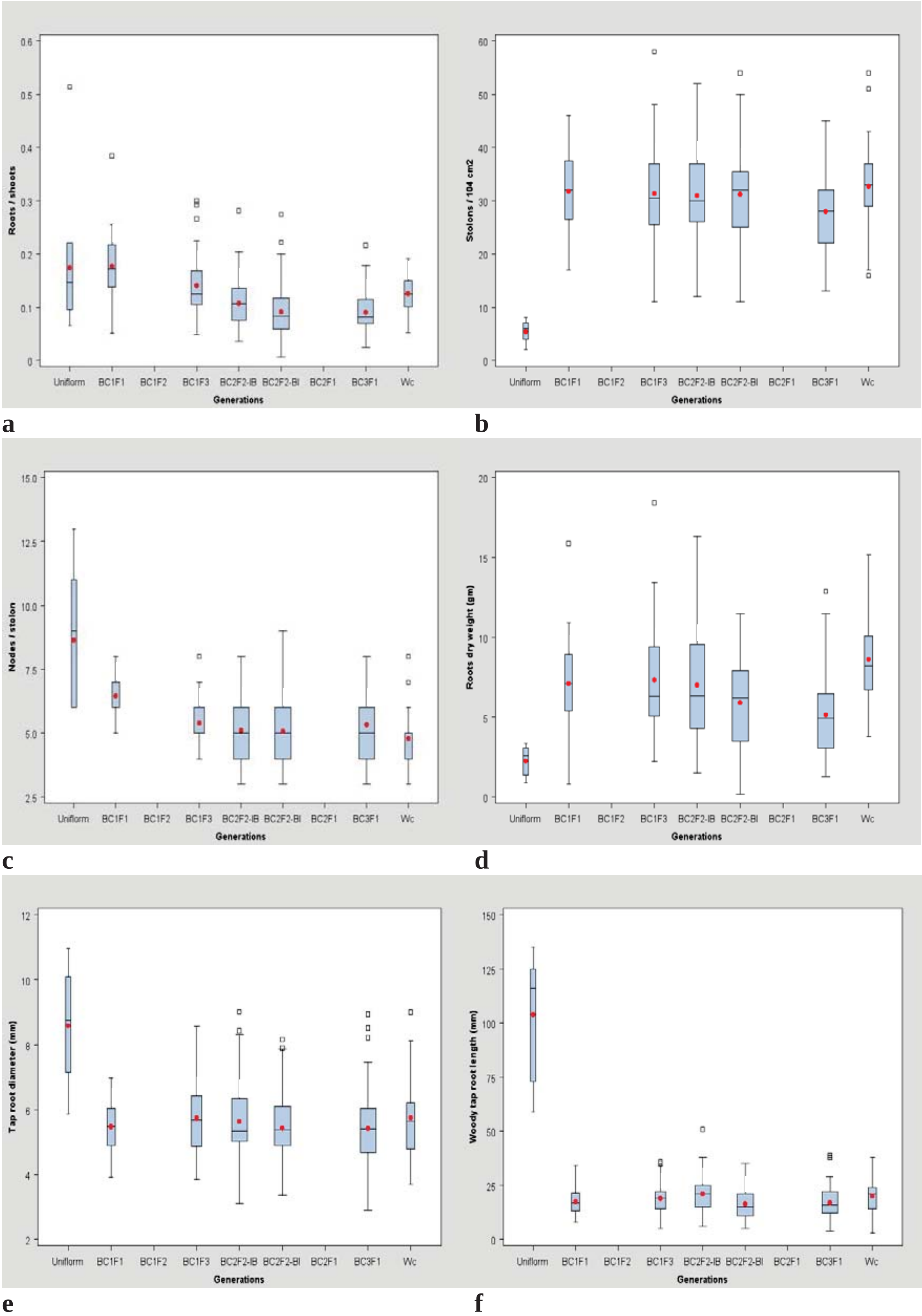


Figure 5.33. Comparison of generations for root to shoot ratio (a), stolons/104 cm^2 (b), nodes/stolon (c), root dry weight (d) tap root diameter (e) and woody taproot length (f). Rest of the details of plots are same as described for Figure 5.30.

5.5 SUMMARY

Three generations of backcrossed and intercrossed interspecific hybrids produced by recurrent selection, and their parental species (white clover and *T. uniflorum*) were used to analyse the effects of different mating designs on seed production traits, in particular and on a few important vegetative and root traits. The hybrids were also explored for influences of different parental types, inbreeding depression, associations among the traits, and heritability of the traits.

T. uniflorum flowered gradually over a long period and showed the earliest flower emergence among all the material, whereas white clover started flower emergence much later than *T. uniflorum* and showed a distinct phase of peak flowering in the last week of December coinciding with the longest day. The hybrids showed wide variation in flower emergence and flowering intensity. Families maintained the flowering patterns of their parents. The hybrids showed normal pollen stainability and production of ovules per ovary. Only 16% of the potential seed yield of the hybrids was actually harvested, whereas it was 31% in white clover. Differences in selection of white clover recurrent parents remained moderately effective in creating significant differences among the progenies at both, cultivar and genotype within a cultivar, levels. Different hybrid parents used in intercrossing were generally less effective in creating significant difference among their progenies. Reciprocal crosses mostly produced similar results.

White clover was more variable than *T. uniflorum* for most of the seed production traits. The third backcrossed generation expressed less diversity than the third intercrossed generation. The hybrids showed significant inbreeding depression for almost all the traits. Peduncle length was the most affected trait, whereas head production was the trait least affected by inbreeding. Comparison among generations showed that white clover had the least inbreeding depression compared to the hybrids, while the intercrossing increased sensitivity to inbreeding.

The general comparison of parents with progenies showed that selection based on individual traits remained mostly effective, and the progenies maintained the status of their parents in most cases. Backcrossing mostly affected the inheritance of derived traits, whereas intercrossing disturbed the inheritance of primary traits. Head size exhibited very high values of narrow sense heritability. Head production also appeared

to be moderate to highly heritable. Seeds per floret and seeds per head expressed moderate heritability, whereas florets per plant and seeds per plant were found to have low-moderate values of heritability. Foliage production and peduncle length showed low values of heritability.

Head production was found to be an important contributor to plant seed production. Head size, peduncle length and foliage production showed strong associations among each other. Head size was also moderately associated with seeds per floret but showed a negative relationship with ovules per ovary. The second source of plant seed production was seeds per head. Number of seeds per floret was a more influential contributor than head size for production of seeds per head. Numbers of seeds per floret were not negatively affected by the numbers of ovules per ovary. A moderate, positive association was observed between foliage production and head production. None of the root traits exhibited a strong negative association with any reproductive or vegetative trait. Generally a positive association was observed between the stolon and root traits. Nodes per stolon appeared to be an important contributor to head production. Stolon production was negatively associated with nodes per stolon. However, total effect of stolon production on head production was negligible. No negative association of head production with foliage or stolon production was observed. A few families belonging to BC_2F_2-BI, BC_3F_1 and BC_1F_3 showed above average counts for all three primary seed production traits, i.e. seeds per floret, florets per head and heads per plant, making these families better in head and seed production traits, compared to those of white clover cultivars.

T. uniflorum showed the highest potential for ovules per ovary, seeds per floret, seed weight, heads per plant, nodes per stolon, root to shoot ratio, tap root diameter and woody tap root length, whereas it was the lowest performer for head size, peduncle length, leaf area, seeds per head, florets per plant, seeds per plant, foliage production, plant cover area, stolon production and root dry weight traits, when compared to the hybrids and white clover. White clover was better in seeds per floret, seeds per head and root weight than all the hybrids. Except for the BC_1F_1 plants, most of the families in different generations were products of hybridization of parents selected for better seed production traits. The hybrid generations were generally intermediate to their primary parental species, and showed a wide range of variation for most of the seed traits. The exceptions were low seed setting per floret of all hybrids as compared to the parents, and better florets per plant of BC_2F_1 and BC_3F_1 as compared to white clover. The BC_1F_1 generation showed the closest resemblance with *T. uniflorum* for head size, ovules per ovary, seeds per head, florets per head, seeds per plant, peduncle length, leaf area, foliage production, stolon weight, root to shoot ratio and nodes per stolon. Except for BC_1F_1, BC_1F_3 and BC_2F_2-IB, the hybrid generations expressed better head numbers than white clover. The BC_1F_3 generation had leaves significantly smaller than the BC_3F_1 and white clover. Both, intercrossing and backcrossing, improved numbers of florets per head and florets per plant, peduncle length, leaf area, foliage production, and stolon weight of the hybrids. However, these breeding strategies were ineffective in improving seeds per plant beyond the second crossing cycle. Backcrossing was more effective than intercrossing in recovering florets per plant and seed weight while the opposite occurred for self-compatibility and root weight. Root to shoot ratio and nodes per stolon became reduced with the progression of both intercrossing and backcrossing. The hybrid generations showed root weight, taproot diameter and woody tap root length similar to that of white clover. However, the BC_2F_2-IB showed significantly longer woody tap root portions than BC_2F_2-BI. *T. uniflorum* appeared to be the most isolated species in cluster analysis. Along with BC_1F_1 hybrids, a few BC_1F_3 families clustered with *T. uniflorum*. White clover cultivars showed close resemblances with a few families of the BC_3F_1, BC_2F_2-IB and BC_2F_2-BI generations.

The progression in hybridization was directed by recurrent selection towards the improvement in seed production traits. Significant improvement along with creation of a wide range of variation in these traits was successfully achieved. Although the hybrids lost the root traits of *T. uniflorum*, no negative association was found between seed production and root traits. Seed production of the hybrids could, therefore, be improved without compromising persistence and foliage production. Hybrid families with valuable combinations of important seed production traits were identified.

Chapter 6

DISCUSSION

The materials analysed in this research were interspecific hybrids between white clover and *T. uniflorum*, developed with the aim to introgress the drought tolerance traits of *T. uniflorum* into white clover. The primary F_1 hybrids exhibited low seed production ability due to the low florets per head of the *T. uniflorum* parent, the interspecific nature of the hybridization and possibly the poor adaptation of *T. uniflorum* to the New Zealand climate. The poor seed production of the hybrid raised many questions such as;

- Why do the hybrids lose reasonable seed production?
- How components of seed production combine and contribute to overall seed production?
- Is there enough variation to select for better seed production?
- Do seed production traits have reasonably high heritabilities to respond to selection?
- Was inbreeding useful or detrimental?
- How do different breeding strategies contribute to the recovery of seed production?

Backcrossing and intercrossing with recurrent selection were used to improve seed production of the hybrids. Study of the drought tolerance properties of these hybrids was not part of this project. However, root traits were studied and no evidence of any negative relationship between root and seed production traits was observed. This eliminated the risk of losing the better root traits of the hybrids due to selection for improvement in seed production ability.

The expected unstable genetics of the interspecific hybrid and complex nature of seed production traits required a consistent and progressive investigation. Experiment-1 was a feasibility study. It started with plant cuttings, conducted under glasshouse conditions and was unreplicated. The main objective was to analyse the variation present in the available BC_1F_1 generation. Variations in seed production traits were found to be reasonably high, justifying further exploitation. Selected genotypes were used to produce the next generations' hybrids. Experiment-2 was managing the available variation to analyse different aspects of seed production. Hypotheses were developed and tested in this experiment under open air conditions with families of two available generations i.e., BC_1F_2 and BC_2F_1. Crosses were made at the end of this experiment to create a range of all possible advanced hybrid generations. Experiment-3 included eight different generations with a diverse range of genetic background. It analysed the hypotheses made and tested during earlier experiments with a wider diversity of plant material. New aspects of seed production were also explored for these hybrids and different breeding strategies were compared in the context of improvement in seed production. In each experiment, besides addition of new hypotheses, earlier ones were further tested using wider diversity. The results mostly remained consistent across the experiments. This made it suitable to discuss the results comprehensively under broad headings in one place.

In white clover, the components of seed production differ in their environmental optima. A duration having overlapping favourable conditions for all seed yield components in a cool temperate climate was recommended to be late spring and early summer (Thomas, 1987). This seasonal span shows a sharp increase in duration of sunshine along with a smooth rise in temperature and consistent moderate rainfall at Palmerston North (Appendix 3 and 4). The plant materials used in this study were very diverse in their flowering patterns. Decisions regarding the last defoliation (closing date), flowering counts and inflorescence harvesting were based on previous studies of white clover in New Zealand, preferably at Palmerston North. Such studies recommended that fields should be closed from grazing during November, giving an expected flowering peak during December and harvesting in late January or early February (Clifford, 1980, 1985; Budhianto et al., 1995; Burggraaf, 2005).

Data for seed production components of these hybrids were positively skewed. This was an expected outcome as one of the parental species i.e., *T. uniflorum*, was extremely poor in seed production and the nature of the cross was interspecific, giving reduced fertility and seed-set (Rieseberg and Carney, 1998). All statistical analyses used in this study were parametric and required normal distribution of the data. Box-Cox transformations were used to normalize data distribution. All statistical analyses were done in Statistical Analysis System (SAS) package 9.2. Path analysis was used with an *a priori* model, based on causal relationships between dependent and independent variables to explore associations among traits. The relationships among different generations, families and traits were also analysed by using Principal Component Analysis (PCA).

6.1 SEED PRODUCTION OF THE HYBRIDS

Seed production depends on the fertility of the plants and its expression in seed setting.

6.1.1 Fertility of the hybrids

The BC_1F_1 generation in experiment-1, under glasshouse conditions, expressed 54% mean pollen stainability (Table 3.5). Family B showed considerably lower pollen viability i.e., 32%. All families including family B, expressed high variability for this trait. Average pollen stainability of family A and S was 64% in experiment-1, whereas it was 76% in experiment-3. The average pollen stainability of all hybrid generations was between 76% and 82% (Figure 5.31a). Abnormalities in chromosomal pairing during meiosis can reduce pollen viability. Pandey et al. (1987) reported 24% on average pollen stainability of BC_1F_1 generations of the similar hybrid nature. The occurrence of low frequency of quadrivalents in pollen mother cells (pmc) of the hybrids e.g., 0.04-0.06 per pmc in BC_1F_1 was contradictory to the absence of this formation in white clover and high rate of quadrivalent pairing in *T. uniflorum*. The disturbed parental chromosome pairing pattern was taken as an indication of hybridity. This disturbance in chromosomal pairing caused by hybridity was also evident from reduction of mean pollen viability from 24% in F_1 to 10% in F_2 generation. (Hussain et al., 1997b) observed in interspecific hybrids between white clover and *T. nigrescens* (2n=16) that chromosome doubling of the triploid improved its average pollen stainability from 10% to 89% in the hexaploid hybrids. Williams and Hussain (2008) also reported less than 50% pollen stainability for most of the BC_1F_1 and BC_1F_2 plants, produced by backcrossing (having white clover as recurrent parent) of octaploid F_1 hybrid between white clover and *T. ambiguum* (4x). Contrary to that, Williams (1978) suggested that the expression of very low pollen fertility (0.96%-2.96%) by interspecific hybrid between white clover and *T. ambiguum* (2n = 32) was not due to gross meiotic irregularities. In a later report (Williams and Verry, 1981) it was shown that the bivalent pairing was most frequent (15.6 per pmc) in these hybrids. However, it was not known whether the bivalent pairing was a result of auto- or allosyndesis nature. The high pollen stainabilities of the hybrids in the current study indicated absence of chromosomal pairing or any other abnormalities affecting pollen viability.

The quantity and quality of ovules, the precursors to seeds, are important components of plant fertility. Ovule production ability of the hybrids was accessed during experiments-2 and 3. In experiment-2, two pairs of BC_2F_1 families were analysed (Table 4.5). The average number of ovules per ovary was 5.7. During experiment-3, a diverse range of hybrid generations were analysed (Table 5.6). The average number of ovules per ovary of the hybrids was 5.4 with a range of 4.5-6.0. White clover control cultivars showed almost similar average potential i.e., 5.1, consistent with the normal ovules per ovary reported for this species (Thomas, 1996). These results showed that the ovule production ability of the hybrids was normal, and not affected by the wide hybridisation.

The range of average seed setting as the result of selfing (facilitated by rubbing of the floral head) was 30-71% (seeds per 100 florets) with an average of 48% for all families under glasshouse conditions (Table 3.5). This was clearly higher than the 5% shown by the parent species white clover. The selfing efficiency increased for white clover to 16% under field conditions during experiment-3 (Figure 5.31c). The environmental conditions and white clover cultivar differences between these two studies might have caused the difference in selfing efficiency of white clover. For example, results of experiment-3 showed that selfing percentage of cultivar Aran was almost three fold (26%) than that of Sustain (8%). The hybrid generations expressed 36% as an average seed setting potential by selfing with a range of mean from 16% in BC_2F_2-IB to 70% in BC_1F_3. Intercrossing favoured self-fertility, whereas backcrossing improved self-incompatibility of the hybrids. Pandey (1957) first reported production of a self-fertile hybrid from a cross between the same two self-incompatible species i.e., white clover and *T. uniflorum*. To explain self-fertility of the hybrid, he proposed that self-incompatibility S alleles might be present on different chromosomes or loosely linked on the same chromosome of the two parental species. Later on, it was described that along with multiples alleles at the S locus causing self-incompatibility, there are self-compatible alleles facilitating self-fertility in white clover (Williams, 1987b). Another explanation can be derived from the work of Brewbaker and Atwood (1952) on octaploid white clover. The octaploid white clover was carrying four S alleles and their pollen grains had two S alleles. The researchers proposed that hetrogenic diploid pollens (e.g., S_1S_2) can cause self-fertility in the case of competitive interactions between the alleles, leading to each one cancelling out the effect of the other or in the case of complete mismatch of alleles between pollen and style. The self-incompatibility mechanism of *T. uniflorum* is not confirmed. Assuming that it has a similar S locus based self-incompatibility mechanism as that of white clover, then the hybrids might have two S loci (one from white clover and the other from *T. uniflorum*). Competition between the S alleles from the two species in a gamete might have led to self-fertility of the hybrids. The two cultivars of white clover involved in the current study showed different selfing performance i.e., 7.5% for Sustain and 26% for Aran. The reason for this difference might be that the European origin of Aran made its incompatibility system more sensitive to high temperatures than the New Zealand origin of Sustain.

These results showed an acceptable level of fertility and breakdown or leakage of the self-incompatibility mechanism in these hybrids.

6.1.2 Differences between potential and actual seed yields

The hybrids showed considerable differences between potential and actual seed yields during both studies. Seed yields from white clover were 31% (about two fold of the hybrids) of its potential. Actual seed yields of 20% to 50% of potential are a well reported phenomenon in white clover (Thomas, 1996). Both genotype and environment contribute to this difference (Williams et al., 1998). The difference between potential and actual seed yield varied with parental compositions and developmental stage of the hybrids (Figure 4.8 and 5.6). The harvestability of potential seed yield was better in BC_1F_3 plants than in BC_3F_1 plants (Figure 5.6). Genetic stability caused by inbreeding and selection might have contributed to this better performance of BC_1F_3 plants as compared to plants of BC_3F_1. This reason was supported by better pollen stainability percentage and ovule production ability of BC_1F_3 than BC_3F_1 (Figures 5.31a and 5.31b). It was suggested by Grant (1966) that selection and genetic drift reduce post zygotic barriers with advancement of generations.

Low performance of hybrids for harvestability of potential seed yield as compared to white clover could be due to the interspecific nature of these hybrids and the poor seed production effect inherited from their *T. uniflorum* parent. No mature seed could be harvested from this species under field conditions. One reason might be poor adaptation of this species to the humid temperate climate of Palmerston North for seed production. The strong activity of bumblebees and the simple flower structure of *T. uniflorum* excluded the possibility of absence of pollination. The presence of abundant well exposed white clover flowers in the vicinity might have made the less conspicuous single flowers of *T. uniflorum* less attractive for pollinators. However, the observed swollen pods and embryos with developed cotyledonary leaves, hypocotyl and radical showed successful pollination, fertilization and embryo development in this species. The pods turned down under the stolons onto the soil surface. It is possible that because *T. uniflorum* is alien to the humid temperate environment of Palmerston North, the highly humid conditions caused decaying of seed pod or immature seeds resulting in rapid seed loss into the soil. This was supported by observation that uplifting of stolons significantly enhanced production of mature seeds (Figure 5.5).

The main reasons for low seed setting of these hybrids could be the same as reported for white clover i.e., infertility of ovules and embryo abortion (Pasumarty and Thomas, 1990). Difficulties in chromosome pairing and genome incompatibility further reduced fertility in the hybrids, so that the seed-set was further halved. This argument is supported by the observation that although availability of gametes in the hybrids was nearly normal, the pollen viability reduced in order of BC_1F_3 > BC_3F_1 > BC_2F_2-BI > BC_1F_1 (Figure 5.31a). An almost similar pattern appeared in harvestable potential seed yield (Figure 5.6), which indicated that all gametes were not fully functional.

There was a clear difference among tested hybrid genotypes for their harvestability of potential seed yields. This indicated the presence of genetic variation for this trait which could further be exploited to improve ovule fertility and retention of embryos (Bogaert, 1977; Pasumarty et al., 1993b).

6.2 ASSOCIATIONS AMONG THE TRAITS

Associations among traits are discussed on the basis of the consistent outcomes of PCA (Table 4.8 and 5.7), path coefficient analysis (Figure 4.11 and 5.7) and correlation studies of all three experiments. *T. uniflorum* was not included in these analyses because of its unavailability or because its data could not be transformed to fulfil the assumption of statistical normality for these tests. Phenotypic correlations were used in experiment-

1 as it was a feasibility study and based on only the BC_1F_1 generation which was expected to be genetically unstable. PCA and Path analysis were used in both experiment-2 and experiment-3 with various levels of generations involved. Associations among traits can be discussed by focussing on seed production, vegetative and root traits.

6.2.1 Reproductive traits

Number of heads produced by a plant appeared to have major direct significant contribution to seed production. The trait of florets per head (head size) showed a moderate effect on seed production. These results are in agreement with general findings of work on seed production of white clover (Hollington et al., 1989; Jahufer and Gawler, 2000; Montardo et al., 2003). The trait of seeds per head contained the accumulated effects of seeds per floret and head size. Improvement in this trait also appeared to be an alternative way to improve seed production, although less efficient, than heads per plant. Binek (1983) showed that reduction in number of heads per plant to 40 and 20 was compensated by increase in seeds per head by 58% and 69%, respectively, in Podkowa white clover cultivar. Weak positive phenotypic correlations appeared between heads per plant and head size in selected families of BC_1F_1 in experiment-1 (Table 3.6). Correlation studies of Cain et al. (1995), Annicchiarico et al. (1999), and Jahufer and Gawler (2000) showed the same results for white clover. This weak correlation indicated that there was scope to select plants with high densities of bigger heads. However, the studies in experiment-2 and 3, which included advanced hybrid generations along with the white clover parent showed slightly negative associations between head size and heads per plant which might be due to negative associations of these traits inherited from their parents. White clover and *T. uniflorum* both showed negative relationships (Figure 5.25) for these two traits. White clover had better head size but low numbers of heads per plant, whereas *T. uniflorum* produced high numbers of heads per plant but with one to three florets per head. The comparison between BC_1F_2 and BC_2F_1 in experiment-2 (Figure 4.17) also showed that the generation having superiority in head size was inferior for head numbers per plant, and vice versa. The expression of previously mentioned weak correlations between these two traits in BC_1F_1 plants of experiment-1 might be due to the unstable nature of this early backcrossed generation, very high selection pressure at this early stage of hybrids or strong environmental influences on this association. The negative association between head size and head numbers per plant found in the advanced generations of this study was never particularly high. The comparisons of families belonging to different generations on the basis of head size, head numbers and seeds per head (Figure 5.11) showed the existence of families having acceptable combinations of these three traits. This showed the potential for improving seed production by combining better floral head density with improved numbers of seeds per head, as recommended by Woodfield et al. (2004). The donor parent, *T. uniflorum* was particularly inferior to white clover in florets per head. This trait was focussed on for correction during development of BC_3F_1 hybrids. Two white clover plants, Will-2 and Aran-802, with big heads, were used as recurrent parents in the third cycle of backcrossing. This resulted in the head size of the BC_3F_1 generation being almost the same as that of white clover (Figure 5.26). The improved head size coupled with more heads per plant, an ability possibly retained from *T. uniflorum*, made the BC_3F_1 generation the highest florets per plant producer, with significant difference from BC_1F_1, BC_1F_3 and white clover.

Head size showed a positive effect on seeds per head. It also expressed a moderate positive relationship with seeds produced by a floret. This was in accordance with the findings that bigger heads produced by large leaved varieties had more seeds per floret, resulting in more seeds produced by a head (Williams et al., 1998). However, head size had a negative association with ovules produced in an ovary. Both head size and seeds per floret contributed to seeds per head. Seeds per floret appeared to be an influential trait, via seeds per head, in plant seed production. This is in agreement with other studies, such as that of Piano and Annicchiarico (1995). Seeds per floret and heads per plant were independent of each other. Williams et al. (1998) also found that these two traits are under different genetic control and can be manipulated independently. Seeds per floret had shown no strong relationship with ovule production. Ovule number was reported as one of the important factors involved in seeds produced by a floret (Thomas, 1987). The absence of the expected positive association between ovules per ovary and seeds per floret, despite the existence of significant variation for ovules per ovary, indicated that factors other than ovule production ability were more influential in seed setting per floret.

Individual seed weight showed no strong associations with any traits except nodes per stolon. It was suggested that a trade-off takes place between 1000 seed weight and the number of seeds formed, due to dependence of seed provisioning on photosynthate availability (Clifford and Baird, 1993; Thomas, 1996). However, no association appeared between 1000 seed weight and the QTL identified for seeds per head (Barrett et al., 2004a). Seed weight was found to be negatively linked with nodes present on a stolon. *T. uniflorum* has both of these traits in positive co-existence. The negative association between them in the studied material comprising of hybrids and white clover cultivars showed weak direct association between these traits. Woodfield et al. (2004) discussed that heritable changes in seed yield per inflorescence is more due to changes in seeds per floret or florets per head than changes in 1000 seed weight.

Peduncle length is an important contributor in pollination and harvestability of the flower head. It has particular importance in white clover x *T. uniflorum* hybrids as the donor parent *T. uniflorum* has a very short peduncle making flowers less conspicuous and prone to lose immature seeds due to the early lodging of the flower head. This peduncle length appeared to be positively associated with foliage production, leaf area and head size. It showed negligible to light and moderately negative association with number of heads per plant and ovules per ovary, respectively, in PCA. However, path analysis explained that head size has a negative association with ovules per ovary, whereas its relationship with seeds per floret is positive. This indicates that selection to improve peduncle length of these hybrids will have two fold benefits. Firstly, it will be associated with improvement in head size, leaf size and foliage production. Secondly, it will help in keeping the flower head above plant canopy for extended time period for better pollination, seed maturation and harvestibility. Marshall (1995) showed that high heads per unit area and head size co-existed with better peduncle attributes i.e., diameter and dry weight, in the white clover cultivar Aberdale.

6.2.2 Vegetative traits

Strong associations were observed among stolon numbers, stolon dry weights and root dry weights in the PCA results (Table 5.7). Path coefficient analysis further explained that high stolon numbers led to high stolon dry weights, which were strongly associated with root dry weight. Leaf area and foliage production were associated with head size. Cain et al. (1995) also reported that head size was positively associated with seeds per floret and biomass production. Leaf area and foliage dry weight also showed positive associations with peduncle length. These observations were in agreement with findings of no negative correlations on genetic, phenotypic or micro-environmental bases among biomass and reproductive growth components of white clover by Cain et al. (1995). Leaf area was found to be slightly negatively influencing heads per plant. Woodfield et al. (2004) observed consistent decrease in leaf size with increase in inflorescence density in white clover. Clifford (1985) reported that increases in leaf size enhanced head size and number of heads per square meter up to an optimum size (5.6 cm^2 in their study). Beyond the optimum, increase in leaf size continued to be associated with head size but head number showed decline, resulting in decrease in seed yield. Individual leaf area showed a moderate association with foliage production. The positive association between foliage and head production got improved with recovery of white clover phenotype by hybridization and selection.

Number of nodes present on a stolon was positively associated with head production per plant. Nodes per stolon were negatively linked with number of stolons. Although, no direct negative relationship appeared between head numbers and stolon numbers produced by a plant, their indirect association via nodes per stolon was negative enough to make the overall relationship negligible (Table 5.9). This finding is partly in agreement with expected negative relationships between seed production traits, especially head production, and persistence (as indicated by dense stolon branching) in ecotypes and natural populations of white clover (Williams, 1987b; Annicchiarico et al., 1999).

T. uniflorum exhibited a prominent feature of low stolon numbers and concentrated nodes per stolon. The nodes are mainly used for head production rather than stolon production in this species. This attribute of *T. uniflorum* seemed to be a valuable contribution to head production of its hybrids with white clover. The improved nodal concentration at a stolon of the hybrids was utilized to produce more floral buds. The absence of a direct negative association between stolons and heads production indicated that the inverse relationship between persistence and head production got diluted to some extent in these hybrids. A high number of stolons produced by a hybrid also improved its stolon dry weight and foliage production. This showed positive associations between dry matter production and persistence. A similar positive relationship was observed by Piano and Annicchiarico (1995) for at least three growing seasons in Regal Ladino genotypes originating from land races and ecotypes. Stolon weight was found to be positively associated with root weight. Pederson (1989) also found a significant positive correlation between shoot and root dry weight in white clover cultivars having a tap root system.

6.2.3 Root traits

Tap root diameter, measured at the base of crown, showed low variation in the studied genotypes. These results supported Nichols (2012) findings that no significant difference existed among basal tap root diameters among *T. uniflorum*, white clover and their BC_1F_1 and BC_2F_1 hybrids having white clover as recurrent parent. However, *T. uniflorum* had significantly thicker tap roots at 20 mm from the base than white clover and the BC_1F_1 and BC_2F_1 hybrids. Nichols (2012) also observed that white clover had significantly narrower tap roots than the BC_1F_1 at the start of the primary root. In PCA, tap root diameter showed moderate associations with root weight, stolon number and stolon weight. However, path coefficient analysis showed no marked relationships of tap root diameter with these three traits. Both analyses confirmed positive effects of diameter on the woody nature of the tap root. Tap root diameter did not show a strong correlation with leaf size. These findings are consistent with the findings of Nichols (2012). This might be due to the low variability of taproot diameter in the studied genotypes. Woodfield et al. (1996) mentioned that a strong relationship between these two traits is reported in studies based on white clover cultivars and ecotypes with highly diversified morphology and origin. Nevertheless, they successfully improved taproot diameter of four large leaved and one medium leaved white clover cultivar without affecting leaf size and stolon numbers.

No negative associations were evident between root and seed production traits. This showed the potential for improving both traits simultaneously. Vegetative traits were also found to be supporting the seed production components. The only exception was the negative association of leaf area with ovules per ovary, detected in PCA. The path coefficient analysis showed that leaf area was linked with head size which further negatively affected number of ovules per ovary. An important result was the absence of a direct negative association between heads per plant and number of stolon, indicating potential for having good seed production attributes without negatively affecting persistence. This has been a much highlighted issue in improving seed production (Gibson, 1957; Piano and Annicchiarico, 1995).

Ideally seed production can be improved by combining the high head production ability of one species with the large head size of other species as demonstrated by Marshall et al. (1998) for white clover x *T. nigrescens* hybrids. However, the results of the current study showed that seed production can be enhanced by improvement in:

a. Head number, which was the most influential contributor in seed production. In this study it was not directly negatively linked with stolon production (potentially the cause of persistence reduction). However, it showed slight negative association with head size.
b. Seeds per floret, which was the second most influential factor in seed production. It contributes in seed production through seeds per head. It appeared independent of ovules per ovary and heads per plant but had a positive association with head size.
c. Head size, which was less efficient than seeds per floret in improving seed production via seeds per head. It also negatively affected number of ovules per ovary. Due to its slight negative association with heads per plant, improvement in this trait can spare more nodes for stolon production, which is expected to result in increased persistence. Head size had also shown positive influence on seeds per floret.

Few heads of medium size with high seeds per floret can help improve seed production without effecting persistence and foliage production. However, the association of the traits with seed production can be exploited by effective selection which depends on available genetic variation and heritabilities of the traits.

6.3 GENETIC VARIATION

6.3.1 Sources of variation

Genetic variation was added into the hybrids at two levels i.e., F_1 and advanced hybrids.

6.3.1.1 Genetic variation at F_1 level

The parental species were the sources of variation in the development of F_1 hybrids. They were expected to contribute high levels of genetic variation to the hybrids because of their heterozygous nature and the wide hybridization.

a. **Heterogeneous nature of the parents**: Both parents i.e., white clover and *T. uniflorum* were predominantly self-incompatible and cross pollinated, resulting in genetic heterozygosity. Especially, white clover has remarkable genetic diversity due to its wide adaptation and outcrossing nature (Williams, 1987b). The diversity comparison showed that the white clover population was more variable than *T. uniflorum* plants for a majority of the traits (Figure 5.28). *T. uniflorum* is also a highly diversified species (Badr et al., 2002; Williams and Nichols, 2011). The crossing between these two heterogeneous parents was expected to result in a hybrid with inbuilt genetic diversity.

b. **Wide hybridization**: White clover and *T. uniflorum* are two distinct but related *Trifolium* species. White clover is adapted mainly to temperate zones, with good vegetative vigour, better seed production ability than *T. uniflorum*, shallow fibrous nodal roots and susceptibility to drought. *T. uniflorum* is adapted to dry climates, has poor vegetative vigour and seed production ability, a strong woody tap root system and drought tolerance. The crossing of these two species resulted in a wide hybridization, combining genes for contrasting traits from diverse origins.

The current study started with plants of the BC_1F_1 generation selected for better agronomic performance.

6.3.1.2 Genetic variation at advanced hybrid levels

Heterogeneity is an essential genetic component of white clover and *T. uniflorum* populations, as in all outcrossing species, and it must be retained in their hybrids (Williams, 1987b). Normally more than eight parents are involved in white clover cultivars (Caradus and Woodfield, 1997). To maintain the heterozygous nature of the hybrid plants and heterogeneous nature of their population, 15 different white clover cultivars were incorporated into the hybridization process as recurrent parents at different stages of development, from production of the F_1 to BC_3F_1 generations in this study. These white clover parents were very diverse in their origin and plant characteristics. The diverse origins of four white clover parents i.e., Durana, Aran, Tienshan and Sustain are given as examples. Durana was an ecotype from Georgia (USA) selected for better persistence than cv. Regal Ladino (Bouton et al., 2005). Aran was bred in Ireland for high dry matter yield from germplasm collected from France and Israel, and Tienshan was a Chinese ecotype, tolerant to drought and UV-B radiation (Caradus and Woodfield, 1997). Furthermore, the diverse germplasm involved in development of these cultivars provided them with inbuilt genetic diversity. For example, Sustain had high inbuilt genetic variation for seed production. The seed production potential of the parental lines varied from 350 kg/ha to 3000 kg/ha (Caradus et al., 1997). Incorporation of a diverse and wide range of white clover cultivars at different stages of this study were supported by the recommendations made by Nichols (2012) who comprehensively studied introgression of root and shoot characteristics in white clover x *T. uniflorum* interspecific hybrids. The high level of observed variation led her to recommend the screening of large numbers of hybrid families with a wide range of genetic backgrounds. The numbers of white clover cultivars used as recurrent parents in the current study was gradually reduced without compromising the heterozygosity of the hybrids. Nine white clover cultivars were involved in production of BC_1F_1 generation. This number was reduced to five with addition of two cultivars (Prestige and Regal Ladino) and an ecotype (Tienshan) during development of the BC_2F_1 generation.

There were two sources of variation among hybrid progenies at advanced levels. One was the different white clover recurrent parents used in backcrossing and the other was various hybrid parents used in intercrossing.

a. **Variation contributed by the white clover recurrent parent:** Significant differences among progenies having common BC_1F_1 hybrid parents crossed with different white clover parents were observed in 46% of the comparisons in experiment-2 (Table 4.10). Use of different plants within a variety appeared to be at least as effective as use of different varieties to create significant differences among progenies (Figure 4.13). The same investigation was repeated in experiment-3 by using more advanced levels of hybrid generations.

The parental influence became improved. There were significant differences in 66% of the comparisons made for different traits of the progenies having different white clover parents crossed with a common BC_2F_1 or BC_1F_2 hybrid (Table 5.10). Nichols (2012) also found that families of this interspecific hybrid with different white clover parental cultivars can significantly differ from each other in phenotypic expression of fully established plants. She recorded significant differences among families of the BC_1F_1 generation for dry matter production. Families of Kopu-II were the highest, whereas the families of the Crusader and Barblanca were the lowest in dry matter production.

The improvement in parental influence with advancement in hybrid generation could be due to reduction in allelic diversity of the hybrid parents. For example, inbreeding and selection reduced heterozygousity in BC_1F_2 plants, compared to the plants in BC_1F_1 generation. A common parent of BC_1F_2 was less absorbent, as compared to BC_1F_1, for the effects of different white clover parents crossed with it. This might have resulted in a greater proportion of significant difference among the progenies when different white cover varieties were crossed with a common BC_1F_2 hybrid, instead of BC_1F_1 hybrid. The other reason might be that white clover varieties AR, CH, LA and DU were used in crossing with BC_1F_1, whereas different set of varieties, i.e. AR, CH and Will-2, were used in crossing with BC_1F_2. This might also have contributed to the difference in results of the two studies.

The effect of the white clover parent in creating differences among progenies varied with the cultivar used. For example in experiment-3, the proportion of white clover cultivar AR_{802} in comparisons with significant differences was 16%, whereas it was 32% for cultivar CH_{13} (Table 5.10). The hybrid parental genotypes appeared influential in creating difference among their progenies. For example, plan-2 and plan-3 had the same white clover parents involved with different genotypes of the BC_1F_1 Cu family (Table 4.9). The difference among progenies in seed components and vegetative traits of plan-2 was significant in 75% of cases, whereas a it was significant in only 38% of the comparisons of plan-3 (Table 4.10). Similar results were obtained with the use of the second backcrossed and intercrossed hybrid parents in experiment-3 (Table 5.10). These results indicate genetic variability and different combining abilities of the hybrids.

Both vegetative and root traits appeared to be less affected than reproductive traits with a change in white clover parent in experiment-2 (Table 4.10). This finding indicates that these hybrids are genetically more variable for seed traits as compared to vegetative and root traits.

b. **Variation contributed by the hybrid parent:** Different BC_1F_1 hybrids crossed with another common BC_1F_1 hybrid (in intercrossing) and a common white clover (in backcrossing) created a significant difference for 36% and 39% of the comparisons, respectively (Table 4.12 and 4.13). The same analysis involving BC_1F_2 hybrids produced similar results (Table 5.11 and 5.12). The advancement in hybrid generation did not improve differences in progenies. This might be due to similar genetics of the hybrid parents. Nichols (2012) observed trends towards a larger shoot weight and root to shoot ratio for the BC_1F_1 families having hybrid 80-2 (F_1 hybrid between white clover and *T. uniflorum*), as F_1 parent, compared to the families having hybrid 900-4 as their F_1 parent.

The effect of parental composition was evident in both analyses. For example, intercrossing among BC_1F_1 hybrids of family A in plan-1 produced significant differences in 80% of the comparisons, whereas progenies of intercrossing among hybrids of S family in plan-2 were statistically similar in all traits (Tables 4.12 and 4.13). Even different parental compositions of the same family A in plan-1 and plan-5 expressed different results, i.e. 80% and 33% cases with significant difference among progenies of these schemes, respectively. The general analysis of results showed that heads and florets per plant were the least, foliage production was moderately and, seeds per floret, florets per head, seeds per head and peduncle length were the most effected traits with change in hybrid parent.

Study of the variation contributed by the parents showed that parental influence was more expressive in the case of backcrossing than intercrossing i.e., white clover varieties created more difference among progenies than different hybrid genotypes. This indicates more genetic difference among white clover varieties as compared to the differences among hybrid parents. The genotypic composition of the parents was the most consistent factor affecting the variation among progenies. Reproductive traits were more sensitive to parental change as compared to vegetative and root traits. Advance hybrids can be crossed with white clover cultivars to create variation in seed production traits to apply selection pressure in a desired direction.

6.3.2 Levels of variation

Variation among these hybrids can be discussed at the levels of within a family, family within a generation and among generations.

6.3.2.1 Variation within a family

Experiment-1 studied the variation within and between families of the BC_1F_1 generation. Although the numbers of samples for different families were not equal, there was no strong correlation (r = 0.12) between number of samples and coefficient of variation. The phenotypic variation, as measured by coefficient of variation, was low to moderate among plants of a family (Table 3.5). The reason might be that the plants represented a highly selected group, and so only represented a small proportion of the phenotypic variation that was available. The data were not subjected to ANOVA as the trial was not in replicated form, based on clonal propagules and part of a feasibility study. However, the ANOVA results of experiments 2 (Table 4.4) and 3 (Table 5.5) confirmed that plants within a family (full sibs) did not significantly differ from each other for all studied traits. Given the heterogeneous nature of the parents, it seemed to be an unexpected outcome.

There can be two explanations for this outcome. One possibility is that all plants of a family used in three replications were grown from seed i.e., were not clones, and had their own distinct genetic composition. These 15 plants were randomly distributed in the three replications. The plants placed at position one (plant-1) in the three replications had a probability of being genotypically as different from each other as any other combination of plants of the three replications. The ANOVA procedure took the average of the family and then compared the average of each plant position present in three replications to that of the family average. The three plants, for example of plant-1 position, were expected to have different values due to difference in their genotypes. This difference of values of these three plants possibly got reduced by averaging their values to make an average of the plant-1. Similarly, values of three genetically different plants were averaged to get their respective mean value for each of the other plant positions. The averaging procedure caused a reduction in diversity of 15 different genotypes into five plant positions. Moreover, this reduction in diversity did not have any sound basis e.g., common parentage, clonal propagules, etc. This might have resulted in the appearance of no significant difference among mean values of the five different plant positions within a family.

The second reason for non-significant difference among full sibs could be that the same parentage had significantly reduced the heterogenosity of the full sibs, which implied that the family could be considered as a stable unit for selection.

6.3.2.2 Variation among families of a generation

The comparison of progenies produced by intercrossing and backcrossing showed variation at family level. For example, comparison of families of generation BC_1F_2 in experiment-2 showed that family group S, involving genotypes of cultivar Sustain as BC_1F_1 parents in intercrossing, produced significantly higher seeds per floret, florets per head, seeds per head, peduncle length and foliage mass than family group C having genotypes of cultivar Crusader as BC_1F_1 parent in intercrossing (Figures 4.15). Family group B, having genotypes of cultivar Barblanca involved as BC_1F_1 parents in intercrossing, was significantly superior to family group A, the product of intercrossing among BC_1F_1 genotypes of cultivar Aran, for florets per head, florets per plant and seeds per plant. In general, family group B had better seed traits and inferior vegetative and root traits than other families involved in this comparison. Similarly there was significant variation among families of the BC_2F_1 generation for 42% of the comparisons made where different BC_1F_1 hybrid families were crossed with cv. Regal Ladino white clover (Figures 4.16). This showed that the involvement of at least one different parent (BC_1F_1 hybrid in this case) resulted in considerable variation. Nichols (2012) observed differences among hybrid families in both, BC_1F_1 and BC_2F_1 generations, for being superior to their white clover parent for vegetative and disease resistance traits.

Blending of different parents involved in developing advanced hybrid generations like BC_3F_1 and BC_1F_3 also created variation among families. For example, the cluster analysis showed that BC_2F_2-IB had family 55 resembling *T. uniflorum* parent, whereas its few other families (12, 36, 29, etc.) were grouped with the white clover parent (Figure 5.14). Families 9, 10 and 15 belonged to the BC_3F_1 generation and had the same plant i.e., Will-2, involved as the recurrent parent in the third backcross. In the flowering pattern study (Figure 5.21), family 9 showed a decline in flowering, family 10 continued flowering with constant intensity; whereas family 15 expressed a new flush of flowering.

6.3.2.3 Variation among generations

Significant variation was also observed among different hybrid generations for most of the seed production traits. A comparison of the coefficients of variation showed that the third intercrossed generation showed more diversity than the third backcrossed generation in the phenotypic expression of seed traits (Figure 5.28). BC_1F_3 was also more variable than white clover for most of the traits. Backcrossing increased the genomic proportion of the recurrent parent (white clover) in the hybrids, resulting in more white clover-like phenotypes and reduction in variability. In a study of hybrids of similar pedigree for vegetative traits, Nichols (2012) found that the BC_1F_1 generation was significantly more variable than white clover for specific leaf area and specific leaf mass but significantly less variable than white clover for internodal length, stolon diameter, petiole length and leaf lamina area. The researcher attributed this unexpected trend to the genotype x environment interactions.

The existence of significant variation at family and generation levels provided opportunity for selection responses. For example, woody tap roots are expected to improve tolerance against pest attack and drought (Williams, 1987b). Significant variation at both family and generation level in experiment-1 for woody tap root diameter (Table 4.4) and for woody tap root length in experiment-3 (Table 5.5) provided opportunity for selection for these traits in the hybrids. The tree diagram in experiment-3 for the combined seed trait data showed two families of BC_3F_1 in close vicinity to the white clover cultivars (Figure 5.14). These families could be selected as an improved product if they also retained the desired traits, particularly drought tolerance, of their donor parent, *T. uniflorum*. Two BC_1F_3 families were found in close vicinity of the donor parent indicating retention of the *T. uniflorum* traits in them. Three cycles of intercrossing were expected to create considerable blending of genes from white clover and *T. uniflorum* in these hybrids. They can serve as a valuable potential starting point of a recurrent selection or backcrossing process to improve hybrids by retaining desired trait(s) of the *T. uniflorum* parent. Similarly, three dimensional scatter plots provided data summaries that allowed selection of families of BC_1F_3, BC_3F_1, BC_2F_2 (both first intercrossed and then backcrossed and vice versa) generations with the desired combination of seed production traits (Figure 5.11). Comparison on the basis of averages identified some families of these generations having above average performance of all three important seed production traits i.e., seeds per floret, florets per head and heads per plant (Table 5.14).

6.4 INHERITANCE OF THE TRAITS

Inheritance of the traits was evaluated by comparison of progenies of different parental groups and through narrow sense heritability estimates.

6.4.1 Progeny comparisons

6.4.1.1 Index selection and florets per plant

This study started with the BC_1F_1 generation in experiment-1. The plants were selected for better agronomic performance. Heritability estimates were unavailable for these plants. Existing information regarding associations among seed production traits and their heritabilities in white clover could not be applied due to the early developmental stage and interspecific nature of these BC_1F_1 hybrids. The BC_1F_1 hybrids were having on average 25% of the *T. uniflorum* genome and each family had a different 25% from every other family. This resulted in considerable variation for all traits both within and among families (Table 3.5). Marshall et al. (1998) found a wide range of variation in the BC_2F_1 generation of interspecific hybrids between white clover and *T. nigrescens*. Plants of this generation showed retention of better flowering of the donor parent (*T. nigrescens*) along with recovery of agronomic potential of white clover, making this generation suitable to apply selection pressure.

Index selection was adopted to avoid strict selection based on one or few traits at BC_1F_1 level. All studied traits were given a weight according to various scenarios relating to their expected contribution in seed production. Four empirical indexes, based on different weight scenarios, were developed and their final outcomes were compared with direct selection for florets per plant. The trait of florets per plant was chosen as a key selection criterion because it combined expression of two important seed production components i.e., heads per plant and florets per head. On the consensus basis of these two criteria, plants of each family were categorized as high, medium and low performing for different traits.

The performance of intercrossed and backcrossed progenies of these selected plants in experiment-2 showed that this selection was quite ineffective (Table 4.20). The phenotypes of plants attained in experiment-1 were not significantly retained in experiment-2. Although comparison in most cases was between progenies of high (positive) and medium performing plants, progenies of high and negative performing plants also could not retain the performance of their parents. This finding was contradictory to the observations in white clover, where progenies retained heads per square meter and seeds per head abilities of their parental combinations (Woodfield et al., 2004). The inability of the progenies to maintain the status of their parents in the current study can be attributed to an expected outcome of a phenotypic selection based on too little information. The crucial information like genetic background of the families, heritability of the traits, flowering pattern, etc., was unavailable for plants of the BC_1F_1 generation. Another possibility can be that phenotypes did not reflect the genotypes because of low heritability caused by the unstable nature of the plants or high environmental influences. There were many differences between experiment-1 and 2. For example, plants in experiment-1 were propagated by cuttings under glasshouse conditions, whereas experiment-2 was a field trial of plants established by seeds. Seed setting by the hybrids was the most weighted criterion in the index selection. In experiment-1, seed setting was a result of manual crossing, whereas in experiment-2, it was a result of natural crossing with bees.

To observe the inheritance pattern at the end of experiment-2, again the high and low performing plants were selected but this time on an individual trait basis. The performance of their progenies in experiment-3 showed retention of the status of their parents in most of the cases. The few deviated results were mostly from plants of the

BC_1F_3 generation. This indicated a contribution of genetic instability in maintaining the status of the hybrids in the next generation.

6.4.1.2 Cytoplasmic inheritance

The cytoplasm of female parents can also contribute as a source of genetic variation along with the environment and nuclear genes. This question was explored by comparing the performances of progenies from reciprocal crosses.

This study was much more comprehensive in experiment-2 than Experiment-3. In experiment-2, 13 combinations of reciprocal crosses between BC_1F_1 families were studied for 12 traits including vegetative and root traits along with seed traits. The seeds traits were affected less (almost half) than those of the vegetative and root traits (Table 4.16). The family effect was also evident as family group C expressed a significant difference in 45% cases which was considerably higher than 14%, 11% and 17% of family group A, S and B, respectively.

The commonality between results of both studies was that in most of the cases i.e., 81% in experiment-2 (Table 4.16) and 83% in experiment-3 (Table 5.13), there were non-significant difference between results of these reciprocal crossings. This suggested an absence of any maternal or paternal influence on the studied traits in these hybrids. The results of Pandey et al. (1987) based on F_1 hybrids of the same interspecific cross showed influence of both factors i.e., parental genotype and the direction of crosses, in the phenotypes of the hybrids. Generally the hybrids were in the intermediate range. However, their results showed an inclination of hybrids towards male parents instead of female parent in the majority of recorded traits that were not intermediate between the parents. In white clover, Hill et al. (1989) also found no maternal effects in the inheritance of peduncle length, floral buds per stolon, florets per head, and floral density per square meter. Similarly, no reciprocal effect was found in studies of responses to phosphorus and *M. incognita* infestation in white clover (Pederson and Windham, 1992; Caradus et al., 1992).

This finding considerably reduces the chances of significant variation in these hybrids caused by the direction of the cross for studied traits. Progenies of two crosses involving the same parents could be compared irrespective of the order of the parents.

6.4.1.3 Inheritance of flowering pattern

Flowering pattern influences overall flowering intensity and time of maximum flowering (flowering peak), two important contributors in seed harvestability. The different geographic adaptation of the parental species i.e., white clover and *T. uniflorum*, used in this hybridization resulted in variation in flowering pattern. *T. uniflorum* showed much earlier emergence of flowering than white clover cultivars (Figure 5.20).

Thomas (1987) explained that species originating from lower latitudes are better adapted to dry summers. Such species initiate flowering earlier at the start of the season during autumn which provides enough time for seed production before the onset of drought in summer. In contrast, species from high latitudes require a longer and more intense period of cold temperature to initiate flower production. They behave like short-long-day species, have a brief period of flower initiation stimulated by spring long days and stop flower initiation with the onset of longer days in summer thereby avoiding investment of energy in late flowers which have less chance to reach maturity before the onset of winter. *T. uniflorum* is adapted to a dry climate (Williams and Nichols, 2011), whereas white clover cultivars Sustain and Aran are bred in New Zealand and Ireland, respectively (Caradus and Woodfield, 1997). The comparatively early and late flowering nature of *T. uniflorum* and white clover cultivars in the current study can be attributed to their dry and temperate cold areas of origin, respectively.

The results for selected families to observe inheritance of flowering pattern in experiment-2 to experiment-3 showed that progenies had clearly maintained the status of their parents for being early, medium or late (Figures 5.22 and plate 6.1). Only two out of fifteen families expressed a deviated flowering pattern from the rest of the families of their respective category. This indicated the high heritability for flowering pattern in the hybrids (Figure 6.1). Studies in other plants like *Cajanus cajan* L. (Gumber and Singh, 1997), *Lotus pedunculatus* (Kelman and Bowman, 1996) and *Poa annua* L. (Johnson and White, 1998) showed high heritability of this trait controlled by one or a few genes. Plants of two generations i.e., BC_1F_3 and BC_2F_2-BI were investigated in the current study. The BC_2F_2-BI expressed high heritability, whereas the generation BC_1F_3 showed a similar decline of flowering in both the early and medium categories (Figures 5.23). This result shoeds that remixing of the genome in the BC_1F_3

generation had disturbed the inheritance of this trait. Marshall et al. (1998) crossed the early flowering *Trifolium* species *T. nigrescens* with the later flowering white clover. The F_1 and BC_1F_1 hybrids (having white clover as recurrent parent) were not significantly earlier to flower than white clover. However, plants of the BC_2F_1 generation were significantly earlier than white clover and later than *T. nigrescens*. This generation also showed large variation for this trait.

Plate 6.1. Difference in flowering times of BC_2F_1 families. Late families are on the left, whereas early families are on the right.

The flowering trends have important consequence in terms of timely harvest to obtain the best proportion of mature heads. In the above mentioned inheritance study, the late flowering group would provide maximum harvestable heads during late March to April. These months can receive significant autumn rains, causing problems in harvesting intact heads. The medium flowering group had a tendency for a long continuous peak of low intensity flowering. This would cause difficulty in deciding the best harvesting time for maximum seed yield. The wider spread of flowering can be beneficial where management as well as seasonal constraints hinder following fixed dates for field closing, getting flowering peak and then harvesting (Pyke et al., 2004). The early flowering group seemed suitable for Palmerston North conditions. It had a concentrated flowering peak leading to a distinct time span of favourable environmental suitable conditions for harvesting maximum seed yield. Detailed study of the flower maturation pattern showed the existence of a wide range of variation, from families having all their heads fully mature to families having a late flush of flowering (Figure 5.21).The existence of variability and high inheritance of the flowering pattern provided opportunity for selecting genotypes for maximum seed yield, according to the management and environmental conditions.

6.4.2 Narrow sense heritability

Due to the unavailability of parents and offspring in a single growing season and the difference between the growing seasons of these generations, full and half sibs were compared in experiment-3 to estimate narrow sense heritabilities. Families belonging to BC_1F_3 (26%), BC_2F_2-IB (22%), BC_2F_2-BI (39%), BC_1F_2 (9%) and BC_3F_1 (4%) generation were collectively used in the study (Table 5.21). The reason to use families of different generations was to have general heritability assessment of the hybrids not restricted to any particular generation. Another reason was to increase the sample size to improve validity of the analysis. All families having one common parent were used in this study irrespective of their generations.

Head size appeared to be the most heritable trait (Table 5.23). This was also the most divergent trait between the parents. *T. uniflorum* had a head size of 1–3 florets, whereas white clover had 70-150 florets per head. The hybrids were expected to have high genetic variation for head size resulting in high heritability (h^2 = 0.93) of this trait. Head size has previously been reported to have high heritability in white clover (Bogaert, 1977; Annicchiarico et al., 1999). High heritability of head size provides an opportunity to make effective selection for improved head size which can contribute to the improvement of seed production by using fewer nodes for head production, thereby allowing more nodes fo stolon production, an important persistence trait.

Numbers of heads per plant expressed moderate-high narrow sense heritability (h^2 = 0.55). This was similar to the findings (h^2 = 0.58) of a previous study in white clover (Annicchiarico et al., 1999). Less emphasis was given on the number of heads as compared to head size during this study. A slight inverse correlation between these two traits in hybrids was evident. The selection procedure focused on improvement in seed production per plant which was the desired trait from the white clover parent. Higher numbers of heads was the desired trait of the *T. uniflorum* parent. However, the genetic proportion of *T. uniflorum* was continuously reduced in the hybrids through the backcrossing with white clover as the recurrent parent and the selection pressure for better head size and seeds per plant in the hybrids.

Seeds per floret and seeds per head showed moderate heritabilities. Hill et al. (1989) found low narrow sense heritability estimates for seeds per floret in white clover and argued that ovule abortion reduces the variability created during ovule formation, resulting in an increased influence of the environment on seeds per floret. Woodfield et al. (2004) mentioned a narrow sense heritability (h^2 = 0.4) for seed yield per head, similar to the value found in the current study. They attribute this lower than expected heritability to the composite nature of this trait. Both peduncle length and foliage production also exhibited very low heritabilities indicating a stronger influence of the environment than genetics on these traits. Hill et al. (1989) also reported a low narrow sense heritability for peduncle length and suggested that non-additive variations for this trait are due to epistasis.

Both the seeds per plant and florets per plant showed low-moderate heritabilities. The composite nature of these traits make them attractive for selection with minimal effort. However, low-moderate heritabilities of these derived traits make them less useful than the basic traits such as florets per head, seeds per floret and heads per plant, which show comparatively high heritabilities for seed production. In white clover, Woodfield et al. (2004) found that selection for heads per plant resulted in doubling the seed yield per plant compared to selection for seeds per head.

6.5 INBREEDING DEPRESSION

The high heterozygosity of out crossing species accumulates a high load of deleterious recessive genes. Inbreeding results in depression due to expression of these genes in the homozygous form (Woodfield and White, 1996). This expression of recessive deleterious genes usually takes place in the early generations of inbreeding (Allard, 1999). Inbreeding can have serious effects on the growth and development in white clover (Williams, 1987b). To observe inbreeding depression, comparison was made between selfed and crossed forms of hybrids and white clover genotypes. It was expected that inbreeding effects would be expressed in this comparison due to high heterozygosity of the hybrids. The results supported this assumption. The comparison among BC_1F_1, BC_2F_2, BC_1F_3 and white clover showed that heterozygosity resulting from intercrossing increased sensitivity to inbreeding (Figure 5.19). White clover showed the least inbreeding depression, whereas BC_1F_3 suffered the most depression due to selfing.

For most of the traits, selfing of the hybrids expressed significant inbreeding depression as compared to their crossed counterparts (Table 5.16). This effect was relatively more consistent for foliage production and its associated trait, the peduncle length. For a few traits, predominantly heads per plant, no significant differences were observed between selfed and crossed progenies. In a few cases even selfed progenies performed better than crossed progenies. Most of these cases were again for heads per plant. This showed that head production ability of a plant appeared to be less affected by inbreeding. No differences appeared among the second generation that were backcrossed or intercrossed. Inbreeding also decreased the harvested proportion of the potential seed yields (Figure 5.6). These results are consistent with the findings of Michaelson-Yeates et al. (1997) who selfed 15 self-compatible white clover plants for five generations. Eleven of these plants showed severe inbreeding depression, especially for seedling vigour and seed production, and the progeny could not survive. They compared the surviving four parental inbred lines, which were significantly diverse among themselves, with their F_1 hybrids. Varied degrees of heterosis were observed for the studied traits. Significant heterosis was observed for dry matter yield in all cases. F_1 hybrids were also superior to the mean performance of their parents for stolon and petiole length in the majority of cases. However, with only one exception, there were no significant differences for both vegetative bud and head production traits between parental mean values and the F_1 hybrid performance.

6.6 EFFECT OF DIFFERENT BREEDING STRATEGIES

Backcrossing and intercrossing based on phenotypic recurrent selection were used to create BC_1F_1, BC_1F_2, BC_1F_3, BC_2F_2-BI, BC_2F_2-IB, BC_2F_1 and BC_3F_1 generations. In the production of BC_2F_2-IB, plants of BC_1F_1 were first intercrossed to produce BC_1F_2 and then in the next year these BC_1F_2 were backcrossed with white clover. The generation BC_2F_2-BI was the product of the reverse crossing sequence, i.e. backcrossing in the first year and intercrossing in the second year. Both parental species i.e., white clover and *T. uniflorum* were included as controls in the comparison of the generations. The following discussion is based on comparisons shown in Figures 5.30 to 5.33. Figures other than these are mentioned specifically.

6.6.1 Parental species

The comparisons among parental species placed them at opposite poles and the hybrids intermediate between the parents for most of the traits (Figure 5.25, 5.27 and 5.30 – 5.33). White clover was the highest and *T. uniflorum* the lowest performer for florets per head, seeds per head, peduncle length, leaf area, foliage production, plant covered area, stolon and roots dry weight. *T. uniflorum* also showed the least potential for florets per plant and seeds per plant. In contrast, plants of *T. uniflorum* were the best for ovules per ovary, seeds per floret, heads per plant and seed weight among all studied generations. In non-reproductive traits, this species expressed highest stolon nodes per unit area, tap root diameter and woody tap root length. White clover, like *T. uniflorum*, was significantly better in seeds per floret than all hybrid generations. White clover also showed, though statistically not significant, better performance for pollen viability and stolons per unit area than other generations. White clover was generally low in heads per plant, seed weight and nodes per unit area of stolon as compared to most of the other generations.

T. uniflorum expressed a typical plant structure adapted to drought tolerance. Dry harsh climates promote energy allocation towards reproductive growth and away from vegetative growth (Hickman, 1975; Hickman and Pitelka, 1975). This plant species has restricted above ground growth with small leaves and has more focus for woody tap root development. Limited availability of soil surface moisture in dry climates discourages nodal root development. The lack of opportunity for vegetative propagation due to limited stolon growth and nodal roots make it more reliant on long term survival, supported by a central woody tap root system and production of high numbers of seeds per unit covered area. The high concentration of nodes per unit area of stolon and small plant stature allows development of only small flower heads i.e., only one to three florets per head. This small head size deficiency is compensated by production of high numbers of heads per plant, ovules per ovary, seeds per floret and big seeds. Big seed size is expected to enhance seedling vigour (Pandey et al., 1987). *T. uniflorum* has a very short peduncle which usually remains hidden under the stipule. The pedicel facilitates exposure of the flower above the leaf canopy. Even the combined length of the peduncle and pedicel is shorter than the adjacent petiole (Table 3.5). The flower head is exposed above the foliage canopy mainly due to the low angular orientation of the adjacent leaf, whereas the flower head remains erect before pollination. The pedicel of a deflexed flower bends down, taking the head under the foliage cover. This brings the head in contact with the soil surface and reduces light availability. In a dry climate, seeds can mature under the leaf canopy but in a temperate humid environment proximity to the moist soil can cause the retardation of seed maturation, decaying of immature seeds or loss of mature seeds under foliage canopy. This is the most probable reason for failing to harvest any mature seed from *T. uniflorum* under the temperate humid conditions of Palmerston North. The lifting of stolons up above the foliage canopy and soil surface resulted in more normal maturation and availability of some ripened seeds (Figure 5.5).

In contrast to *T. uniflorum,* white clover has vigorous growth supported by stolon and nodal root production due to its adaptation to a temperate humid climate. It allocates a smaller proportion of resources to reproductive (flowers and seeds) growth due to the availability of a vegetative strategy of propagation and survival through stolons (Turkington and Cavers, 1978). White clover breeding has focussed on foliage production and persistence as primary aims. Seed production is a secondary objective in cultivar development and utilization (Davies, 1984; Pyke et al., 2004; Abberton and Marshall, 2005). Consequently at the plant level, the majority of the nodes are used for stolon production, giving fewer reproductive nodes producing few flower heads. The lower number is compensated by the large size of the heads. The peduncle is generally longer than the petiole of the adjacent leaf and keeps the flower head well above the foliage canopy, resulting in normal seed maturation and harvestability in white clover.

Both parental species are reported to be self-incompatible (Pandey, 1957). They showed higher pollen viability and seeds per floret than all hybrid populations. However, well developed embryos of *T. uniflorum* could not be harvested in mature form. The reasons have been discussed in section 1.2. White clover genotypes appeared to be more variable than *T. uniflorum* plants for the majority of the traits (Figure 5.28).

The comparison between *T. uniflorum* and white clover in this study showed that the more vigorous plant growth of white clover with fewer but bigger heads made it a better seed producer than *T. uniflorum* on a per plant basis. Very few florets on a head of *T.*

uniflorum can improve nutrient allocation, resulting in higher seeds per floret, however the small peduncles can reduce the pollinator's visit and harvestibility of the mature heads.

6.6.2 Hybrid generations

Different breeding strategies successfully created variation among mean performances of hybrid generations for almost all studied traits. The exceptions were woody tap root length and stolon density per unit area. Tap root diameter was measured at the base of the crown and *T. uniflorum* was found to have considerably thicker tap roots than the hybrids. (Nichols, 2012) studied the same interspecific hybrids and parental species under field conditions at Lincoln. She also observed no significant difference among *T. uniflorum*, BC_1F_1, BC_2F_1 and white clover for tap root diameter at the base of the crown. However, she reported a decrease in tap root diameter in the order of: *T. uniflorum* > BC_1 > BC_2 > white clover. The difference became wider and more significant with increase in distance from the crown. The significantly thicker tap root of *T. uniflorum* in the current study might be due to the use of potting mix instead of soil.

6.6.2.1 First backcross (BC_1F_1)

The BC_1F_1 hybrid theoretically contained 25% of the *T. uniflorum* (donor parent) genome. This was the hybrid generation with the greatest loss of the donor parent (50% to 25%) and so plants of this generation were expected to show the most visible signs of elimination of the donor parent phenotype. This happened for the traits including seed weight, stolons per unit area, plant covered area, root dry weight, tap root diameter and woody tap root length. The rapid shift away from the donor parent could also be attributed to a speedy recovery of interspecific hybrid towards its recurrent parent i.e., white clover, caused by a more rapid elimination of *T. uniflorum* genes than expected, for these traits. For most of the traits i.e., florets per head, seeds per head, florets per plant, seeds per plant, leaf area and foliage production, nodes per unit area of stolon and root to shoot ratio, this generation remained significantly intermediate between the donor parent and, other advance hybrid generations and the recurrent parent. Williams and Hussain (2008) observed the best expression of root thickness trait of the donor parent (*T. ambiguum*) in the BC_1F_1 and BC_1F_3 generations of interspecific hybrid between *T. ambiguum* and white clover. This is in contrast to findings of the current study where BC_1F_1 could not retain root traits of the donor parent. Both of these studies were based on unselected plant material.

This BC_1F_1 generation showed two important outcomes. The first outcome was the production of fewer seeds per floret than both parents. This trend was also present in all other hybrid generations to varying degrees. It might have been due to genetic disturbances caused by the interspecific nature of the cross or, alternatively, to the inheritance of poor seed maturation ability of the *T. uniflorum* parent under the humid temperate (Palmerston North) conditions. The BC_1F_1 generation was expected to express more of the high seeds per floret ability of the *T. uniflorum* than the advanced hybrids which, on average, had less *T. uniflorum* genetics. Most of the BC_1F_1 families studied in experiment-1 had peduncle to petiole ratios of less than one (Table 3.5). This showed that the flowers could not get above the plant canopy unless they were large enough to overcome the short peduncle length, or the orientation of the head was much more vertical than the adjacent leaf. Successful seed production required long and strong peduncles to keep the flower heads above the foliage canopy for an extended period of time to facilitate efficient pollination, seed maturation and harvestability (Marshall, 1995). Reduction in light intensity has been reported to have a negative effect on the fertility of ovules and their development into mature seed (Pasumarty and Thomas, 1990). Shading effect triggers elongation in peduncles resulting in increased allocation of photoassimilates to the peduncle at the expense of the flower head (Pasumarty et al., 1991). In the current study, the BC_1F_1 plants might have reduced ovule fertility, pollination and successful development of the ovules into mature seeds due to their short peduncles which kept them under foliage canopy.

The second important outcome and unexpected, result in these BC_1F_1 hybrids was the low head production, similar to that of the recurrent white clover parent. Nichols (2012) reported the same finding while working on similar BC_1F_1 plant material during 2008-09. In the following year a subset of the BC_1F_1 population produced more flowers than white clover. The difference in results might have been caused by the different selection of BC_1F_1 plants used in 2009-10 trial. However, even in the trial with BC_1F_1 hybrids producing more flowers than white clover, the BC_1F_1 plants produced fewer flowers than BC_2F_1 plants. Higher flower production was a trait of the *T. uniflorum* parent and it was expected to decline with the reduction of the *T. uniflorum* genomic proportion from the BC_1F_1 to BC_2F_1. This result suggested that the high flowering potential of *T. uniflorum* was suppressed in the BC_1F_1 generation. It cannot be attributed to the more rapid elimination of *T. uniflorum* genes than expected as, in both the current study and the study of Nichols (2012) the advanced backcrossed and intercrossed hybrid generations showed head production better than the BC_1F_1 and the recurrent parent. For some unclear reason head production ability became suppressed in the BC_1F_1 generation but was successfully recovered by selection in the advanced hybrid generations.

White clover **_T. uniflorum_**

F_1

BC_1F_1

Plate 6.2. Peduncle length and position of inflorescence in relation to the foliage canopy.

Both seeds per floret and heads per plant are the most important contributors in seed production of a plant. However, their low expression in the most basic hybrid generation of this study (BC_1F_1) may be taken as a post reproductive barrier, limiting the propagation ability of the interspecific hybrids. The recovery of these traits in successive generations showed the efficiency of the selection procedure in overcoming this post fertilization barrier. Li et al. (1997) observed that incompatible epistatic gene interactions resulted in post reproduction barriers by affecting fitness traits like heading date and floret number per panicle in F_4 interspecific hybrids between *Oryza indica* and *O. sativa*.

6.6.2.2 Second backcrossed and intercrossed generations (BC_2F_1 & BC_1F_2)

Plants of BC_1F_2 generation theoretically retained 25% *T. uniflorum* genome, whereas the BC_2F_1 plants were expected to retain 12.5% of the *T. uniflorum* genome. These generations showed inverse trends for two important seed traits i.e., head size and numbers. Backcrossing improved the head size and traits like foliage production, leaf area and peduncle length which is associated with head size in path analysis (Figure 5.7) and PCA results (Table 5.7). Intercrossing improved heads per plant. Almost the same relationships were observed between these two generations for most of the traits in experiment-2 (Figure 4.11 and 4.12). In experiment-3 both generations expressed higher seeds per floret potential than their preceding and following hybrid generations. The results showed that second intercrossed and backcrossed population were better for seeds per plant than their next generations. Their mean seed production per plant was also similar to that of white clover. Williams and Hussain (2008) also found the best flower production was shown by the BC_2F_1 generation in interspecific hybrids between *T. ambiguum* and white clover.

6.6.2.3 Intercrossed generations (BC_1F_1 to BC_1F_3)

The BC_1F_1, BC_1F_2 and BC_1F_3 generations theoretically had the same proportion of donor chromosomes as that in BC_1F_1 generation i.e., 25%. Being intercrossed these generations had progressive mixing of genomes of the two parental species in each breeding cycle expected to result in genetic recombination. Intercrossing was expected to increase variation from BC_1F_1 to BC_1F_3 due to creation of new genetic combinations and segregation. This phenomenon was prominent in all traits except for seed weight and root to shoot ratio. Increase in variation was progressive from BC_1F_1 to BC_1F_3, despite the application of selection pressure beyond BC_1F_1 which was mainly for improved seed traits. This increased variation provided scope for selecting new combinations of favourable genes having complementary effects and resulting in superior transgressive segregates. Hussain and Williams (2008) also observed significant positive variation among segregating generations of white clover x *T. uniflorum* interspecific hybrid. Significant improvement from BC_1F_2 to BC_1F_3 was observed for florets per head and its associated trait of peduncle length. Foliage production also showed the same trend but this was not statistically significant.

Selection was made for traits in favour of seed production at the end of every experiment. The worst plants in that respect were always ignored for the next experiment. The comparison between progenies of high and low performing plants was actually a comparison between progenies of high and medium performing plants (Table 5.19). This selection pattern always improved seed production traits and shifted the population towards the better seed producing parent, white clover. Bigger head size was the most prominent seed production attribute of white clover. It showed significant improvement from BC_1F_1 to BC_1F_3. Traits allied with head size i.e., seeds per head, florets per plant, seeds per plant, peduncle length, foliage production and plant covered area, showed significant improvement with progression of intercrossing. Higher head numbers per plant was a trait of *T. uniflorum*. This trait significantly declined from BC_1F_2 to BC_1F_3. The BC_1F_1 generation showed lower head production than the BC_1F_2 generation which expressed the highest mean head production among all hybrid generations. The second highest generation in this respect was BC_2F_2-BI. Both of these generations had the common feature of first backcrossing and then intercrossing. This indicated that white clover suppressed higher head production in backcrossed hybrids, which was recovered by a later cycle of intercrossing among the hybrids. However, this reason should have made BC_1F_3 the highest head producing generation, which was not the case. Another reason might be that BC_1F_1 plants were unselected and expressed a moderate level of post reproductive barrier to interspecific hybridization success by reduced head production. The most advance intercrossed generation i.e., BC_1F_3 showed best pollen stainability and self-compatibility. This is consistent with findings of Williams and Hussain (2008) with the white clover x *T. ambiguum* hybrids.

6.6.2.4 Backcrossed and intercrossed generations (BC_2F_2-BI and BC_2F_2-IB)

The generations BC_2F_2-IB and BC_2F_2-BI contained the same theoretical mean genomic composition (12.5% *T. uniflorum* : 87.5% white clover) but differed in the alternate sequence of backcrossing and intercrossing used in their formation and in the use of different white clover cultivars as recurrent parents. The strategy of first intercrossing and then backcrossing created more variation than the alternative pattern for most of the studied traits. Higher variation in BC_2F_2-IB was important as these families had more chances of interspecific introgression due to the presence (on average) of 25% of the *T. uniflorum* genome at the time of intercrossing.

The typical inverse relationship between head numbers and size was apparent in comparisons of these two formations. The mean performance of BC_2F_2-IB was significantly superior for head size and inferior for head number to that of BC_2F_2-BI. The better performance of BC_2F_2-IB for head size also applied to foliage production, peduncle length, leaf area, root to shoot ratio and woody tap root length. Except for the last one, all these traits were associated with superior head size of white clover. The better head size of white clover plants used in the production of BC_2F_2-IB than white clover plants used in BC_2F_2-BI might have contributed in this relationship. For the remainder of the studied traits, there were no significant differences between mean values of these two generations. In general, any of these mixed crossing generations did not outperform pure intercrossed or backcrossed generations for any traits except stolon dry weight. However, better introgression chances, higher variation and head size of the BC_2F_2-IB generation, made it more attractive for selection as compared to the BC_2F_2-BI generation.

6.6.2.5 Backcrossed generations (BC_1F_1 to BC_3F_1)

The BC_1F_1 BC_2F_1 and BC_3F_1 theoretically contained 25%, 12.5% and 6.25% genomic proportion of the donor parent, respectively. Contrary to the intercrossed generations, they were expected to express more phenotypic resemblance to their recurrent parent i.e., white clover. This was observed for florets per head, self-compatibility, peduncle length, leaf area and foliage production traits. Although these generations showed a progression, they were significantly lower than white clover for seeds per floret, seeds per head and stolon dry weight. Both the BC_2F_1 and BC_3F_1 were significantly better than white clover for heads per plant and seed weight. Higher numbers of heads per plant of BC_3F_1 were counter-intuitive, because the amount of *T. uniflorum* was only 6% and yet the influence was still large. This indicates possible introgression of this ability from *T. uniflorum* into white clover

Backcrossing increased the genomic proportion of white clover in the hybrids and coupled with the selection process generally led hybrids closer to white clover. Moreover, the use of the large head sized Will-2 and AR-802 plants as recurrent white clover parents in the production of the BC_3F_1 focussed on improvement of head size. This emphasis on improvement of head size was combined with inherited trait of high head production from *T. uniflorum* parent and resulted in significantly higher florets per plant in BC_3F_1 than the recurrent parent i.e., white clover.

For most of the studied traits the variation within a generation increased with the progression of backcrossing. This showed that increase in genomic proportion of white clover had effectively increased the variation. This is inconsistent with the expectations that loss of *T. uniflorum* alleles should lead to reduced variation. Increase in the variation was more visible from BC_2F_1 to BC_3F_1 than from BC_1F_1 to BC_2F_1. This might be due to larger sample size differences between BC_2F_1 and BC_3F_1, whereas both BC_1F_1 and BC_2F_1 had similar and small sample sizes. Nichols (2012) reported similar findings in comparisons among BC_1F_1, BC_2F_1 and parental species for heads per plant, peduncle length, leaf area and foliage production. However, she found non-significant difference for head production between white clover and the BC_1F_1. In the current study the BC_1F_1 produced significantly fewer heads than white clover. This contradiction could be due to differences in plant genotypes and environmental conditions of the two studies as explained earlier.

6.6.3 Important generations

The fertile and hybrid nature of unselected plants of the BC_1F_1 generation for most of the traits showed evidence of compatibility and chances of introgression in this interspecific hybridization. Both recurrent crossing strategies i.e., intercrossing and backcrossing, remained effective for the improvement in most of the reproductive and vegetative traits. The strategies mainly recovered the white clover phenotype. Backcrossing improved head size in particular. It also retained the better head producing ability of the *T. uniflorum* parent to some extent. This resulted in similar mean seed production of BC_2F_1 and BC_3F_1 as that of white clover. Despite the small sample sizes and presence of selection pressure, an increase in variation was observed among segregating generation for valuable traits like numbers of heads per plant, florets per plant, seeds per plant, ovules per ovary and self-compatibility (Figures 5.30 – 5.33). The diversity analysis also showed that BC_1F_3 was more diverse than BC_3F_1 for most of the seed production traits (Figure 5.28). This revealed the potential for selection for improved recombined interspecific genotypes. The second backcrossed and intercrossed generations expressed high production of seeds at both floret and plant level, along with reasonable variation. This indicated potential of getting desirable genotypes in second backcrossed and intercrossed generations. Marshall et al. (1998) also recommended applying selection pressure in the BC_2F_1 generation to gain improvement in economic as well as reproductive traits of the interspecific hybrids.

Marshall et al. (2002) observed that in white clover x *T. nigrescens* hybrids improved head size and numbers did not result in the backcrossed generations being better seed producers than their recurrent parent white clover. Number of seeds per floret and their harvestability were found to be important contributors in determining seed production. The current study also indicated that the real problem in seed production was the inability to materialize the seed setting potential. All hybrid generations remained significantly poor in seed setting per floret. Environmental factors seemed to be important, as nutrition and pollination were normal. Mature seeds could not be harvested from *T. uniflorum* under the humid temperate climate of Palmerston North. This adaptation problem of the *T. uniflorum* parent might have been inherited into the hybrids and their complex interspecific genetics might have further aggravated the already established issue of ovule infertility and early abortion. The potential for numbers of seeds per floret of *T. uniflorum* was much higher than that of white clover. If the hybrids had retained this ability, even at the white clover level, the seed production of BC_1F_2, BC_2F_1 and BC_3F_1 would have been significantly better than white clover as all of these hybrids also showed higher numbers of heads and florets per plant than white clover.

Chapter 7

CONCLUSIONS

7.1 CONCLUSIONS

The dry climatic origin and woody tap root system of *T. uniflorum* makes it a valuable source for the improvement of the drought tolerance ability of white clover through wide hybridization. The small head size of *T. uniflorum* and interspecific genetics result in poor seed production of the early hybrids. This serious hurdle for wide dissemination and commercial success of these hybrids can be overcome by recurrent intercrossing and backcrossing.

Vigorous plant growth of white clover with fewer but bigger heads make it a better seed producer than *T. uniflorum*. The few florets per head of *T. uniflorum* produce more ovules per ovary. However, its short peduncle is not only unfavourable for pollinator visits and harvestability of the mature head, but also fails to keep the flower head at an adequate height above the plant canopy and soil surface to prevent immature seed loss under humid temperate conditions. The interspecific hybrids of these two species expressed significant variation at both, the generation and the family levels for most of the traits. No cytoplasmic influence appeared in expression of the traits, eliminating any risk of significant difference in these hybrids caused by the direction of the crosses. The hybrids produce viable gametes and showed "leakage" (partial breakdown) of the self-incompatibility mechanism.

Heads per plant was found to be the most influential trait in seed production. This trait showed moderate-high heritability. Higher nodes per stolon enhanced head numbers but were associated with reduced stolon production which could possibly limit persistence of the plant. The trait of seeds per floret was an effective contributor to seed production. This is a much emphasized trait for improvement in seed production, as infertility of ovules and embryo abortion are well established causes of the difference between potential and actual seed set in white clover. This problem was also observed in the hybrids, with a higher intensity. The seeds per floret trait showed no negative association with ovule and head production. Its moderate heritability indicated that genetic as well as environmental factors were influential and selection might be less effective to improve this trait, compared to head size and head numbers.

Florets per head (head size) was very influential trait in seed production. Although this trait exhibited a negative association with ovule production, it showed positive relationship with seeds per floret. This trait showed the highest heritability, making it the most responsive to selection. It had a slight negative association with head numbers. Selection for head size could spare more nodes for stolon production resulting in improved persistence. However, the absence of a strong negative association between head size and number showed potential of this interspecific hybridization to improve seed production by combining high head production ability of *T. uniflorum* with the large head size of white clover. The results showed that families with acceptable combinations of these traits along with seeds per floret could be identified. Improvement in peduncle length was associated with head size, leaf size and foliage production. However, this trait had low heritability. Selection based on seeds per head and seeds per plant can be less responsive due to their derived nature and moderate to low-moderate heritabilities. The trait of flowering pattern showed considerable variation and heritability, providing opportunities to select early, medium and late flowering genotypes, according to the environmental and management needs.

No negative correlation was apparent between root and seed production traits. Tap root diameter, nodal root diameter and woody tap root length were positively associated with each other. Nodes per stolon promoted head production but were negatively associated with stolon production. Leaf area was positively associated with head size. Foliage production was associated with size as well as number of heads.

The number of heads per plant and seeds per floret were lower than expected in the BC_1F_1 generation. However, further crossing and selection recovered these traits in advanced hybrids. This BC_1F_1 generation was the most inferior as well as the most responsive to selection for most of the seed traits, foliage production, leaf area and stolon dry weight. Intercrossing coupled with selection increased variation, pollen stainability, self-compatibility and realization of potential seed yield of the hybrids. It also improved heterozygosity resulting in increased inbreeding depression of BC_1F_3 plants as compared to white clover. First intercrossing and then backcrossing (BC_2F_2-IB) of BC_1F_1 plants created more variation than the alternative pattern (BC_2F_2-BI). Backcrossing coupled with recurrent selection recovered head size, peduncle length, leaf area, foliage production and self-incompatibility of the hybrids. It also improved variation in the hybrids. Bigger head sizes coupled with more heads per plant, an ability possibly retained from *T. uniflorum*, made the BC_2F_1 and BC_3F_1 generations significantly superior in florets per plant than BC_1F_1, BC_1F_3 and white clover. The second backcrossed and intercrossed generations produced high numbers of seeds per floret and plant. However, selection for these traits could be less responsive due to their low heritabilities.

The backcrossing and intercrossing coupled with recurrent selection resulted in an overall improvement in seed production traits. The shift in population distribution, with each breeding cycle, showed a difference between the practical outcome with selection on a limited population (Figure 7.1) and theoretical output without selection for florets per head in a large population (Figure 1.2).

Both the second backcrossed and intercrossed generations are expected to have acceptable level of fertility and, expression and variability of seed production traits. A large population of these generations should be subjected to drought stress. From the surviving population, the plants having better seeds per floret and, number and sizes of flower heads should be used for further crossing. The BC_3F_1 generation is capable of showing seed production traits similar to white clover. Having chances of retaining an average of only 6% *T. uniflorum* genome, this generation should be subjected to field evaluation for drought tolerance. Plants of BC_1F_3 generation would have undergone three cycles of genomic recombination of the parental species. These should also be screened for drought tolerance as well as root traits desired to be introgressed from *T. uniflorum*.

White clover and *T .uniflorum* have contrasting growth habits and habitat adaptations. Their primary interspecific hybrids significantly lose seed production. However, this wide hybridization creates a wide range of variation. This variation enables both reproductive and vegetative traits to be improved by recurrent crossing and selection. Important seed production traits of head number and size have reasonably high heritabilities, whereas seeds per floret is a moderately heritable trait. High selection pressure on the available extensive variation for these traits can restore seed production ability of the hybrids.

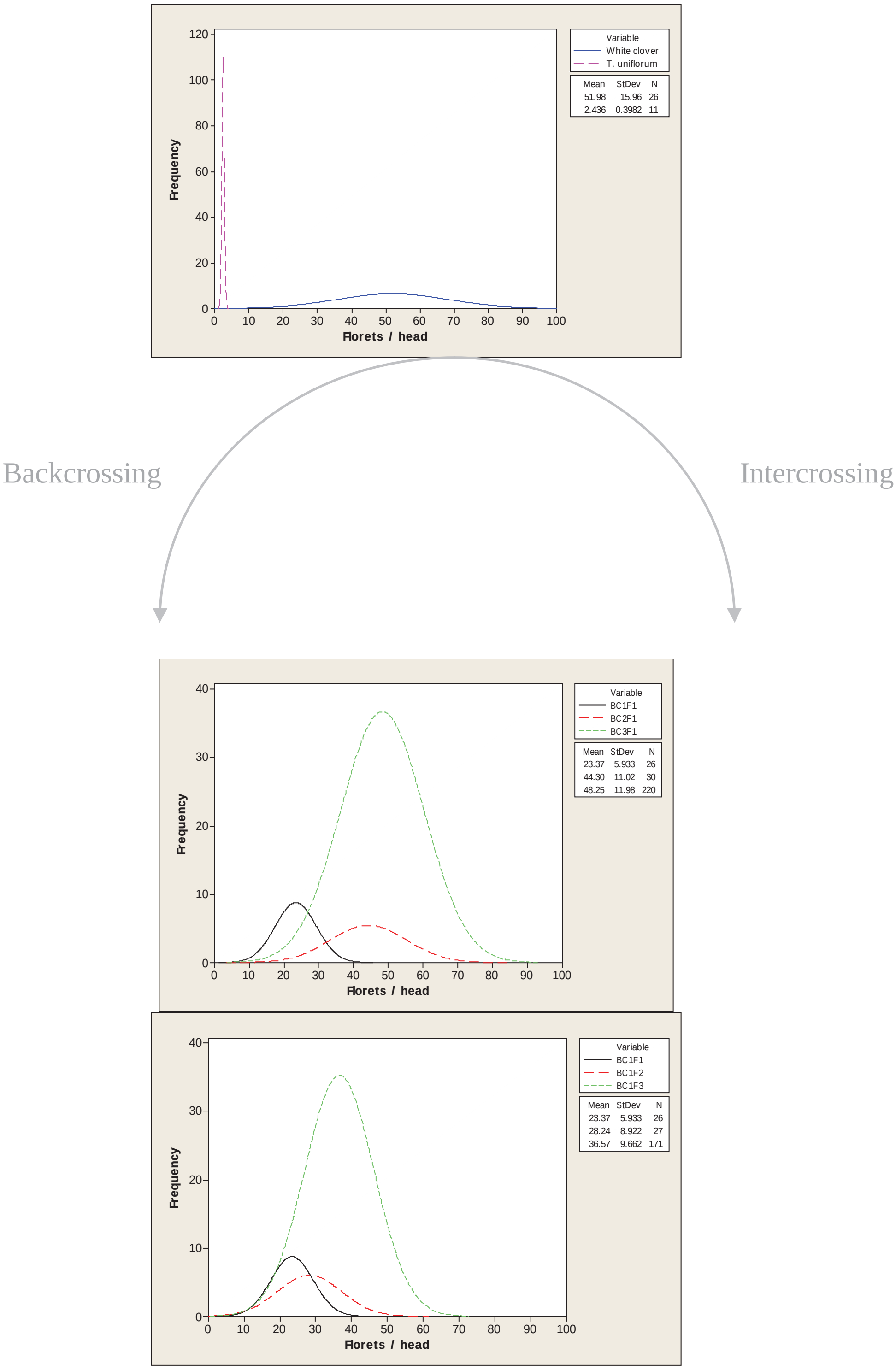


Figure 7.1. Population distributions for florets per head. The distribution is derived from histograms with fit distribution.

No negative correlation between root and seed production traits was observed in these hybrids, showing the possibility of selection for a drought tolerant hybrid with acceptable seed production. Such a hybrid will be a highly valuable product for agricultural production and seed sales in drought affected areas.

7.2 FUTURE WORK

Future work could explore the following areas:

1. Performance of the advance hybrid generations in field conditions under drought stress and in competition with grasses.
2. Seed production of *T. uniflorum* with special emphasis on seed maturation and harvestability. There were indications in the present work that *T. uniflorum* seeds did not mature in the humid environment of Palmerston North, possibly because excessive moisture prevented adequate seed drying and maturation.
3. Study of the BC_1F_1 generation to investigate causes of its fewer seeds per floret than both parents and low head production similar to that of white clover.
4. Leakage of self-incompatibility mechanism in the hybrids.
5. Causes of ovule infertility and embryo abortion to reduce difference between potential and actual seed yield.
6. QTL study for head size.
7. The evolutionary aspects of differences in plant structure of white clover and *T. uniflorum*.

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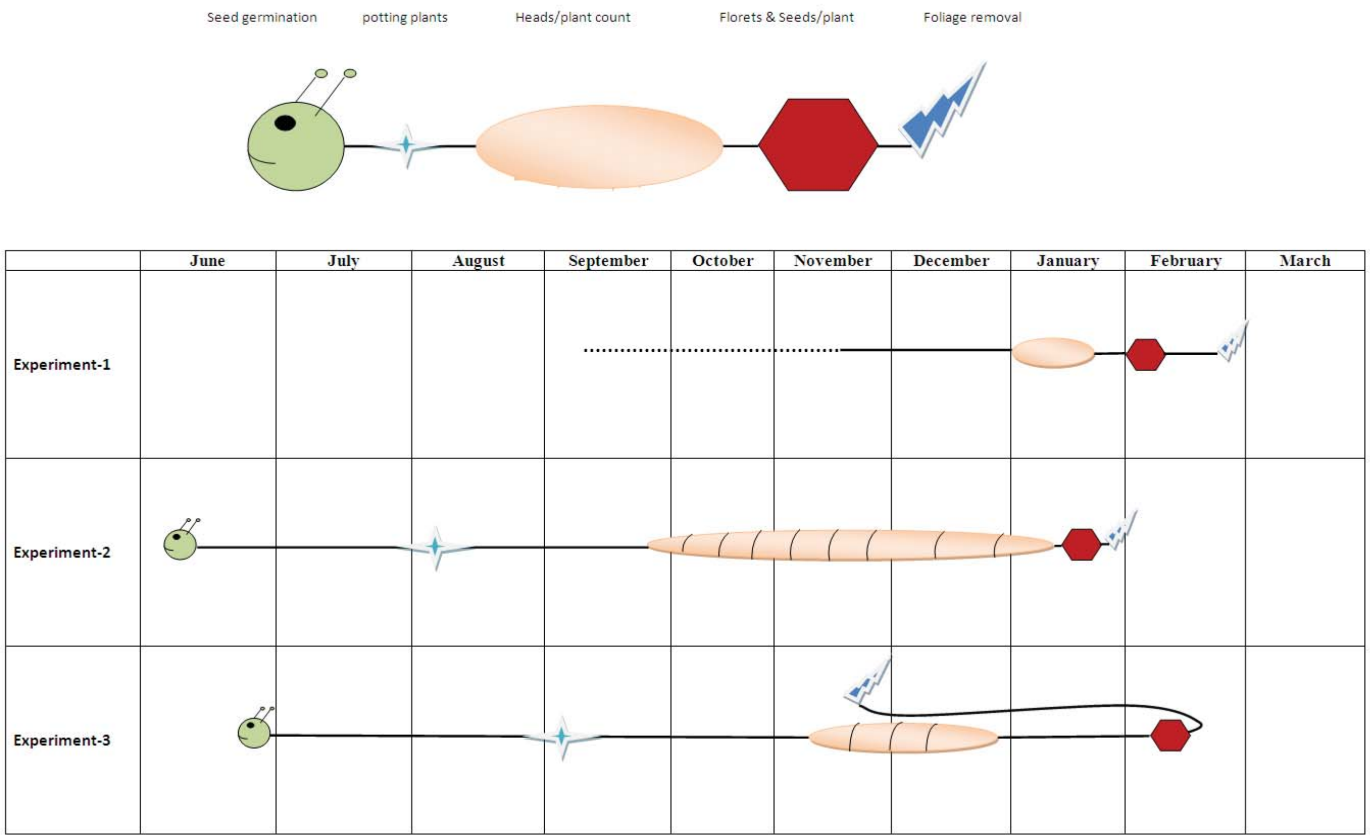
APPENDICES

Appendix 1. Sequence of main steps for all experiments. The experiment-1 started in November 2008 with plant cuttings received from Lincoln. The figure at top shows experimental stages as represented by the body parts of an imaginary insect.

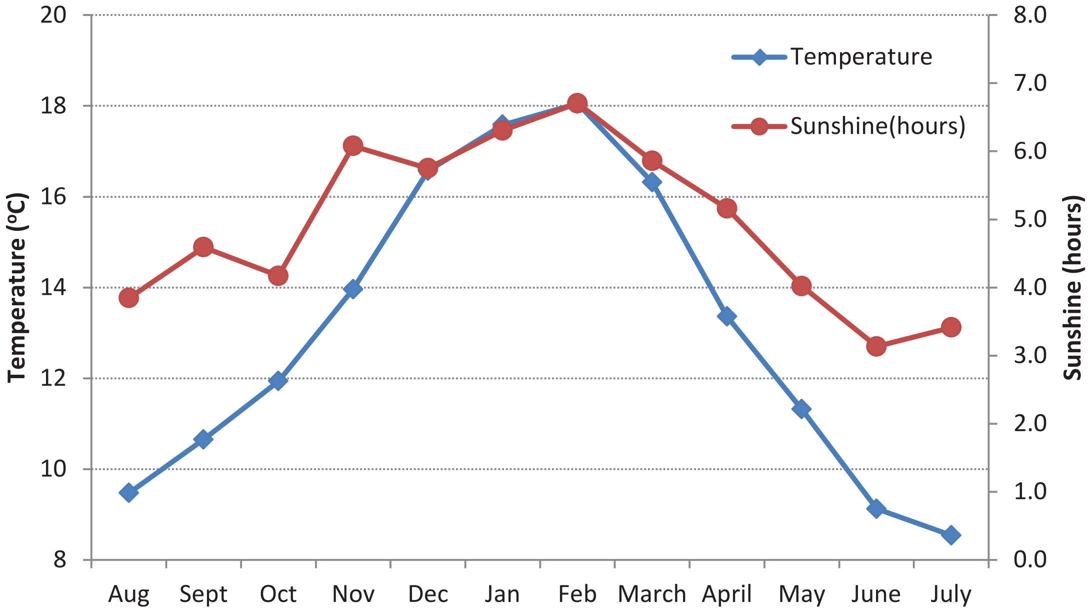


Appendix 2. Details of families involved in the study of inheritance of flowering pattern.

Category	Family	Generation	Pedigree	
			female	male
EARLY	8	BC_1F_3	{Barbalanca x (Kopull-2, Pitau-1 x T66-6)} X {Barbalanca x (Kopull-2, Pitau-1 x T66-6)}	{T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)} X {T3L607, -608, -609 X (Kopull-2 x Az4383-11)}
	30	BC_2F_2	{D44} X {Aran x (Aran-, Tribute-1 x T66-3}	{D57} X {Aran x (Kopull-2, Pitau-1 x T66-6)}
	38	BC_1F_3	{Aran x (Kopull-2, Pitau-1 x T66-6)} X {Aran x (Aran-, Tribute-1 x T66-3)}	{T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)} X {T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)}
	41	BC_2F_2	{Ch5} X {Crau x (Pitau-1, Sustain-1 x T66-6)}	{D45} X {Aran x (Kopull-2, Pitau-1 x T66-6)}
	43	BC_2F_2	{D44} X {Aran x (Aran-, Tribute-1 x T66-3)}	{L3} X {Crau X (Kopull-2 x Az4383-11)}
	51	BC_2F_2	{D57} X {Aran x (Kopull-2, Pitau-1 x T66-6)}	{Ch5} X {Crau x (Pitau-1, Sustain-1 x T66-6)}
	66	BC_2F_2	{D44} X {Aran x (Aran-, Tribute-1 x T66-3)}	{D44} X {Aran x (Kopull-2, Pitau-1 x T66-6)}

	86	BC_1F_3	{Barbalanca x (Kopull-2, Pitau-1 x T66-6)} X {Barbalanca x (Kopull-2, Pitau-1 x T66-6)}	{Crusader x (Kopull-2 x Az4383-11)} X {Crusader x (Sustain-1x Az4437-3)}
MEDIUM	50	BC_1F_3	{Aran x (Kopull-2, Pitau-1 x T66-6)} X {Aran x (Kopull-2, Pitau-1 x T66-6)}	{Crusader x (Kopull-2 x Az4383-11)} X {Crusader x (Kopull-2 x Az4383-11)}
	52	BC_1F_3	{T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)} X {T3L607, -608 X (Sustain-1, Tribute-1 x T66-3)}	{Aran x (Kopull-2, Pitau-1 x T66-6)} X {Aran x (Kopull-2, Pitau-1 x T66-6)}
	62	BC_1F_3	{Crusader x (Sustain-1x Az4437-3)} X {Crusader x (Kopull-2 x Az4383-11)}	{T3L607, -608 X (Sustain-1, Tribute-1 x T66-3)} X {T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)}
	72	BC_2F_2	{A28} X {Crau x (Pitau-1, Sustain-1 x T66-6)}	{Ch4} X {Aran x (Kopull-2, Pitau-1 x T66-6)}
	74	BC_2F_2	{L3} X {Trophy x (Kopull-2, Pitau-1 x T66-6)}	{D21} X {Aran x (Kopull-2, Pitau-1 x T66-6)}
LATE	20	BC_2F_2	{L4} X {T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)}	{L5} X {Trophy x (Sustain-1 x Az4437-3)}
	48	BC_2F_2	{Ch6} X {Crau X (Kopull-2 x Az4383-11)}	{L4} X {T3L601, -607, -608, -609 X (Kopull-2, Pitau-1 X T66-6)}

Appendix 3. Mean daily temperature and sunshine data for 2007-2011.



Appendix 4. Mean daily temperature and rainfall data for 2007-2011.