Genotypic Variation among a Diverse Collection of Red Clover (*Trifolium pratense* L.) Germplasm and *In-vitro* Techniques for Screening Resistance to *Sclerotinia trifoliorum* Erikks in Red Clover

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ABSTRACT

Red clover (*Trifolium pratense* L.) is the world's second-ranking most important forage species after alfalfa (*Medicago sativa* L.) based on seed volumes produced, marketed and cultivar availability. In New Zealand, red clover account for 23% of legume sales by volume after white clover (62%) and contributes significantly to primary industries by providing high quality feed for livestock. However, production is currently constrained by several limiting factors. In order to address some of these limitations, there is a need to broaden the genetic base of existing cultivars through breeding by introducing sources of genetic diversity from germplasm representing different geographical regions.

In the study presented, two major experiments were carried out aiming to investigate:

1. The genetic diversity among 40 selected world source of red clover germplasm together with 3 local cultivars for ten important morphological traits under field condition across three seasons. Univariate and multivariate analysis were used to estimate genotypic variation for each trait and assess inter-relationships for a range of traits respectively so as to identify distinct germplasm accessions based on seasonal morphological measurements. Results from variance component analysis indicate significant genotypic variation as well as moderate to high repeatability among the yield related traits. This indicates potential underlying additive genetic variation among the 40 germplasm accessions for yield related morphological traits.

2. The response of eleven selected New Zealand commercial cultivars to clover rot disease (*Sclerotinia trifoliorum* Erikss) through artificial inoculation under high disease pressure in glasshouse to identify source of resistance for further breeding purposes. In order to facilitate the glasshouse artificial inoculation and identify the sources of resistance, this

experiment used the *in-vitro* culturing procedures for production of *S. trifoliorum* ascospores to inoculate red clover plants using two locally sourced *S. trifoliorum* isolates. Although *in-vitro* production of sclerotia has been successful, our attempt to produce ascospores was not possible leading to delay in artificial inoculation of plants in glasshouse. This result suggests the need to improve on *in-vitro* techniques for ascospore production, understand appropriate culture condition and determine right choice of *S. trifoliorum* isolate to facilitate fertilization.

Generally, both experiments under this study has provided valuable source of information and identify potential untapped germplasm material for future prospects of red clover breeding to develop new cultivars with improved yield, persistence and other desirable traits suitable for New Zealand condition.

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Chapter 1.0 GENERAL INTRODUCTION

1.1 Introduction

Red clover (*Trifolium pratense* L.) is the world's second-ranking most important forage species after alfalfa (*Medicago sativa* L.) based on seed volumes produced, marketed and cultivar availability (*Boller et al*., 2010). It is an important forage crop in temperate regions around the world that provides high quality feed for livestock. Red clover has high yield potential as well as the ability to restore soil nitrogen and resist drought under pure or mixed pasture swards.

In New Zealand (NZ), red clover account for 23% of legume sales by volume followed by alfalfa with nearly 12% of total volume (Morrison, 2017). Red clover contributes significantly in the mixed sward pastures but it is not as popular as the white clover (Martin, 2014) that comprises 62% of legume sales (Morrison, 2017). New Zealand's grazing system is traditionally dominated by a mixture of perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.). However, white clover production is often scarce under prolonged dry summer particularly in drought prone areas (Martin, 2014). It is also predicted that NZ will experience significant warming over the next 50 or more years with frequency of severe drought expected across many parts of the country (Mullen *et al*., 2008, Nottage *et al*., 2010). Livestock grazing in NZ dominates land use by area (Watt *et al*., 2008). These changes are expected to have significant negative effect on pasture production, thus will test the adaptability of farmers to diversify pasture species and shift production zones within the region. Red clover's ability to persist through summer to produce adequate yield and feed quality is an important criterion that makes it an ideal forage species for short-term pasture for livestock fattening as compared to white clover (Kemp *et al*., 1999). It is also a suitable alternative forage species to sustain productivity of pastoral farming in NZ to mitigate impacts of changing climate, particularly with increased frequency of high temperatures.

Despite the importance of red clover in NZ, limitations such as poor persistence, shorter lifespan, poor plant survival particularly in mixed pasture under grazing pressure and poor seed yield are potential problems (Martin, 2014; Ford & Barrett, 2011). Infertility issues in ewes and bloat in cattle feeding on red clover mainly under pure swards are additional limiting factors (Frame, 2005; Ford & Barrett, 2011). Breeding efforts to improve red clover yield and persistency for New Zealand's grazing systems began in the 1930s with some significant progress made so far (Ford & Barrett, 2011). Attempts to select cultivars suitable for NZ in terms of persistency under grazing and high forage yield remains a major challenge. Likewise, resistance to fungal diseases and low oestrogenic levels are additional challenges in order to make red clover competitive against other forage legumes.

The overall progress in genetic improvement of red clover characters is slow due to little attention given to understanding their genetics and inheritance, interrelationship between characters, environmental effects on characters (Hossein, 1993) as well as genetic variability in conserved germplasm. Furthermore, there is need to broaden the genetic base within breeding programs by introducing new germplasm to facilitate the development of new cultivars with improved yield, persistency and disease resistance.

While persistence is currently the number one issue that needs to be tackled with some progress already made (Ford & Barrett, 2011), other problems are being addressed by various breeding programmes around the world including NZ. Utilization of international germplasm have so far made positive progress in improving yield and persistency under mixed swards in recent NZ cultivars such as Relish, Broadway and Sensation (Claydon *et al*., 2003). The persistency and high yield of red clover under heavy grazing in mixed swards as well as minimizing economic diseases of red clover such as clover rot in pure swards are important

considerations for improving productivity of red clover in NZ (Ford & Barrett, 2011). Characterization of diverse red clover germplasm accessions with origin from different geographical regions of the world provides the opportunity to identify suitable germplasm for developing cultivars with improved performance and persistency.

Although, red clover is ideal for summer production in pure swards for short-term pasture and conservation feeds, intensification in production can possibly trigger outbreak of certain pests and diseases such as the clover rot disease (*Sclerotinia trifoliorum* Erikss.). Besides, incidence of existing pests and diseases are likely to increase in the next decades and onwards due to unpredictable changes in New Zealand's weather patterns caused by climate change (Nottage *et al*., 2010, Hennessy *et al*., 2007). Development of Sclerotinia pathogen is highly sensitive to variation in environmental condition, thus, any change in climatic condition that is right for the pathogen could positively prompt the clover rot epidemic.

Clover rot is known to be widespread in the northern hemisphere particularly in European and Mediterranean regions where red clover originates (Bolton *et al*., 2006). Since most of the modern cultivars of red clover in NZ have their parentage from these regions (Taylor, 2008), it is important to know the clover rot susceptibility status of current commercial cultivars and germplasms for disease inheritance.

1.2 Experiment overview

From the genetic point of view, this investigation has taken into consideration a wide range of available red clover genetic diversity in NZ to evaluate for desired characteristics. The study was planned and executed in two experiments to identify potential parents with desirable genes for future prospects of red clover breeding. The first experiment was on genotypic variation among a set of 40 selected world-sourced accessions of red clover germplasm.

Second experiment was on screening for clover rot (Sclerotinia) resistance in 11 commercial red clover cultivars.

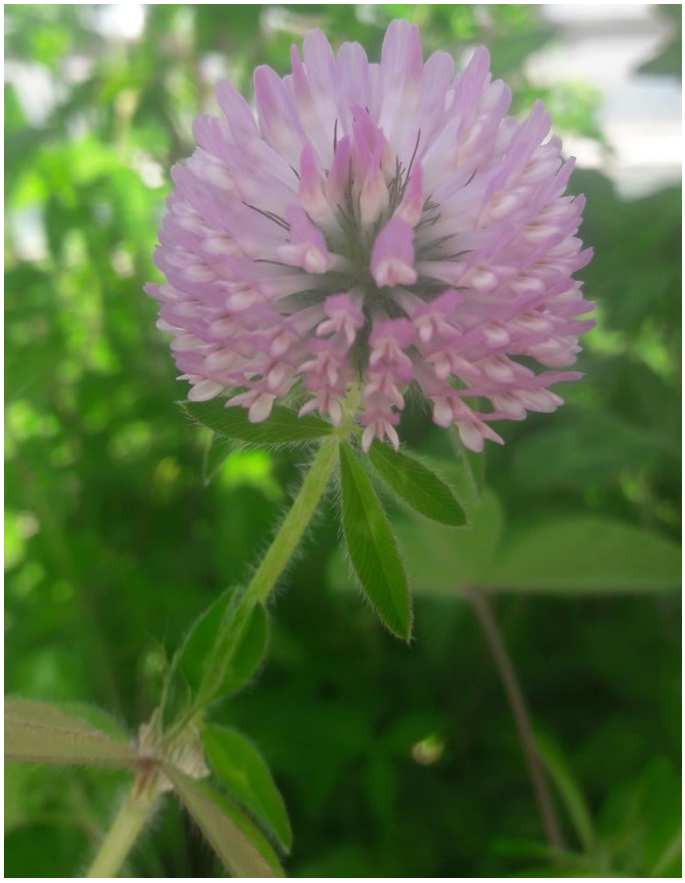
The overall aim of this study was to select diverse germplasm with high forage yield and resistance to Sclerotinia clover rot disease that is expected to form the basis for red clover cultivar development. The first study objective was to estimate the level of variation for key morphological traits among 40 red clover germplasm accessions, the relationship between them and the effect of the environment, in order to identify potentially valuable sources of genetic diversity for future breeding programs. The experiment was done specifically to generate information on genotypic mean performances, variance components, broad-sense heritability, correlation between characters and clustering of accessions based on yield related traits and morphological characteristics. The second experiment was aimed at investigating the variation in clover rot susceptibility among eleven commercial cultivars to identify sources of resistance for development of resistant cultivars. As red clover production increases in New Zealand's pastoral system, incidence of clover rot - the most destructive disease of red clover - is likely to increase, and prevalence further exacerbated by seasonal changes in local climate. Identification of resistant alleles and their use in breeding to develop clover rot resistant cultivars can be considered a vital objective for red clover breeding in NZ to mitigate potential outbreak of clover rot disease induced by climate change. To facilitate artificial inoculation and selection of resistant cultivars, the *in-vitro* techniques to produce *S. trifoliorum* ascospore were employed in this experiment.

Chapter 2.0 LITERATURE REVIEW

2.1 Red clover

2.1.1 Origin, distribution and adaptation

Red clover (*Trifolium pratense* L.) is believed to have originated along the Mediterranean Sea in Eurasia and then spread through colonial contact to the Americas, Africa and other parts of the world (Fergus & Holowell, 1960). Information on the exact centre of diversity is not yet established due to the early spread of red clover across wider regions of the world.



Its importance as a forage legume is recognized in most of the temperate regions around the world. It is well established and widely cultivated in Europe, particularly across the Mediterranean region to the North of Scandinavia, countries in North and South America while its use in Australia, New Zealand, Japan and China is recently gaining momentum (Taylor & Quesenberry, 1996).

Figure 2.1 Picture of red clover (*Trifolium pratense*) plant in full bloom from cultivar Broadway under glasshouse at the Plant Growth Unit, Massey University.

Red clover has spread over many regions of the world today due to ecotypes and cultivars evolved over time and adapted to a wide range of climates, soils, temperatures and elevations. The red clover plant (Figure 2.1) adapts well and is highly productive under temperate

climates without much extremes in temperatures (hot and cold) (Boller *et al*., 2010). It prefers equally distributed rainfall throughout the year and well-drained soils with optimum pH of 6.6 to 7.6 (Boller *et al*., 2010; Taylor & Quesenberry, 1996).

2.1.2 Economic importance

The exact area under cultivation for red clover in most parts of the world is not documented in order to estimate the total production and device appropriate strategies to utilize it in the forage industry. However, an indirect estimation of area under cultivation has been derived by using the red clover seed production records in many countries. For example, in 2005 to 2007 annual estimation, 2.8 million kg of red clover seed was produced globally which is adequate to cover about 4 million hectares per year (Taylor & Quesenberry, 1996). Red clover is utilized to feed animals as fresh herbage by grazing or cut for haylage, hay or silage which is usually grown in pure stands or mixed swards (Undersander *et al*., 1990; Lacefield & Ball, 1999). Red clover can be harvested three to four times in a year from the time of sowing to first flowering usually at an interval of 35 to 40 days, especially to be used as animal fodder. Red clover usually produces approximately 12-16 t/ha of dry matter yield per year under mixed swards particularly with perennial ryegrass (*Lolium perenne* L.) (Brown *et al*., 2000; Conaghan & Clavin, 2017). Typical dry matter yield of red clover in New Zealand is about 17 t/ha/year in pure swards under well irrigated conditions but is reported to be lower under mixed pasture (DairyNZ, 2019). The potential of red clover to yield adequate dry matter is due to its ability to withstand shade, poorly drained soils, low soil fertility and pH, and rapid establishment and regrowth after sowing and grazing (Fergus & Hollowell, 1960; Smith *et al*., 1985; Taylor *et al*., 1997; Undersander *et al*., 1990). Red clover is initially considered as a short-term perennial, but its expected durability to stand out in the field has

been increased up to four years due to breeding and selection in the past century (Smith, 2000).

The use of red clover in agriculture systems is considered as a viable and most profitable option for providing animals with protein feed as well as soil nitrogen particularly in rotational systems of grazing (Burdine *et al*., 2005). Similarly, red clover is an excellent cover crop while also providing adequate biomass for green manure in organic farming (Knorek & Staton, 1996; Sullivan, 2003). It is sometimes used in rotation with other crops especially grains and nurse crops. Besides, red clover has a specific role in rotation system for use as a trap crop to break the cycle of pathogens and pests (Chen *et al*., 2006). Red clover is an alternate option to be used as a winter annual especially in warmer climates (Quesenberry & Blount, 2006).

2.1.3 Uses of red clover in New Zealand

Red clover is an important legume component of the New Zealand (NZ) pastoral system for animal production and the seed industry. It represents about 23% of the forage seed exports and covers 120, 000 ha of the sown pastures (Morrison, 2017). Grown in pure stands or in mix swards with other grasses, red clover adapts well across a range of soil types, management practices and environmental conditions. In NZ white clover is more important than red clover due to its long-standing history and significant contribution to the livestock industry in terms of improved production and product quality. However, white clover hardly tolerates intense grazing during dry summers (Widdup & Barrett, 2011). As pointed out by Brazendale *et al. (*2011), the economic return on livestock farming that feeds on forage depends primarily on pasture persistence. Nevertheless, the integration of red clover under grazing system offers specific advantage to farmers. Red clover provides short-term pastures with high herbage and fodder yield to meet high protein requirements for fattening of animals

(Skipp & Christensen 1990). Red clover also has the ability to withstand prolonged dry summer compared to white clover (Rumball *et al*., 1997). Consequently, several research and development projects are focused on improving red clover production as well as persistence through breeding for adaptation under NZ farming and environmental conditions (Brazendale *et al.,* 2011; Ford & Barrett, 2011; Widdup & Barrett, 2011; Claydon *et al.,* 2003; Rumball *et al.,* 2003; Hyslop *et al.,* 1999; Rumball *et al*., 1997; Skipp & Christensen 1990).

2.2 Taxonomy

Taxonomically, red clover is a dicotyledon plant, a member of the Leguminosae family in the genus *Trifolium* and given the species name as *Trifolium pratense* L. (Zohary & Heller 1984; Ellison *et al*., 2006). With more than 250 known species in the genus *Trifolium*, about 11 of the species are recorded to have some economic importance and are utilized in agriculture (Vleugels, 2013; Taylor & Quesenberry, 1996). It is classified under perennial diploid species (2n = 14) with predicted genome size of 435Mb (Sato *et al*., 2005). Most of the cultivated varieties around the world including NZ are diploids while several tetraploid varieties evolved via chromosome doubling are reported to be used in Europe (Taylor & Queensberry, 1996).

Kingdom	Plantae – Plants
Division	Magnoliophyta – Flowering plants
Class	Magnoliopsida – Dicotyledons
Order	Fabales
Family	Fabaceae (Leguminosae)
Genus	*Trifolium*
Section	*Trifolium*
Subsection	*Trifolium*
Species	*Trifolium pratense* L.

Infrastructure and methodologies on genomic research devised for other closely related species of forage and legumes like *Trifolium repens, Medicago truncatula, Medicago sativa* and *Lotus japanicus* are relevant to red clover genomic studies in most cases (Choi *et al*. 2004). According to phylogenetic analysis with molecular markers by Ellison *et al*. (2006) red clover is grouped with *T. diffusum, T. andricum* and *T. pallidum,* all with base chromosome number of 2n = 14. Several attempts to develop interspecific hybrids in most closely related red clover species with base chromosome number of 2n = 16 is not proven feasible specifically due to differences in chromosome number. Variation in chromosome numbers between the red clover subsections often result in interspecific cross-incompatibility and non-pairing of chromosome in hybrids (Taylor & Quesenberry, 1996). However, interspecific hybridization between red clover and *T. medium* have been widely studied and contributed to expand the genetic base of red clover (Abberton, 2005).

Cytogenetic studies show that red clover, the only species in its section has seven chromosome pairs while the rest have eight pairs of chromosomes. Regardless of the differences in number of chromosomes, red clover possibly can hybridize with other *Trifolium* species such as *T. pallidum* and *T. diffusum* (Phillips *et al*., 1992; Zohary & Heller, 1984). This is made possible due to high degree of chromosome homology between these species at the meiotic stage (Taylor & Quesenberry, 1996). However, such interspecific hybridization often undergo embryo rescue due to sterile hybrids and low production of fertile seeds (Taylor & Quesenberry, 1996; Phillips *et al*., 1992).

2.3 Physiology and morphology

Red clover is not only used as animal feed but also improves soil by fixing nitrogen (N) through mutual association with rhizobium bacteria, that benefits companion grasses as well as providing N to succeeding crops (Bowley *et al*., 1984).

The stem of red clover grows from the crown just above the ground level and becomes hallow when developed fully. The plant varies in size and can reach a height up to 80 cm depending on genotypes (Vleugels, 2013; Taylor & Quesenberry, 1996). Red clover is an alternate-leaved plant, with three leaflets (trifoliate) usually green in colour and lightly pubescent with short non-glandular trichomes and have a distinguished light crescent (V-mark) visible in most cases depending on the genotypes (Bowley *et al*., 1984). Flowers of red clover are produced towards the end of the main and auxiliary stems with timing for floral initiation depending highly on genotype-by-environment interaction. Flowers are typically pink in colour with floral heads covered with dense florets (up to 300 florets) that attract mostly bumblebees and honeybees that facilitate pollination (Bowley *et al*., 1984). Generally, flowering in red clover is induced by long day length and time to flowering for each genotype: photoperiods of 9 and 16 hours are sufficient to initiate flowers in early and late varieties, respectively (Bowley *et al*., 1984; Taylor & Quesenberry, 1996).

Seeds of red clover are kidney shaped that measure up to 2.2 × 1 mm in size and are botanically classified as pyxidia (Taylor & Quesenberry, 1996). Taylor and Quesenberry also reported that seeds are usually dicotyledonous, but occasionally they can produce up to six cotyledons and such multi-cotyledonous combinations are controlled by genetic factors.

Root structure and depth of red clover depends entirely on genetic and environmental factors such as soil condition and density, growth type, plant density and competition for water and nutrients. Red clover is generally known for its robust primary taproot system that can reach a depth of 3 m in light density and well drained soils for a mature taproot (Vleugels, 2013; Taylor & Quesenberry, 1996). However, variation in root structure is eminent in which plants with erect upright position generally have deep taproot, while plants with prostrate growth

habit have adventitious root systems with shorter taproot structures (Taylor & Quesenberry, 1996). Deep taproots and more fibrous root systems growing from the upper part of the crown are important traits that give red clover its longevity in the field and vegetative persistence during prolonged dry seasons. Since red clover is a leguminous species, the roots have ability to form nodules with bacteria *Rhyzobium trifolii* in a symbiotic relationship. Red clover roots are capable of fixing up to 389 kg of soil nitrogen per hectare yearly. This enhances soil nitrogen and partly benefits companion grasses (Taylor & Quesenberry, 1996). However, nodulation and N fixation decelerate from this symbiotic process when soil pH is below 6 (Frame *et al*., 1998; Taylor & Smith, 1979; Taylor & Quesenberry, 1996).

2.4 Reproductive systems

Red clover plant is a cross-pollinated (allogamous) diploid species with a single set of 14 chromosome pairs (2n = 14). Tetraploids with double set of chromosomes have being developed either naturally or through ploidy manipulation. Tetraploid varieties have added advantage over diploids in forage production because they are generally larger. However, seed production with tetraploids remains a problem especially due to large flowers which requires bees and bumblebees with long tongues.

The undifferentiated red clover anther undergoes cell division and develops into microspores or pollen after have gone through cell differentiation at various development stages (Hindmarsh, 1964). As the anther matures, the cell walls of the endothecium **(**the layer of cells lying beneath the epidermis of the wall of the anther**)** often thickens that probably aids dehiscence (Taylor & Quesenberry, 1996). The mother cells of microspore become apparently spherical and increase in size before undergoing the process of meiosis, following the normal diploid pattern (Hindmarsh, 1964).

Red clover ovary commonly has two ovules of which one of them normally evolves into a seed upon fertilization. Occasionally, mature embryo sacs can still develop from both ovules and bear two seeds, particularly in some high seed yielding plants (Taylor & Quesenberry, 1996). Cell division and tissue enlargement results in differential growth of ovule that facilitates megasporogenesis (Hindmarsh, 1964). Megasporogenesis is the development of megaspores (the larger spore that gives rise to female gametophyte) inside the ovules of seed plants. A diploid cell in the ovule, called a megasporocyte or a megaspore mother cell, undergoes three stages of meiosis and gives rise to four haploid megaspores at each end of the embryo sac (Taylor & Quesenberry, 1996). Further development of each nucleus from each embryo sac merges to form binucleate endosperm cell, which enlarges over the egg cells prior to fertilization (Hindmarsh, 1964).

Red clover has an Onagrad type of embryo development in which the development of embryo is endoscopic i.e., apex is towards inside (Mackiewicz, 1965). Three days after pollination, the intraspecific embryos form spherical shape but gradually changes to heart and mostly toperdo shaped after five days (Armstrong, 1968). At this stage, embryos of hybrid sometimes abort as the result of incompatible endosperm in which viable plants can still be developed through the embryos rescue technique. Cotyledon differentiates on the eighth day after fertilization and significantly enlarged on the eleventh day. Between 14 to 17 days after pollination, embryo begins to reach their full size where it requires additional 7 to 10 days for seed maturity (Mackiewicz, 1965). Generally, embryos of interspecific crosses are slower than intraspecific embryos (Taylor & Quesenberry, 1996).

The red clover stigma becomes receptive to pollen at the stage when flower petals are fully expended. An entasis that slightly swollen below the stigma develops then leads to the opening of cavity containing a watery secreation (Heslop-Harrison & Heslop-Harrison, 1982). Red clover has a strong gametophytic self-incompatibility system, a plant mechanism that prevents self-fertilization and encourages outcrossing. The response of self-incompatibility is genetically controlled by one locus (S-lucus), and relies on a series of complex cellular interactions between the self-incompatible (S alleles) pollen and pistil (Taylor & Quesenberry 1996). The governing rule for this system being that a pollen tube growth is slowed down or stopped in a style if the particular S factor exists in both pollen and style (Fyfe, 1966). Self-fertilization is made quite difficult from the same plant due to prevention of pollen to penetrate the hard layer of the ovules. Consequently, red clover depends on insect pollination from other cross-compatible plants. Individuals selected for breeding can be inter-crossed through open pollination by naturally occurring bee and insect pollinators with reasonable spatial isolation or using artificially controlled system such as bee cages. Although honey bees (*Apis mellifera* L.) are frequent pollinator of red clover, their shorter tongue length prevents them from reaching the corolla tubes and can only be pollinated efficiently by Bumble bees (*Bombus* spp.). However, a phenomenon known as pseudo-self-compatibility or reduced expression of self-incompatibility occurs sometime which can be induced under high-temperature treatment. Although growth of pollen tube is ruled by strong self-incompatibility system, pollen germinates without difficulty on the stigma of either compatible or incompatible intraspecific genotypes (Taylor, 1982). However, the incompatible pollen types usually stops further growth shortly after their rapid growth in the entasis region of the style just before reaching it.

2.5 Genetic diversity and germplasm

Genetic diversity as a result of natural or artificially induced variation in a population is prerequisite for any successful breeding program. Precise methods of identifying and characterizing plant genetic resources provide information for classifying genetic resources (Smith & Smith, 1989) for effective conservation and sustainable use (Arif *et al*., 2010). Consequently, efficient methods were developed via morphological, phenotypic, biochemical and DNA or molecular markers. The analysis of red clover genetic diversity done by morphological and molecular characterization in various sets of accessions and cultivars indicate large genetic diversity among and within populations (Dias *et al*., 2008; Greene *et al*., 2004). Breeders always look out for large genetic variation which presents an advantage for a breeding scheme. However, dealing with large diversity also presents greater challenge in terms of evaluation and conservation. A review by Taylor and Quesenberry (1996) show that several valuable traits such as forage yield and quality, pests and disease resistance and dry matter show large genetic diversity, thus, deserve careful phenotypic assessment. Genetic variability in red clover have been described successfully (Ahsyee, 2013; Asci, 2011; Dias *et al*., 2007; Greene *et al*., 2004).

Populations of wild red clover naturally evolving in stable grassland as well as cultivars and landraces are important materials for genetic improvement and conservation. In order to facilitate red clover breeding and conservation around the world, a total of 10,113 accessions of *Trifolium pratense* were reported to be held by various international gene banks and database catalogues managed by the European Internet Search Catalogue (EURISCO), germplasm resources information network (GRIN) and system-wide information network for genetic resources (SINGER) databases. This information system provides information on the current state of collected red clover germplasm (Abberton & Thomas, 2010). New Zealand

via the Margot Forde Forage Germplasm Centre at the AgResearch Grasslands Research Centre, maintains a significant genebank of clover. A total of 1,569 accessions of red clover, representing breeders' lines and wild populations, collected across different geographical origins are currently held at this centre (Abberton & Thomas, 2010). Exploring these germplasm accessions and identifying agronomically superior material has had a positive contribution to the New Zealand pastoral industry, particularly with new cultivars such as the Grassland Sensation, Grassland Relish and Reaper among others.

Evaluation of genetic diversity among and within germplasm accessions facilitates the identification of novel alleles. This also enables potential identification of association between specific plant types and collection environments/geographic centres of origin. Although red clover originated from the Mediterranean region and was initially domesticated in Belgium (Taylor & Quesenberry, 1996), the relationship of existing cultivars to their wild relatives is yet to be established (Isobe *et al*., 2014). Distinct characteristics of wild red clover populations are valuable resources that could be utilized in breeding to broaden the genetic base of red clover for the development of new cultivars with adaptation to specific environmental conditions and uses (Herrmann *et al*., 2005; Mosjidis *et al*., 2004).

A significant challenge to red clover breeders is to develop new cultivars with increased forage yield, feed quality, vegetative persistence, low oestrogens, resistance to pests and diseases and adaptation to local environments (Taylor & Smith, 1979). Information on genetic diversity for these key traits, their heritability and correlation provides a useful guide in assisting red clover breeders to design efficient breeding methods to effectively use genetic resources for development of new and novel cultivars.

2.6 Genetic variability, phenotypic correlation and heritability

Red clover plays a significant role in providing high quality protein for ruminants in most of the temperate countries around the world due to its adaptability to wide environmental conditions. Since red clover is a self-incompatible cross-pollinating species, its ability to produce self-seed under natural conditions is very limited, hence, producing highly heterogeneous genotypes (Taylor & Quesenberry, 1996). The presence of the one-locus, gametophytic S-allele system prevents red clover from self-fertilizing due to slow progress of the pollen tube growth through the style (Taylor & Smith, 1980). This mechanism, in a similar manner, prevents fertilization from crossing between plants with the identical S-allele genotype. Due to this specific mechanism, red clover has high genotypic diversity for most agronomic traits within and between populations.

Estimation of genetic parameters such as genotypic variation, correlation among traits, heritability and genotype-by-environment interaction (GEI), provide guidance for optimizing breeding programs (Dudley & Moll, 1969; Moll & Stuber, 1974). Previous studies in red clover indicates high genotypic variation in forage and dry matter yield, plant height, leaf width, leaf length, leaf size, growth habit, time to flowering (Little *et al*., 2017). A study by Herrmann (2006) on selected Swiss Mattenklee red clover revealed significantly high genotypic variation and heritability for most of the seed yield components. Dry matter and growth habit had high heritability values (Lawson, 1971). Moreover, a study by Anderson (1960) on seven non-inbred progenies derived from diallel crosses show high hebritabilities for yield, persistence, growth habit and time to flowering. A study on selected tetraploid red clover by Jaranowski and Broda (1977) reported high broad-sense heritabilities for qualitative while low values for quantitative traits. Studies of phenotypic correlations among red clover traits show positive association between yield traits and selected morphological traits such as plant height, number of branches, medial leaf length and width, time to flowering and number

of internodes (Ahsyee, 2013; Tucak *et al*., 2013; Ross & Pagano, 2005; Sanja *et al*., 2006). The same authors also reveal that the above mentioned traits are found to be positively associated with each other. On the contrary, many of the red clover traits show weak phenotypic correlation particularly between forage yield and quality traits (Drobna, 2009; Riday & Krohn, 2010; Tucak *et al*., 2013).

The existence of significant genotype-by-environment interaction (GEI) complicates genetic analysis and the selection process in plant breeding programs (Comstock & Moll, 1963; Byth, 1981; Kroonberg & Basford, 1989). Red clover has substantial genotype main effects for most agronomic traits as indicated by several studies done in the past (Little *et al*., 2017; Tucak *et al*., 2013; Sanja *et al*., 2006; Hossein, 1993). The GEI in red clover as compared to other crops is reported to be given much less attention due to heterogeneous populations among cultivars than homogenous pure lines (Hossein, 1993). Having said that, red clover is reported to show low GEI for most of the traits due to the heterogeneous nature of majority of the cultivars (Hossein, 1993) except for dry matter yield (Montardo *et al*., 2003; Eberhart & Russell, 1996; Hossein, 1993). A better understanding of the magnitude and significance of genetic variation, multi-variate information on the association among traits and the stability of trait expression across environments could provide useful guide for optimizing red clover breeding methods (Muntean & Savatti, 2003).

2.7 Breeding of red clover

2.7.1 Breeding objectives

Some of the key objectives in red clover breeding programs are; fresh herbage and dry matter yield, vegetative persistence and resistance to common diseases. Although red clover is known for its high forage yield, its vegetative persistence under intensive grazing and cutting is poor as compared to other forage plants (Boller *et al*., 2010). Poor vegetative persistence in

red clover is further caused by its sensitivity to a range of biotic and abiotic stresses, combined with the species regrowth abilities after grazing or cutting (Ravagnani *et al*., 2012; Boller *et al*., 2010). Nematodes such as *Dithylenchus dipsaci* is known for causing significant damage in red clover, which negatively affects vegetative persistence (Boller *et al*., 2010). Some of the important red clover diseases include clover rot or crown rot (*Sclerotinia trifoliorum)*, northern (*Kabatiella caulivora*) and southern anthracnose (*Colletotrichum trifolii)* and powdery mildew *(Erysiphe polygoni).* Apart from the biotic stresses, the most important abiotic factors that influence red clover growth and vegetative persistence includes drought, low temperatures, flooding, competition among other plant species, grazing or cutting frequency (Ravagnani *et al*., 2012; Bosworth & Stringer, 1985).

Breeding programs around the world have contributed significantly to improving herbage yield and vegetative persistence of red clover over the last decades. Consequently, improved varieties selected from these dedicated breeding programs are able to extend red clover stand duration reliably from two initially to four seasons (Boller *et al*., 2010). Further improvement using Swiss Mattenklee germplasm was achieved with increased vegetative persistence of early flowering and multiple-cut types (Boller *et al*., 2010). Selection of plant type with increased vegetative persistence to withstand high grazing pressure needs to focus on using specific plant morphology such as fine prolific stems with prostrate growth, high vegetative and reproductive characters. Improved cultivars previously released for high persistence and increased dry matter yield includes Crossway, Broadway and G27 (Ford & Barrett, 2011). However, slow progress was made in improving forage yield which is evident in a number of new cultivars failing to outperform the old cultivars. The induction of polyploidy in red clover for development of tetraploids has resulted in substantial forage yield increase, improved resistance to diseases and better persistence compared to the original diploids (Boller *et al*., 2010). Several of the released cultivars possess good resistance to northern and

southern anthracnose as well as powdery mildew. Boller and his colleagues (2010) also pointed out that some of these cultivars show breakdown in their disease resistant ability probably due to aggressiveness of current pathogen races over time. Taylor (2008) stated that breeding for clover rot has made limited progress due to difficulty in breeding for this trait.

High seed yield is another breeding objective that has high relevance particularly for seed companies to achieve market success for red clover. In most cases yield of red clover seed is low and fluctuates. Low seed yield is common especially in tetraploid cultivars, which are often characterized with large flowers that make pollination difficult (Vleugels *et al*., 2014; Monks *et al*., 2010; Rattray, 2005; Taylor & Quesenberry, 1996).

Finally, breeding for forage quality receives more attention and becomes an important breeding objective nowadays. Although red clover is rich in protein, it is not highly digestible in comparison to forage grasses due to less soluble carbohydrates. Even though these issues can be addressed by growing red clover in companion with highly digestible grasses (Boller *et al*., 2010), breeding for forage quality is now a vital component (Vasiljevic *et al*., 2009).

In addition, plant secondary metabolites are important because red clover possess significant phytoestrogens, mainly formononetin, which disturb the usual reproductive cycle in sheep. Hence, it is important to develop cultivars with low levels of phytoestrogenic compounds. There is also a growing interest in breeding cultivars with high polyphenol oxidase activity (Boller *et al*., 2010; Vasiljevic *et al*., 2009). Polyphenol oxidase is an enzyme in red clover that inhibits lipids from degradation in silage. It also leads to increased production of polyunsaturated fatty acids in ruminant products including milk and meat (Van Ranst *et al*., 2011), which is of benefit to consumer health.

2.7.2 Methods of hybridization and primary trait selection

Success in target breeding of red clover for traits of economic importance depends on utilization of genetically diverse germplasm and application of appropriate selection methods such as mass and phenotypic recurrent selection. Red clover has a self-incompatible system that inhibits the maintenance of genotypes through seeds by selfing, but can be maintained vegetatively using cuttings. Mass and family selection methods using open pollination are frequently used in red clover breeding while polycross and strain building are not regularly used (Taylor & Quesenberry, 1996).

Mass selection is actually straightforward but proven as an effective method in red clover breeding, particularly for highly heritable traits. This method is appropriate for breeding cultivars with resistance to disease and low temperature, particularly winter hardiness (Taylor & Quesenberry, 1996). Usually, large breeding populations of adequate genetic variation are generated. Seeds from this population are sown to identify superior plants with desirable characters in which their seeds are collectively harvested in bulk and become planting stock for the next season (Gupta, 2010). This method can be done over several cycles or as a single cycle depending on the accumulation of desirable characters (Gupta, 2010; Taylor & Quesenberry, 1996; Julen, 1959).

Family selection, sometimes referred to as recurrent phenotypic selection has similar approach to mass selection. The key difference to this method is the strict maintenance of family history obtained from the open-pollinated plants or half-sibs instead of being bulked (Taylor & Quesenberry, 1996; Julėn, 1959). Progenies of families with improved characters are evaluated individually and seeds are harvested using recurrent phenotypic selection. Plants with even more desirable characters are again selected and allowed to cross among themselves under isolation before their progenies are evaluated (Vleugels, 2013). This

process is repeatedly done for several cycles until most of the desirable traits are fixed, which results in identification of best progenies. The cycles of recurrent selection should remain at minimum possible, typically less than three cycles, especially among the related individuals in order to prevent depression as a result of inbreeding (Vleugels, 2013; Boller *et al*., 2010; Taylor & Quesenberry, 1996; Julėn, 1959).

Classical breeding approach using polycross method involves the maintenance of parental genotypes vegetatively in the process of progeny testing. The use of this method is aimed at identifying clones with best general combining ability that can be intercrossed to generate improved cultivar (Boller *et al*., 2010). Polycross breeding is more appropriate to improve complex traits such as winter hardiness and forage yield, since the progenies developed are put through testing, as opposed to mass selection (Boller *et al*., 2010; Taylor, 2008). Nevertheless, a major drawback for polycross method is the length of time required and costs involved for several selection cycles (Boller *et al*., 2010; Taylor, 2008; Taylor & Quesenberry, 1996).

Similar to polycross is a method known as strain building. The only difference is that parental genotypes are maintained by seeds in strain building instead of clones (Taylor, 2008). In the process of testing, Taylor (2008) further states that new populations are developed and separately maintained until the most superior populations are identified and subsequently intercrossed after testing.

2.8 Major diseases of red clover

Red clover can be infected by various pathogens including fungal diseases such as southern anthracnose (*Colletotrichum trifolii*), northern anthracnose (*Kabatiella caulivora*), powdery mildew (*Erysiphe polygoni*), rust (*Uromyces trifolii*), target leafspot (*Stemphylium*

saracinaformae), clover rot (*Sclerotinia trifoliorum)* and root rot (*Fusarium* spp.) (Öhberg *et al*., 2005; Taylor & Quesenberry, 1996; Julén, 1959). Viral diseases on red clover are also known for causing significant damage and their spread is facilitated mainly by aphids and other insect vectors (Barnett & Diachun, 1986). The seven known virus of economic importance to red clover are: the peanut stunt cucumovirus (PSV), the red clover vein mosaic carlovirus (RCVMV), the clover yellow vein potyvirus (CYVV), the bean yellow mosaic virus (BYMV), the alfalfa mosaic ilarivirus (AMV), the pea streak carlovirus (PStrV), and the white clover mosaic potyvirus (WCMV) (Taylor & Quesenberry, 1996; Barnett & Diachun, 1986). These viruses systematically affect plants by creating an entry point for other pathogens, while obstructing the plant cells' machinery and defence mechanisms. Barnett and Diachun (1986) further reports that red clover plants infected by virus hardly recover due to lose of vigour, reduced nodulation, vulnerability to other forms of stresses and ultimately die.

Diseases in red clover caused by nematodes include root lesion nematodes (*Pratylenchus penetrans*), root-knot nematodes (*Meloidogyne sp.*), the clover stem nematode (*Ditylenchus dipsaci*) and the clover cyst nematode (*Heterodera trifolii*) (Taylor & Quesenberry, 1996). Plants infected by nematodes often wilt away in field and damage can be severe under highly infectious field. Diseases caused by bacteria sporadically occur in red clover whereas phytoplasma (*Phytoplasma trifolii*) gradually turn out to be an important disease in red clover (Hiruki & Wang, 2004). Hiruki and Wang (2004) further stated that phytoplasma in red clover are transferred by phloem-feeding insects which affect plant growth and ability to produce seeds and radical decrease of persistence.

2.8.1 Clover rot (*Sclerotinia*)

Clover rot, also known as crown, stem and root rot is among the most destructive diseases of red clover in Europe, particularly in cooler regions characterized by high rainfall and snow

cover. This disease is caused by two primary species of the genus *Sclerotinia viz. Sclerotinia sclerotiorum* and S*clerotinia trifoliorum* (Saharan & Mehta, 2008; Boland, 1992). Nevertheless, other soil-borne secondary pathogens and *Fusarium* are also capable of causing crown and root rot, which is often referred to as root rot with symptoms similar to clover rot (Öhberg, 2008).

2.8.2 Origin of *Sclerotinia* family

The *Sclerotiniaceae* belongs to the family of phytopathogenic fungi, order Helotiales and phylum Ascomycota, typically characterized by development of sclerotia (dark resting bodies) in its life cycle. There are more than sixty diseases caused by these groups of pathogens including clover rot, white mould, watery soft rot, root rot, stem rot, crown rot, etc. The *Sclerotinia* genus is one of the 33 genera presently documented in the *Sclerotiniaceae* family. The ability for long term survival accompanied by great reproduction potential make sclerotia an important element in the diseases epidemiology of the species *Sclerotinia.* According to Bolton *et al*. (2006), there are three valid species in the *Sclerotinia* genus *viz. S. trifoliorum* Erikks., *S. sclerotiorum* (Lib.) de Bary and *S. minor* Jagger. These three species are group of necrothrophic fungi that can be grown on different artificial media (Wong & Willetts, 1979). *S. minor* has a total of four chromosomes whereas *S. sclerotiorum* and *S. trifoliorum* have eight chromosomes (Wong & Willetts, 1979). *S. homeocarpa, S. asari* and *S. nivalis* currently under *Sclerotinia* genus are distinct species that needs to be reclassified (Bolton *et al*., 2006). Most *Sclerotiniaceae* fungi probably originate from the Northern hemisphere where they are currently found in abundance. This area has typical temperate climate accompanied by long, cold and wet winter with ice and snow cover in certain areas. In summer, temperature is often mild to cold only for a short time with extended day length and frequent precipitation with high humidity (Bolton *et al*., 2006; Willetts, 1997).

2.8.3 Description, classification and identification of *Sclerotinia trifoliorum* Erikks

Clover rot was first reported in 1849 from Britain (Lawes & Gilbert, 1860) and the later cases were reported in the 1870 from Denmark and Germany (Eriksson, 1880). In Sweden it was initially reported in 1878 after devastation of red clover fields in some parts of the south Sweden (Eriksson, 1880), in which fungus of the causal agent was already reported in Germany and given the name Peziza ciborioides. Based on the earlier reports on clover rot, Eriksson (1880) reclassified the pathogens in to *Sclerotinia* genus and gave a new name *S. trifoliorum* Erikks, which is still in use up until today.

Clover rot is present in the temperate regions of the world mainly in areas with heavy snow covers and mild winters, yet been considered highly destructive disease of clovers in the northern Europe (Hanson & Kreitlow, 1953). Red clover is a highly susceptible host for clover rot with great economic importance, although it has a wide range of hosts including the forage legumes that grow through the winter.

2.8.4 Life cycle of *Sclerotinia trifoliorum*

The *Sclerotinia trifoliorum* is an ascomycete that usually forms the black fungal resting bodies called sclerotia. Mature sclerotia are typically characterized by black coating and white medulla that differs in size (0.3-10 mm), from which brownish fruiting bodies known as apothecia usually grows (Figure 2.2).

The life cycle of *Sclerotinia trifoliorum* is slightly different from the other *Sclerotinia* spp where it forms apothecia towards late summer and autumn. *S. sclerotiorum*, on the contrary, initiates the production of apothecia towards the end of spring and summer (Williams & Western, 1965a; Kohn, 1979). *S. trifoliorum* uniquely displays bipolar heterothallism where ascospores in each ascus segregate between small and large in a 4:4 ratio (Kohn, 1979). The

term heterothallism is used to describe the sexual reproduction in certain fungi that possess sex organs in different individuals, which requires two compatible partners to produce sexual spores.

Small and big ascospores are equally capable of infecting plants, undergo mycelial development and produce sclerotia. However, homothallic isolates that come from large spores can only form apothecia without mating (Uhm & Fujii, 1983a), because they have both male and female structures in the same isolate that allows them to reproduce sexually. Microconidia from *S. trifoliorum* mycelia derived both spores type are incapable of infecting plant tissue but only function as spermatia (Uhm & Fujii, 1983b; Björling, 1942). Production of apothecia are facilitated with isolates derived from progeny genetically vary from their parental isolates and each other when mating between small and large spore spermatia occurs (Rhenstrom & Free, 1993). Moreover, isolates of *S. trifoliorum* which are genetically different evidently show variation in their pathogenicity, which results in plants differ in their level of resistance depending on the isolates used (Halimi *et al*., 1998; Dixon & Doodson, 1974; Björling, 1939).

The *S. trifoliorum* life cycle and plant infection caused by this pathogen was well studied and described by several authors (Raynal, 1985; Loveless, 1951; Dillon *et al*., 1946; Björling, 1942; Eriksson, 1880). The development of *S. trifoliorum* apothecia from sclerotia in soil starts from late summer to autumn. The matured apothecia begin to produce ascospores (spores) usually dispersed by wind and infect plants. The infection in host plants begins with small dark necrotic spots localized on leaves and stems (Figure 2.2) and further develops systematically within the plant under favourable weather conditions (Dijkstra, 1966).

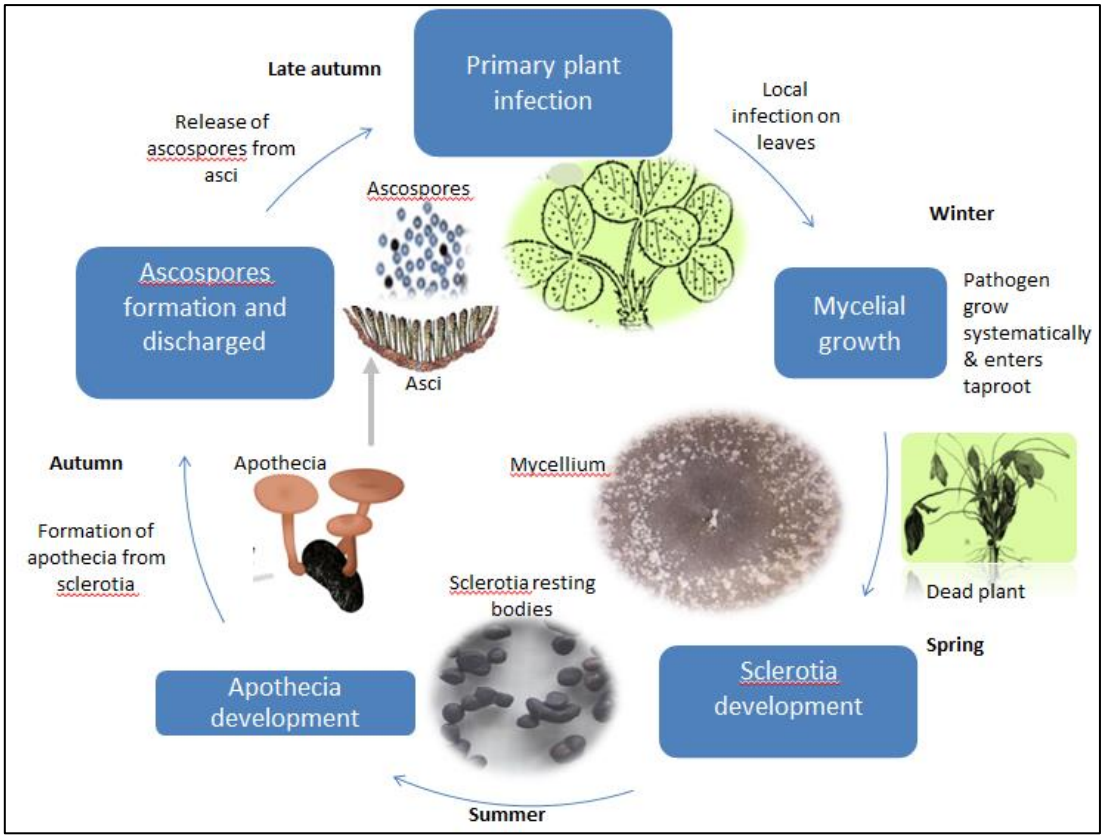


Figure 2.2 The development cycle of *Sclerotinia trifoliorum* on red clover plants in field.

Favourable conditions that encourage fungal growth includes temperatures above 0 °C and below 15 °C and high humidity sooner after snowfall or frost from late autumn to beginning of spring. The leaves of infected plants change color to olive-greyish-brown prior to wilting and ultimately engulfed by mycelium growth. The mycelium growth spreads from the leaves to petioles and stem, then takes control of the crown and finally invades the taproot (Hesselman, 1962).

2.8.5 Host effects and clover rot-red clover interactions

Infection by clover rot could also spread among plants through mycelial growth in field which eventually colonizes and kills the plants. Dead and infected plants are covered with

mycelium in spring after snow melt, then dark sclerotia starts to form within the taproots of dead plant tissue at soil level (Figure 2.3 B).

Matured sclerotia are highly adaptable and can survive long periods without losing its viability. Experimental study on sclerotia viability by Pape (1937) indicated that sclerotia still remained viable after burial in soil for 7½ years while Dillon *et al*. (1946) reported clover rot outbreaks on fields where non-host crops were cultivated for at least 8 years. Soil conditions seem to play a major role in determining the sclerotia viability. Dillon *et al*. (1946) reported poor rate of survival under flooded and dry soils while related study by Williams and Western (1965b) and Björling (1942) concluded that scleroria placed just above the soil surface (1-5 cm) produce more apothecia than those further below.

Figure 2.3 A. Young red clover plants in field infected by *Sclerotinia trifoliorum* ascospores ("Plant Disease: *Sclerotinia trifoliorum* Erikss", 2020). **B.** White mycelium later produced from ascospore infection and colonized the red clover plants.

The mortality rate of red clover in field caused by clover rot increases towards the end of winter and progresses in to spring in which farmers are often misguided by the symptoms of physical damage from winter stress (Hanson & Kreitlow, 1953; Dillon *et al*., 1946). The incidence and magnitude of red clover plant mortality is not so much influenced by the differences in topography within the field, but is highly dependent on autumn and winter

weather conditions which greatly varies from year to year (Dijkstra, 1964; Loveless, 1951; Dillon *et al*., 1946).

2.9 Distribution and economic importance of clover rot in New Zealand

Clover rot disease caused by *Sclerotinia trifolium* Erikss can be destructive in red clover swards in other parts of the world particularly in the northern Europe, but is considered to be moderately important in New Zealand (NZ) under current climate. Thus, the effect of *S. trifoliorum* on red clover in pasture has not been adequately investigated under NZ conditions as compared to Europe. Although its prevalence is low, clover rot incidence was reported in a number of clover fields. A study on the incidence of clover rot among the available white clover cultivars in NZ has shown differences in susceptibility (Watson, 1988). Sclerotinia clover rot was reported to be an issue in red clover pastures in certain parts of NZ in the 80's, which prompted greenhouse screening for the pathogen (J. Ford, Personal communication, June 03, 2020). Significant reductions in plant numbers in the year following establishment were noted by Ledgard *et al*. (1988) at Waikato dairy pasture. Incidence of clover rot was later reported in several red clover fields and evaluation trials in NZ from time to time, but is very random and its effect is not line specific (J. Ford, Personal communication, June 03, 2020). This indicate that certain parts of NZ, especially, on the South Island that experiences mild winters and snow at high altitudes with high precipitation and humidity could favour the spread of *S. trifoliorum* and reduce persistence and productivity of red clover. It is also possible that incidence of clover rot could increase as red clover production further intensifies in NZ farms and variations in weather patterns are unpredictable due to climate change.

Although existence of *S. trifoliorum* was reported in some New Zealand's fields, it is not widespread probably due to a number of reasons. Apart from the variation in conducive environment that encourages clover rot incidence, other possible reasons for low incidence

could be low intensity of red clover production, popularity of white clover over red clover and mixed swards perennial ryegrass clover based pastoral system. Control with fungicide can be possible with a number of products on the market for *Sclerotinia spp.* like Rovral, Carbendazim and Benomyl if incidence is high enough. The *S. trifoliorum* has a broad range of host limited to Leguminous crops with high sensitivity to environment and can trigger the disease outbreak anytime when the condition is right.

2.10 Climate Change Effects on Pastoral Systems in New Zealand

New Zealand (NZ) agriculture contributes about 56% of total export value and dairy products 27%; 95% of dairy products are exported (SNZ, 2012). Agricultural production is sensitive to climate especially drought (Webb *et al*., 2012; Darbyshire *et al*., 2013). Because the region is a major exporter—providing, for example, more than 40% of the world trade in dairy products—changes in production conditions in the region have a major influence on world supply (OECD, 2011). This implies that climate change impacts could have consequences for food security not just locally but even globally (Qureshi *et al*., 2013).

New Zealand's land use by area is dominated by livestock grazing. According to the National Institute of Water and Atmosphere (NIWA) climate modelling, pasture production in NZ is projected to decline at an average of 4% by 2030 (Watt *et al*., 2008). Studies on modelling seasonal changes in fodder supply show greater sensitivity in animal production to climate change and elevated carbon dioxide (CO^2) with some impacts expected even under modest warming in NZ (Lieffering *et al*., 2012).

The principle of climate change induced by the fluctuations in greenhouse gas is firmly established now. Based on the past and present changes in climate, modellers are now more confident of their predictions that NZ will experience significant warming over the next 50 or

more years (IPCC, 2007). Climate predictions by Mullan *et al*. (2008) have produced a detailed account of the consensus view of climate change over the next 80 years based on 12 global climate models and a finer scale regional climate model for additional information. These models summarized that NZ will generally experience increased frequency of high temperatures (1°C increase by 2040 and 2°C by 2090), high frequency of extreme daily rainfalls, an increase in stronger winds, decreased seasonal snow cover and reduced frost risk. The frequency of severe droughts is projected to increase across many eastern parts of NZ by 2080, such as inland and north Otago, eastern Canterbury and Marlborough, parts of Wairarapa, Hawke's Bay, the Bay of Plenty, the Coromandel and Northland. Droughts may happen in spring and autumn, not just summer.

Changes in temperature and rainfall patterns may also alter the spread and distribution of existing pests and diseases, and enable the emergence of new diseases (Nottage *et al*., 2010), as they are very strongly influenced by rainfall and to a lesser extent temperature changes (Watt *et al*. 2008). Also, the biology and potential risk from invasive and native pathogenic species is highly expected to be altered by climate change, but impacts may be positive or negative depending on the particular system (Roura-Pascual *et al*., 2011). Moreover, climate change and elevated CO^2 impacts on pests, diseases and weeds are highly uncertain (Nottage *et al*., 2010). Therefore, developing appropriate adaptation strategies now to mitigate the impacts of climate variability in future is particularly important. Breeding for climate smart crops and forage species with effective plant resistance mechanisms against potential biotic and abiotic stresses induced by climate change should be a vital mitigating response to ensure sustainability in food production in to the future.

2.11 Resistance breeding against clover rot

Role of plant breeding in controlling the devastating effect of clover rot is highly recommended. Since clover rot is the most destructive disease of red clover, development of effective plant resistance mechanisms is particularly important to mitigate potential outbreak of clover rot disease induced by climate change. Selection for clover rot resistance can significantly be increased under field condition of naturally infected plants (Frandsen, 1946), thus early breeding for disease resistance was done through this method. Alternatively, artificial inoculation with fungal spores (ascospores) and mycelium has long being practiced in red clover for selection of resistant cultivars. Numerous laboratory techniques for inoculation using mycelium have been developed. Breakthrough in experimenting with *in-vitro* production of ascospores and apothecia on larger scale was made possible by Dijkstra (1964) for use in artificial inoculation. Procedure for red clover artificial inoculation with ascospores was later developed by Marum *et al*. (1994). Even though considerable improvement was made over the years for selection of resistant cultivars, progress in identifying completely resistant cultivars is slow (Taylor, 2008; Taylor & Quesenberry, 1996).

The use of restricted gene pool comprising mainly of cultivar germplasm is believed to be a main reason for slow progress in resistant breeding. The possibility of incorporating valuable resistant genes from wild germplasm often affect yield and other desirable traits know as linkage drag, due to deleterious genes introduced in to new breeding population with beneficial genes. Conventional breeding methods can be used to improve disease resistance and get rid of unwanted genes via several phenotypic backcross selections. Nowadays, resistance breeding against clover rot and several other pathogens can easily be achieved using molecular breeding such as marker assisted selection (MAS) (Vleugels, 2013).

2.11.1 Types of disease resistance

Plant breeders generally prefer plant type with inherent disease resistance that is genetically determined while temporary control of disease can also be done by fungicides and certain cultivation practices. Selection for disease resistance started in the era of plant domestication and continues up until today due to population of pathogens under selection pressure as the

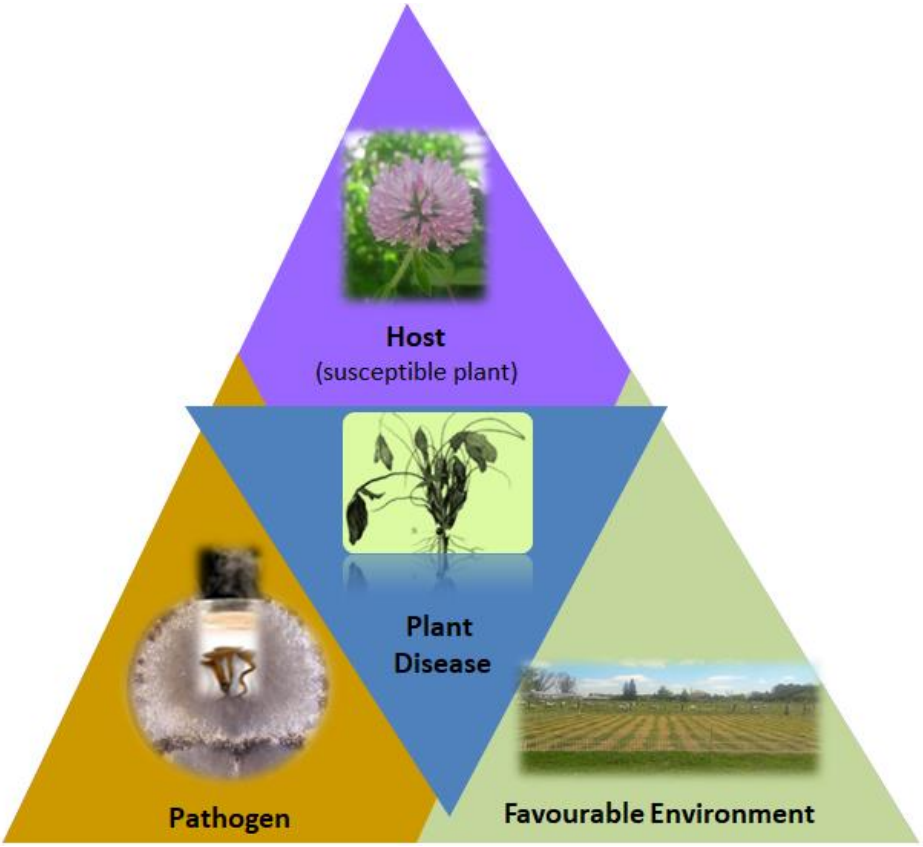


Figure 2.4 Disease triangle illustrating the interaction between the virulent pathogen, susceptible host and favourable environment for disease development to occur.

result of increase in virulence, emergence of new pathogens, changing cultivation practices and climate variability (Stuthman *et al*., 2007). Breeding for disease resistance incorporating resistance genes can disrupt other economically important traits. Also, development of cultivars with adequate resistance to a certain pathogen species or strains may succumb to others. Although cultivation of resistance genotypes is the most economical way of minimizing damage caused by plant diseases, breeding processes involved in developing such

a plant type is often cumbersome and time consuming due to the above mentioned reasons. As illustrated in Figure 2.4, plants react differently when in contact with pathogens and rate of infection is determined by the interaction of pathogens, host plants and environment condition, which is known as the disease triangle ("Cropwatch: Plant Disease - Pathogens and Cycles", n.d.). A disease resistant plant is completely immune from the effects of pathogens while maintaining production level at economic threshold levels, whereas disease tolerance refers to plants that exhibit disease damage to some extent although being exposed to considerable pathogen levels ("Plant Disease Resistance," 2020).

There are several plant specific mechanisms that determine the type of resistance in plants. Plants with basal resistance safeguard them from non-host pathogens by using preformed structures such as hard cell walls and anti-microbial enzymes or defence-activating compounds, e.g. phytoanticipins and phytoalexins (Poland *et al*., 2009). Qualitative or host specific resistance is a gene-for-gene effect mediating resistance allele (single gene resistance) that protects host plants from attack by adapted pathogens or pathotypes (Gururani *et al*., 2012; Kou & Wang, 2010). Resistance conferred by single gene can easily experience breakdown in resistance within few years. Plant disease resistance safeguard plants from diseases through pre-formed structures or barriers and responses of immune system induced by pathogens. Nevertheless, there are certain levels of quantitative disease resistance (QDR) typically observed in plants usually controlled by several genes with partial effect that are referred to as quantitative resistance loci. Interaction effect of such loci (epistasis) combined with environment usually has effect on multiple traits known as pleiotropy (Kou & Wang, 2010; Saint Clair, 2010; Poland *et al*., 2009). Breeding for QDR allows breeders to combine several resistances of different types of disease that slows down the progress of plant infection due to several resistance forces acting against the pathogen. The form of resistance

desired by breeders is QDR, which gives plants a long-lasting resistance particularly against necrotrophic pathogens (Poland *et al*., 2009).

Several studies in red clover genotypes showed significant differences in red clover susceptibility to clover rot with disease severity index (DSI) ranged from 2.0 (resistant with slight necrosis) to 5.0 (highly susceptible or dead) (Yli-Mattila *et al*., 2009; Öhberg, 2008; Dabkevienė & Dabkevičius, 2005; Delclos & Duc, 1996; Marum *et al*., 1994). This indicates that *Sclerotinia spp.* are necrotrophic pathogens, meaning that there are sources of quantitative resistance present among the red clover without the existence of totally resistant population (Poland *et al*., 2009). The only red clover cultivar that was identified to be highly resistant to clover rot with DSI of 1.0-1.5 is 'Vanessa', a European tetraploid cultivar (Vaverka *et al*., 2003).

Although not much information is available on factors that affect susceptibility to clover rot, isoflavones in red clover such as biochanin A, formononetin and genistein possibly play a role in basal disease resistance (De Rijke *et al*., 2001; He *et al*., 1996), however may not be effective against *Sclerotinia spp*. (Debnam & Smith, 1976). A study by Mullaney *et al*. (2000) indicated significant levels of biochanin A, formononetin, and genistein in 39 of the 1093 red clover accessions held by the USDA National Plant Germplasm System (Kouame & Quesenberry, 1993).

Effects of diverse germplasm and variety type from different geographical sources for clover rot resistance are an important research gap which is not fully explored. Breeding for resistance often relies on genetic variation among landraces and new cultivars, whereas incorporation of wild germplasm usually require substantial breeding work to eliminate

unwanted related characters (Boller *et al*., 2010; Taylor & Quesenberry, 1996) known as the linkage drag. Susceptibility to clover rot is likely to be determined by ploidy level as tetraploids are more resistant to diploids (Yli-Mattila *et al*., 2009; Öhberg *et al*., 2005; Vaverka *et al*., 2003). Clover rot susceptibility may also be affected by certain plant morphology such as growth habit, branching level, herbage yield and time to flowering. Varieties with high level of branching and creeping type are reported by Taylor and Quesenberry (1996) to show some level of tolerance to clover rot. Cultivars with late flowering are shown to be less susceptible than the early flowering type (Öhberg *et al*., 2005). Besides, the resistance to other diseases like rust, mildew, leaf spot or viral diseases might influence the susceptibility to clover rot.

2.11.2 Management of clover rot disease in red clover

Preventative measure using certain management practices is the best strategy to minimize the incidence and spread of clover rot disease. In areas where clover rot is a problem, spring sowing and crop rotation are found to be effective than autumn sowing as it increases the risk of young plants being attacked by the disease as the result of winter stress. Deep ploughing to ensure deep burial of sclerotia in the soil, depriving the growth of apothecia due to lack of sufficient light is another control measure often used. Use of fungicides is widely used to prevent the primary infection even though no visible damage occurs (Raynal, 1989). However, fungicide treatment at later stages of infection often fails to prevent plant from clover rot disease any longer. Even though fungicide can effectively control clover rot, it is not sustainable and often quite expensive (Raynal *et al*., 1991). Also, there is currently no specific fungicide registered for use in red clover except the broad spectrum fungicide such as Carbendazim 50% WP and Benomyl 50% WP (Raynal, 1989).

An agent for biological control (biocontrol agent) known as Contans® WG was recently made available. Contans® WG has spores of active substance *Coniothyrium minitans*, a parasitic fungus that is naturally present in some fields and breaks the cycle of *Sclerotinia* spp. by attacking the sclerotia in the soil when conditions are right (Tribe, 2012; Öhberg, 2008). This biocontrol agent (Contans® WG) can minimize damage on red clover caused by clover rot if carefully applied as per the recommendations.

2.12 Clover rot - review conclusion

Clover rot is among the most abundant and destructive diseases of red clover in temperate regions of the world, mainly in areas with heavy snow covers and mild winters. Although clover rot has a wide range of hosts including forage legumes, red clover is known to be a highly susceptible host that causes significant economic loss. Sclerotinia spp has a systematic life cycle that is highly sensitive to environmental condition. Usually the apothecia develop from sclerotia in soil from late summer to autumn. Matured apothecia start to produce ascospores that are usually dispersed by wind and eventually infect plants from late autumn to beginning of spring. Clover rot infection could also spread among plants through mycelial growth and finally colonizes and kills the plants. Taproots of infected dead plants start to form sclerotia at soil level in spring. Sclerotia when matured are highly adaptable and can survive up to seven years without losing its viability. The ability for long term survival accompanied by great reproduction potential make sclerotia an important element in the diseases epidemiology of the species *Sclerotinia.* Red clover plants react differently in the presence of *Sclerotinia* pathogen and the infection rate is determined by the interaction of the pathogen, host plant and environment condition known as the disease triangle.

The *Sclerotinia* spp is present in New Zealand and its prevalence in red clover fields is at random across the country. Although red clover in New Zealand is not as popular as the white clover and mostly grown in mixed swards, yet significant damage by clover rot was observed in field under red clover pure sward at times. Considering the theory of the disease triangle, there is possibility of incomplete interaction between pathogen, host and environment under NZ condition which could impede the progression of disease. However, as red clover uses and farm sizes increase given the favourable climatic conditions induced by climate change, it is likely that clover rot prevalence would be triggered.

Clover rot can be effectively controlled using certain management practices, fungicide treatment, biological control and resistant cultivars. Currently, there are no resistant cultivars of red clover selected for clover rot disease, and even the status of NZ commercial cultivars to clover rot is unknown. The NZ bred red clover cultivars and germplasm are not only used locally but have been exchanged widely around the world in major red clover growing regions such as the Mediterranean and Europe where clover rot disease is prevalent. Currently NZ exports seeds of cultivar Sensation in to UK market while other varieties are in evaluation in different parts of Europe (J. Ford, Personal communication, May 10, 2021). This justifies the need for identifying resistant genes among diverse germplasm and utilize in breeding program to derive resistant red clover cultivars. Development of cultivars that express certain levels of quantitative disease resistance is an important mitigating strategy to prevent against the spread of clover rot in disease prevalent regions of the world as well as safeguard red clover from uncertainty of any future disease outbreak locally.

Chapter 3.0 GENOTYPIC VARIATION AMONG A WORLD COLLECTION OF RED CLOVER GERMPLASM

3.1 Abstract

Red clover in New Zealand contributes significantly to primary industries by providing high quality feed for livestock in mixed sward pastures. Although significant progress were made to select cultivars suitable for New Zealand's grazing systems, poor persistence and plant survival under grazing pressure, shorter lifespan, high oestrogenic levels, susceptibility to various fungal diseases and low seed yield are still major limitations that requires further research and development. In order to address some of these limitations, there is a need to broaden the genetic base of existing cultivars by introducing sources of genetic diversity from germplasm representing different geographical regions. Likewise, better understanding on red clover genetics and inheritance, genetic variability, genotype-by-environment interaction effect, relationships between key characteristics are of great importance for efficient breeding. This experiment was aimed at investigating the genetic diversity among 40 selected world source of red clover germplasm together with 3 local cultivars for ten important morphological traits under field condition across three seasons (winter, spring and summer) using spatial row-column design. Univariate and multivariate analysis were used to estimate genotypic variation and identify distinct germplasm accession groups based on seasonal morphological measurements for a range of traits, respectively. Results from variance component analysis indicate significant genotypic variation as well as moderate to high repeatability among the yield related traits. This indicates potential underlying additive genetic variation among the 40 germplasm accessions for yield related morphological traits that could provide the genetic diversity for red clover cultivar development. Phenotypic correlation among traits varied in response to seasons, however, strong positive correlations

were generally noticed for most of the yield related traits. Cluster analysis revealed widely divergent germplasm for use in breeding, although the germplasm responded differently due to seasonal effects. Principal component analysis (PCA) for the first two PCs accounted for 75 % of the total variation which indicates sufficient genetic variation among the studied germplasm and relative contribution of various traits to total variability. Multivariate pattern analysis using PC corresponded well with analysis for variance components, cluster and phenotypic correlation coefficients. This confirms the usefulness of PCA in providing a quick glance into germplasm characterization and diversity, components of variation to the total variability and inter-relationship among studied traits prior to breeding.

3.2 Introduction

Red clover (*Trifolium pratense* L.) is considered as a significant forage perennial legume for livestock and is extensively grown in most parts of the world particularly in temperate climates. It belongs to the family *Fabaceae* and genus *Trifolium* with its origin from the Mediterranean and centre of diversity in Europe (Radinovic *et al*., 2017). The genus *Trifolium* has more than 250 species but only few, about 10% are useful in the pastoral industry (Kiran *et al*., 2010). Red clover is an important plant as it has wide adaptation in nature or under cultivation as compared to other forage legumes (Taylor, 2008). Red clover also improves the soil by fixing atmospheric nitrogen through its symbiotic association with *Rhizobium* bacteria (Yates *et al*., 2014). Moreover, red clover produces high forage yield with increased protein content similar to white clover (*Trifolium repens* L.), with some cultivars potentially being able to produce herbage yields higher than alfalfa (*Medicago sativa* L.) (Drobna & Jancovic, 2006).

White clover is associated with a superior feeding value, high acceptability by stock and fixation of atmospheric nitrogen (Crush, 1987; Caradus, 1991). However, as widely reported, the lack of vegetative persistence of white clover under grazing in mixed swards, especially in ryegrass/white clover pastures, is a major production challenge (Archer & Robinson, 1989; Hutchinson, 1993). As reported by Brazendale *et al. (*2011), economic return on livestock farming dependent on forage is strongly influenced by vegetative persistence. Consequently, there has been considerable investment into improving white clover dry matter production as well as vegetative persistence through breeding for adaptation under New Zealand (NZ) farming conditions. Unlike red clover, breeding efforts in white clover have resulted in several improved varieties with the ability to persist under heavy grazing and long-term mixed swards while still maintaining optimum herbage yield (Widdup & Barrett, 2011). In NZ grazing systems, the uptake of red clover is slow largely due to the lack of vegetative persistence (Ford & Barrett, 2011).

Due to increased usage of red clover in livestock farming, genetic improvement has been mostly focused on yield, protein content, persistence and resistance to various biotic and abiotic factors (Repkova *et al*., 2006). In order to further develop new varieties with improved yield, quality and adaptive traits associated with vegetative persistence, the availability of novel genetic diversity for red clover breeding programs is vital.

Red clover is naturally a diploid species with base chromosome number of n=7, but recently tetraploid varieties have been developed through various ploidy manipulation techniques in commercial breeding programs. According to Sattler *et al.* (2016), induced tetraploid varieties show enhanced performance by increasing persistence, hardiness to winter, drought tolerance, disease resistance and forage dry matter yield compared to diploids. Red clover

populations are highly heterozygous that leads to high levels of genetic variation in and among the populations (Tucak *et al*., 2009), due to the gametophytic self-incompatibility system (Riday & Krohn, 2010). Its outcrossing nature (allogamy) contributes to high genetic diversity within populations (Tanhuanpaa & Manninen, 2012). Due to high natural variation in red clover, prior understanding of the morphological and genetic variation within and among breeding material for key traits is a prerequisite for any genetic improvement programme. Determining the inter- and intra-population genetic variation of this species is necessary for preservation of germplasm and its utilization in breeding, particularly for cross-pollinated species like red clover which is more disadvantaged by inbreeding depression (Radinovic *et al*., 2017). Although red clover has gained a dominant status in livestock production, its wide genetic diversity both in nature and among cultivars is not well studied (Dias *et al*., 2008). To optimize the use of red clover genetic resources, it is important to characterize the morphological and genotypic variation among germplasm accessions in breeding programs (Strauss *et al.* 1988).

Despite the specific advantages of red clover vegetative persistence, particularly under grazing, low seed yield (Clifford & Scott 1989) and vulnerability to fungal diseases (Skipp & Christensen 1990), present major constraints to herbage production. For the genetic potential of red clover to be realized in NZ grazing systems, it is crucial to develop new improved varieties focused on overcoming these major constraints. Research and development for improving vegetative persistence in red clover in NZ was initiated in the 1930s and mainly focus on plant physical structure (morphology) and phytochemistry (Ford & Barrett, 2011). This work resulted in the release of first commercial varieties Grasslands Hamua and Turoa followed by Pawera with some level of persistence. However, problems associated with oestrogenic compounds, known to affect fertility in grazing animals, in these varieties raised

concern. This resulted in breeding to develop improved material with low phytoestrogen expression. Follow up efforts were focused on highly productive plants with prostrate growth habit that could be vegetatively propagated from stems while possessing low levels of phytoestrogens (Hyslop *et al.,* 1999). Varieties derived from this breeding effort were G27 (Rumball *et al*., 1997), Grassland Sensation, Crossway (Claydon *et al.*, 2003), Broadway (Rumball *et al.,* 2003), Tuscan and Colenso all having high dry matter yielding ability with increased vegetative persistence and low phytoestrogens. Recently, the variety Grassland Relish was specifically released for pasture mixes with significantly high survival rate and vegetative persistence, optimum production over a period of 3-4 years, resulting in high weight gain in sheep compared to existing NZ commercial varieties (Ford & Barrett, 2011). Although the development of Relish is a significant breakthrough in red clover genetics in NZ and globally, particularly for its persistence under grazing, research is still ongoing at AgResearch NZ to identify new genetic diversity for further improvement through breeding.

The success of a breeding program largely depends on diversity present in the genetic resources which provide the foundation for genetic improvement. According to Marshall (1989), the lack of information such as characterisation data from germplasm collections is a technical limitation to the effective use of the genetic resources. Germplasm characterisation offers useful information on the strengths and weaknesses in collections to help direct future breeding work as well as management of genetic resources (Bunting, 1983). The Margot Forde Forage Germplasm Centre (Fu & Hampton, 1996) at the AgResearch, Grasslands Research Centre at Palmerston North, New Zealand, contains a globally sourced collection of legume germplasm which also includes red clover. This chapter is focused on a field based morphological characterization trial, carried out over a period of 8 months, on a set of forty

red clover germplasm accessions representing nine different geographic origins around the world.

3.2.1 Objectives

The aim of this research is to estimate the level of variation for key morphological traits among 40 red clover germplasm accessions, the relationship between them and the effect of the environment, in order to identify potentially valuable sources of genetic diversity for future breeding programs.

3.3 Materials and Methods

3.3.1 Red clover germplasm

Forty red clover germplasm accessions representing seven different countries of the Southern Mediterranean region were used in this study (Figure 3.1). These countries include Armenia, Azberbaijan, Caucasus, Georgia, Greece, Portugal, Spain and Tajikistan that experience the Mediterranean climatic condition similar to New Zealand. Accessions selected from these regions were expected to adapt well under New Zealand's condition. Seeds of these accessions were obtained from the Margot Forde Forage Germplasm Centre at the Grasslands

Figure 3.1 Map showing the countries (indicated by blue dots) of red clover collection evaluated in this study.

Research Centre at AgResearch in Palmerston North, New Zealand. The accessions were evaluated under field conditions together with three NZ commercial cultivars; Relish, Broadway, Sensation, which were used as checks. A detailed description of these germplasm accessions with regards to their geographic origins is presented in Appendix 1.

3.3.2 Seed germination and preparation of seedlings

The germination and preparation of seedlings were done according to procedures suggested by Jahufer *et al*., (1997). Fully developed seeds from each accession were randomly sampled, placed in petri dishes on moist filter paper and incubated in dark at 25˚C for three days in order to overcome seed dormancy and induce germination. Following germination, they were planted in to nursery trays containing potting mix and maintained in a glasshouse for 8 weeks before being transplanted out in to the field.

3.3.3 Experimental site and design

The field experiment was established at the experimental field site of the Grasslands Research Centre at Agresearch in Palmerston North, (latitude 40°21′S, longitude 175°37′E, altitude 31 masl) New Zealand (Figure 3.3). The field trial was planted on the 02nd of May in mid-autumn of 2019. Seedlings of each accession were transplanted into the field. The trial was planted according to a row-by-column experimental design containing two replicates (Figure 3.2).

The three check cultivars repeated within each replication would provide an opportunity to reduce spatial trends within each replicate. Perennial ryegrass was sown, 21 days after planting the red clover seedlings, at the rate of 18kg/ha to establish a perennial

ryegrass-based mixed sward in the trial. Due to the establishment of ryegrass not being successful, the trial was continued as a monoculture planting. In every replicate each accession was represented by a row consisting of ten random plants, each spaced at 0.25 m from each other. There was also 0.25 m between the accession rows. Standard cultivation and management practices of clover were followed during the trial. Manual weeding was done once a month after planting the trial, and herbicide Glyphosate 360 (360g/L isopropylamine salt) was applied in between rows once in spring and summer to keep out infestation by weeds

	Column 1	Column 2	Column 3	Column 4	Column 5	
Row 1	29_2507	37_3942	12_3269	Relish	14_4088	
Row 2	Relish	31_2647	6_3898	7_3470	5_3895	
Row 3	20_4084	21_4085	8_3473	33_3591	Broadway	
Row 4	23_2496	4_3885	25_3511	30_2538	Sensation	Rep 1
Row 5	Sensation	32_3590	35_3935	40_3949	26_3513	
Row 6	17_4077	27_3514	36_3941	9_3474	3_3862	
Row 7	34_3594	28_3592	Relish	19_4080	2_3860	
Row 8	18_4079	11_2465	Broadway	15_4095	1_3857	
Row 9	38_3945	Sensation	16_4097	Broadway	10_3476	
Row 10	13_3270	Relish	22_4087	39_3947	24_2498	
Row 1	27_3514	39_3947	29_2507	25_3511	Broadway	
Row 2	15_4095	34_3594	24_2498	Sensation	12_3269	
Row 3	6_3898	Sensation	18_4079	37_3942	9_3474	
Row 4	40_3949	36_3941	Relish	16_4097	21_4085	Rep 2
Row 5	10_3476	1_3857	30_2538	32_3590	Relish	
Row 6	26_3513	33_3591	11_2465	Relish	23_2496	
Row 7	8_3473	22_4087	Sensation	2_3860	7_3470	
Row 8	Broadway	5_3895	19_4080	17_4077	13_3270	
Row 9	28_3592	Broadway	31_2647	14_4088	35_3935	
Row 10	Relish	38_3945	3_3862	20_4084	4_3885	

Figure 3.2 Field layout of the 10 row-by-5 column experimental design containing the 40 germplasm accessions and 3 repeated commercial check cultivars across the trial. The trial consisted of two replicates.

Figure 3.3 Newly established field trial of red clover at the Agresearch Grasslands Research Centre in Palmerston North, New Zealand.

3.3.4 Soil and climatic conditions

The experimental site had soil type characterized as Kairanga silt loam that is still the same as the classification done by Cowie (1974). Soil samples were collected using a 75 mm core a month before planting for soil analysis. The results are presented in Table 3.1.

Table 3.1 Soil test results based on the samples taken from the red clover field trial at the Agresearch Grasslands Research Centre, Palmerston North.

SOIL TEST RESULTS	Units	Results	Soil Range	Soil Fertility Desired
NU015 pH	pH	6.7	5.8~6	
◆NUD09 Effective Cation Exchange Capacity	cmol+/kg	18	12~25	
◆NU388 Volume Weight	g/ml	0.81		
ANIONS				
NU252 Olsen Phosphorus	mg/l	65	20~30	
NU342 Sulfate Sulfur	mg/kg	13	10~12	
CATIONS				
NU057 Calcium MAF QT	MAF QT	15	4~10	
◆NUD04 Exchangeable Calcium	cmol+/kg	14.4		
NU189 Magnesium MAF QT	MAF QT	21	8~10	
◆NUD05 Exchangeable Magnesium	cmol+/kg	1.13		
NU280 Potassium MAF QT	MAF QT	12	5~8	
◆NUD06 Exchangeable Potassium	cmol+/kg	0.76		
NU326 Sodium MAF QT	MAF QT	6	5~20	
◆NUD07 Exchangeable Sodium	cmol+/kg	0.14		
BASE SATURATION				
◆NU051 Calcium Base Saturation	%	80	60~75	
◆NU217 Magnesium Base Saturation	%	6.3	6~15	
◆NU171 Potassium Base Saturation	%	4.2	2~5	
◆NU234 Sodium Base Saturation	%	0.8	1~2	

Results from laboratory analysis showed: pH 6.7, cation exchange capacity (CEC) 18 cmol+/kg, Olsen Phosphorus 65 mg/l, calcium 15 MAF QT, potassium 8 MAF QT, sulphate sulphur 13 mg/kg, magnesium 21 MAF QT, and sodium 6 MAF QT (Table 3.1). Before planting the trial, the soil preparation was done by ploughing and tilling to produce an even seed bed.

Information on the monthly weather conditions for the trial site is presented in (Figure 3.4). Total rainfall distribution during the experimental period from May to December 2019 was 804.7 mm. Mean air temperature was 11.36 ºC while the maximum and minimum air temperatures were 16.0 and 7.01 ºC, respectively.

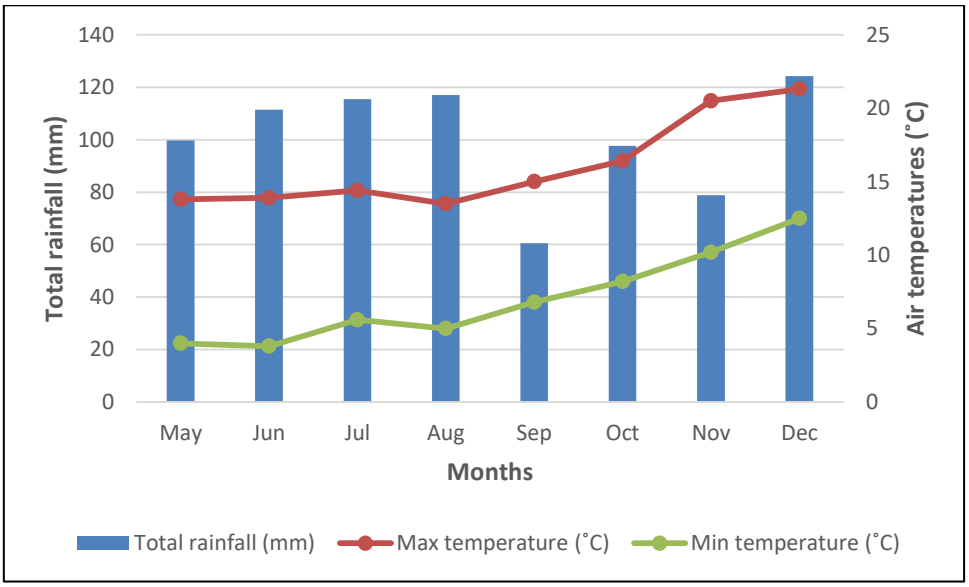


Figure 3.4 Total monthly rainfalls (mm) and mean maximum and minimum monthly air temperatures (℃) at AgResearch Glassland Research Centre, Palmerston North, for the trial duration from May to December 2019.

3.3.5 Assessment methods

Morphological trait measurements were conducted on a seasonal basis. A total of three assessments were conducted. The trial was grazed for 2 to 3 hours by sheep following each assessment. The sheep were removed from the trial area immediately after grazing. The first

assessment was done two months after planting in late winter. The second assessment was carried out towards the end of spring in October 2019. The final assessment was done in early summer in December 2019.

Assessment for semi-quantitative morphological traits: The morphological traits measured were; plant growth habit (PGH) (Figure 3.5), plant growth for visually assessed forage yield (PLG) (Figure 3.5), leaf size (LS), leaf colour (LC), intensity of white colour on leaves (IWC), intensity of anthocyanin pigmentation on stem and leaf (IAN), shape of medial leaflet (SML), tendency to flower (TFL) (Figure 3.6), time to first flowering (TFF). These traits were studied to help with classification of the new germplasm based on their structural and physical features. Only results of the traits PGH, IAN and SML that showed significant ($P<0.05$) differences among the 43 entries are presented in this thesis. A detailed description for assessment of these traits is presented in Table 3.2.

Figure 3.5 Picture of red clover accessions showing variation in their morphological features and growth habit in late spring (6 months after planting) from our field experiment.

Measurement of quantitative traits: The morphological/phenotypic characters that were quantitatively assessed include; stem number (STN), medial leaf length (MLL) and medial leaf width (MLW), plant height (PLH), plant survival (PLS) and area of plant spread (APLS). Since these traits are generally associated with red clover forage yield, indirect measurements for these traits could possibly provide an indication of the germplasm's yield potential. A detailed description of the assessment method used for each trait is presented in Table 3.2. Since genotypic variation among the 43 entries for the trait plant survival (PLS) was not statistically significant (P>0.05), due to high and uniform plant survival after planting, the result is not presented in the thesis.

Table 3.2 Description of the semi-quantitative and quantitative morphological traits assessed in the investigated red clover population.

Traits	Description	Reference
Assessment for semi-quantitative morphological characters		
Plant growth (PLG)	Visually assessed on a scale of 1-9. Scored against a well-established plot/line of plants assigned a score of 9 as reference point. 1 = extremely poor growth, 3= poor, 5= moderate, 7=vigorous growth, 9= highly vigorous	Asci (2011) UPOV (2001)
Plant growth habit (PGH)	Visually assessed according to five levels of scoring (1= erect, 3= semi erect, 5= intermediate, 7= semi prostrate and 9= prostrate). Visual estimate was taken of the angle that the outer shoots make with the horizontal.	Ahsyee *et al*. (2014)
Tendency to flower (TFL)	Ability to flower was assessed based on population, as visually estimated in the field scoring (1= no flower, 3= poor, 5= moderate, 7= profuse flower). Refer to Figure 3.6	Asci (2011) UPOV (2001)
Time to first flower (TFF)	Measured in number of days after planting (DAP). Observed regularly to record initiation time to first flowering	

Leaf size (LS)	Visually assessed on group of plants. Scores 3=small, 5=medium, 7=large	UPOV (2001)
Shape of medial leaflet (SML)	Visually assessed based on illustration of medial leaflet shapes. Leaflet shapes 1= elongated, 2= ovate, 3= rounded.	UPOV (2001)
Leaf color (LC)	Visually assessed where 3= light green, 5= medium green, 7=dark green)	UPOV (2001)
Intensity of white marks on leaves (IWM)	Leaf mark determined as absent or present based on visual assessment by a single observation of a group of plants in each column within the rep.	Ahsyee *et al*. (2014)
Intensity of anthocyanin colouring on stem and leaf (IAN)	Assessed visually on group of plants and scored according to 3-point scale. Scores 1=no to very weak anthocyanin present, 2=weak to moderate, 3=moderate to strong	
Measurement of quantitative characters		
Plant height (cm) (PLH)	Measured distance at ground level to the tip of the plant without stretching using meter scale. Measured on four inner row plants on each column within each replicate during or just before grazing	Asci (2011)
Area of plant spread (APLS) (cm^2)	Plant spread was estimated as the area of a circle using the mean of two measurements of plant diameter taken perpendicular to each other.	Jahufer *et al*. (1997)
Number of stems (STN)	Manually counted primary branch per planton four inner row plants	Asci (2011)
Width of medial leaflet (MLW)	Measured width (mm) on four inner row plants on each column within each replicate using venier calliper.	Asci (2011) UPOV (2001)
Length of medial leaflet (MLL)	Measured length (mm) on four inner row plants on each column within each replicate.	Asci (2011) UPOV (2001)
Plant persistence (PPE) or plant survival (PLS)	Persistency in field was measured in terms of plant survival (%) count in each plot based on initial number of plants planted.	Ahsyee *et al*. (2014) Ford & Barrett (2011)

Figure 3.6 Picture of red clover accessions at flowering stage from our field trial in mid-summer at 7 months after planting. The ruler placed in front of the red clover plants was used to measure plant height.

3.3.6 Statistical analysis

Statistical analysis of all yield related traits; plant growth (PLG), leaf size (LS), plant height (PLH), number of stems (STN) and area of plant spread (APLS) were carried out using DeltaGen (v 0.03) (Jahufer & Luo, 2018), R (v 3.5.3) (R Core Team, 2019) and GenStat 17th (VSN, 2016) statistical software. Quantitative traits with multiple data points, for instance, PLH, STN, APLS, medial leaf width (MLW), medial leaf length (MLL) and plant survival (PLS) measured on several plants within a row were averaged in Ms Excel Spreadsheet to come up with one measurement then analysed using accession as treatment and replicate as block. Normality test was done with GenStat 17th (VSN, 2016) before the actual data analysis in order to make sure that the data is distributed normally. Analysis of variance (ANOVA) for each season and pooled across three seasons was performed separately using one-way and two-way ANOVA respectively using DeltaGen (v 0.03). Data for each trait was

analysed separately to provide outputs for means, least significant difference ($LSD_{0.05}$) and F pr values as tests of significance at 95% confidence interval. Further statistical test using multivariate analysis procedures (cluster analysis, principal components analysis (PCA), variance component analysis, phenotypic correlation) were done on traits that show significant ($P<0.05$) genotypic variation in the population. Cluster analysis was done with a hierarchical agglomerative classification procedure with squared Euclidean distance as a measure of dissimilarity and Ward's minimum variance method for clustering. Analyses were performed using the 'hclust' function in R version 3.5.3 (R Core Team, 2019). Prior to cluster analysis the BLUP values from REML for traits were scaled to have a mean of zero and a variance of one. Genotypic means of population from ANOVA and their respective LSDs and F pr values for the five yield related traits were plotted on boxplot using R to graphically depict performance and variability of each accession/cultivar. The lollypop chart was performed using the phenotypic means generated for each country from the ANOVA in R.

3.3.6.1 Residual maximum likelihood analysis

Variance component analysis based on Residual Maximum Likelihood (REML) was conducted for all measured traits within each season and across all three seasons. The variance component analysis was done in order to assess the significance and magnitude of genotypic variation among the accessions and cultivars. The data were analysed using the procedures for variance component analysis, in the univariate REML option, in DeltaGen software (v 0.03) (Jahufer & Luo, 2018). Analyses were performed using a linear mixed effects model with season as a fixed effect, particularly for analysis across seasons. Variance components were generated from random variables with associated standard errors.

Linear model 1 (Equation 3.1) was used in the REML analysis for the traits measured in each separate season based on individual plant or plot (row). Linear model 2 (Equation 3.2) was

used for trait measurements pooled across all three seasons. In both linear models, "accession" refers to both germplasm and commercial check cultivars.

The random linear model used in the analyses for within individual season trait expression:

$$\boldsymbol{Y_{ijkl} = M + g_i + b_j + r_{jk} + c_{jl} + \varepsilon_{ijkl},} \quad (3.1)$$

Yijkl is the value of an attribute measured from sample of plant *i* in row *k* and column *l* of replicate *j*; $i=1,...,n_g$, $j=1,...,n_b$, $k=1,...,n_r$, $l=1,...,n_c$, where *g*, *b*, *r* and *c* are accessions, replicates, rows and columns respectively. *M* is the overall mean and g_i is the random effect of accession *i*, $N(0,\sigma^2_g)$. The initial b_j represents the random effect of replicate *j* $N(0,\sigma^2_b)$ while r_{jk} is the random effect of row *k* within replicate *j*, $N(0,\sigma^2_r)$. The random effect of column *l* within replicate *j*, $N(0,\sigma^2_c)$ is represented by $c_{jl.}$ Initials ε_{ijkl} is the residual effect of accessions *i* in row *k* and column *l* of replicate *j*, $N(0,\sigma^2_\varepsilon)$.

The linear random model used to analyse accession performance across seasons:

$$\boldsymbol{Y_{ijklm} = M + g_i + s_j + (gs)_{ij} + b_{jk} + r_{jkl} + c_{jklm} + \varepsilon_{ijklm},} \quad (3.2)$$

Yijklm is the value of an attribute measured from sample of plants *i* in row *l* and column *m* of replicate *k* nested in season *j* and $i=1,...,n_g$, $j=1,...,n_s$, $k=1,...,n_b$, $l=1,...,n_r$, $m=1,...,n_c$, where *g*, *s*, *b*, *r* and *c* are accessions, seasons, replicates, rows and columns respectively. *M* represents the overall mean whilst g_i and s_j are the random effect of accessions *i*, $N(0,\sigma^2_g)$ and season *j* $N(0,\sigma^2_s)$ respectively. Effects of interaction between accessions *i* and season *j*, $N(0,\sigma^2_{gs})$ is denoted by $(gs)_{ij}$. Represented by $b_{jk,}$ $r_{jkl,}$ c_{jklm} are the random effects of replicate *k* within season *j* $N(0,\sigma^2_b)$, row *l* within replicate *k* within season *j*, $N(0,\sigma^2_r)$ and column m within replicate *k* within season *j*, $N(0,\sigma^2_c)$ respectively. ε_{ijklm} is the residual effect of accessions *i* in row *l* and column *m* of replicate *k* in season *j*, $N(0,\sigma^2_\varepsilon)$.

3.3.6.2 Best linear unbiased predictor (BLUP)

Best Linear Unbiased Predictor (BLUP) values were generated for all measured traits from REML analysis when the 43 accessions were considered as random.

3.3.6.3 Repeatability on an accession mean basis

Repeatability among the 43 accessions was determined to estimate the upper limit on broad-sense heritability, because it includes genetics and environmental sources of variation (Falconer, 1989).

Repeatability (R^2) based on accession mean was estimated for each trait when there was significant (P<0.05) genotypic variance (σ_g^2) from REML analysis. Genotypic (σ_g^2), genotype-by-season interaction (σ_{gs}^2), and experimental error (σ_ε^2) variance components obtained from REML analysis were used with the number of replications (n_b) and the number of seasons (n_s) where applicable, to estimate repeatability. Repeatabilities (R^2) were estimated for trait expression within individual seasons and across seasons, respectively, using equations 3.3 and 3.4.

$$\boldsymbol{R_1^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_\varepsilon^2}{n_b}} \qquad (3.3)$$

$$\boldsymbol{R_2^2} = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_{gs}^2}{n_s} + \frac{\sigma_{gb}^2}{n_b} + \frac{\sigma_\varepsilon^2}{n_s n_b}} \qquad (3.4)$$

3.3.6.4 Multivariate pattern analysis

BLUP values for all traits were used to construct a two-way accession-by-trait matrix which was used in pattern analysis, a combination of principal component analysis (PCA) and

cluster analysis. Pearson correlation among the traits was also estimated. All analyses were conducted using DeltaGen (v0.02) software (Jahufer & Luo, 2018).

3.4 Results

3.4.1 Differences in yield related traits under different seasons

The means and associated LSD (least significant difference) estimates for the accessions/cultivars for plant growth and yield related morphological traits (leaf size, stem number, plant height, area of plant spread) evaluated in winter, spring, summer and also across the 3 seasons are summarized in Figures 3.7 to 3.21.

3.4.1.1 Plant growth (PLG)

Significant ($P \leq 0.05$) differences among populations were detected for the plant growth (PLG) in winter, spring and summer. This was clearly obvious from the picture of the red clover showing variation in their growth at AgResearch field experiment as shown in Figure 3.5.

The value of PLG ranged from 1.0-8.5, 3.0-9.0 and 4.5-9.0 in winter, spring and summer, respectively. High PLG mean of 7.66 was recorded in summer followed by spring (7.24) and winter (4.5). Plant growth was generally high in summer which was indicated by the grand mean of 7.66 (Figure 3.7).

Accessions17, 18, 19, 25 and Broadway were among the 13 accessions/cultivars which show significantly ($P \leq 0.05$) higher mean plant growth in winter including the check cultivars (Relish and Sensation). It is evident that the accessions with origin mainly from Spain and Portugal have shown vigorous growth and are highly rated for PLG in winter. However, majority of the accessions from Greece and Tajikistan show significantly ($P \leq 0.05$) poor

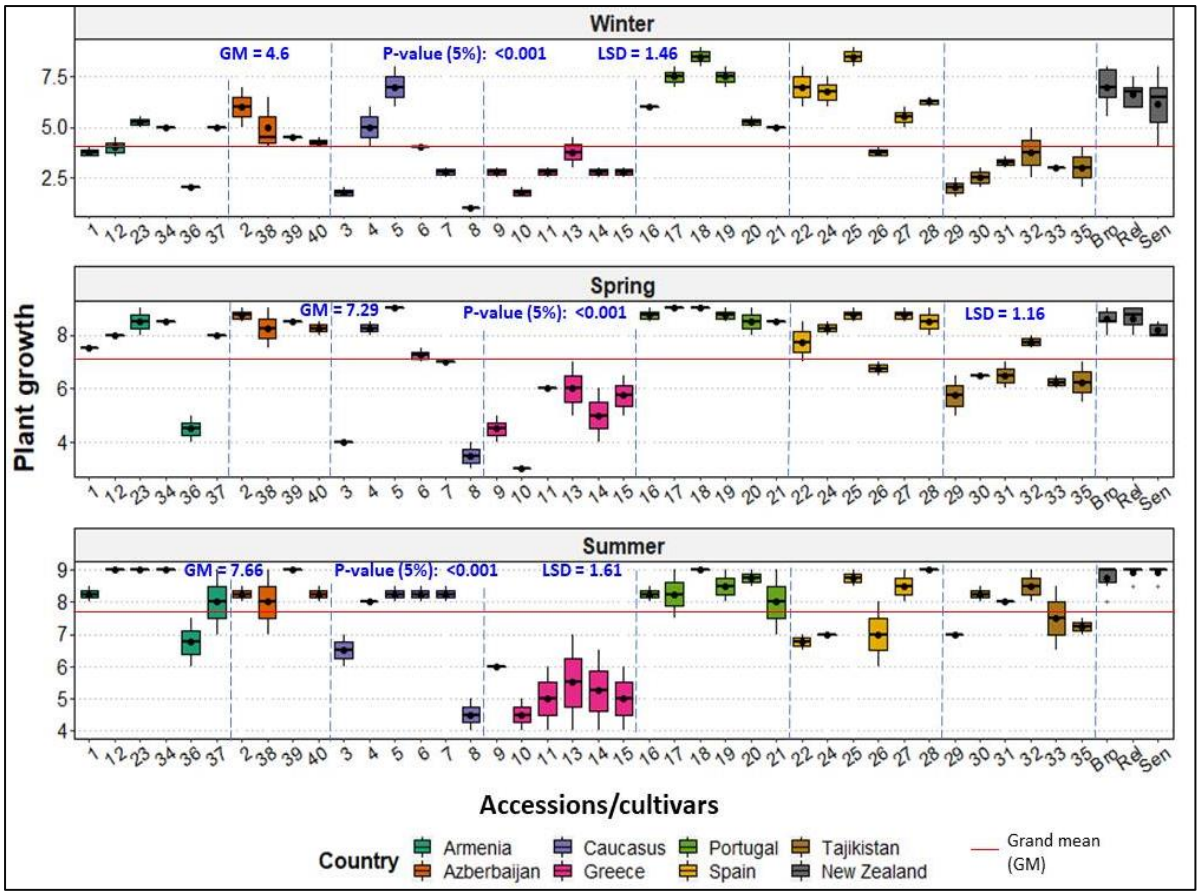


Figure 3.7 Boxplot showing variation in the mean plant growth (PLG), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars recorded in winter, spring and summer.

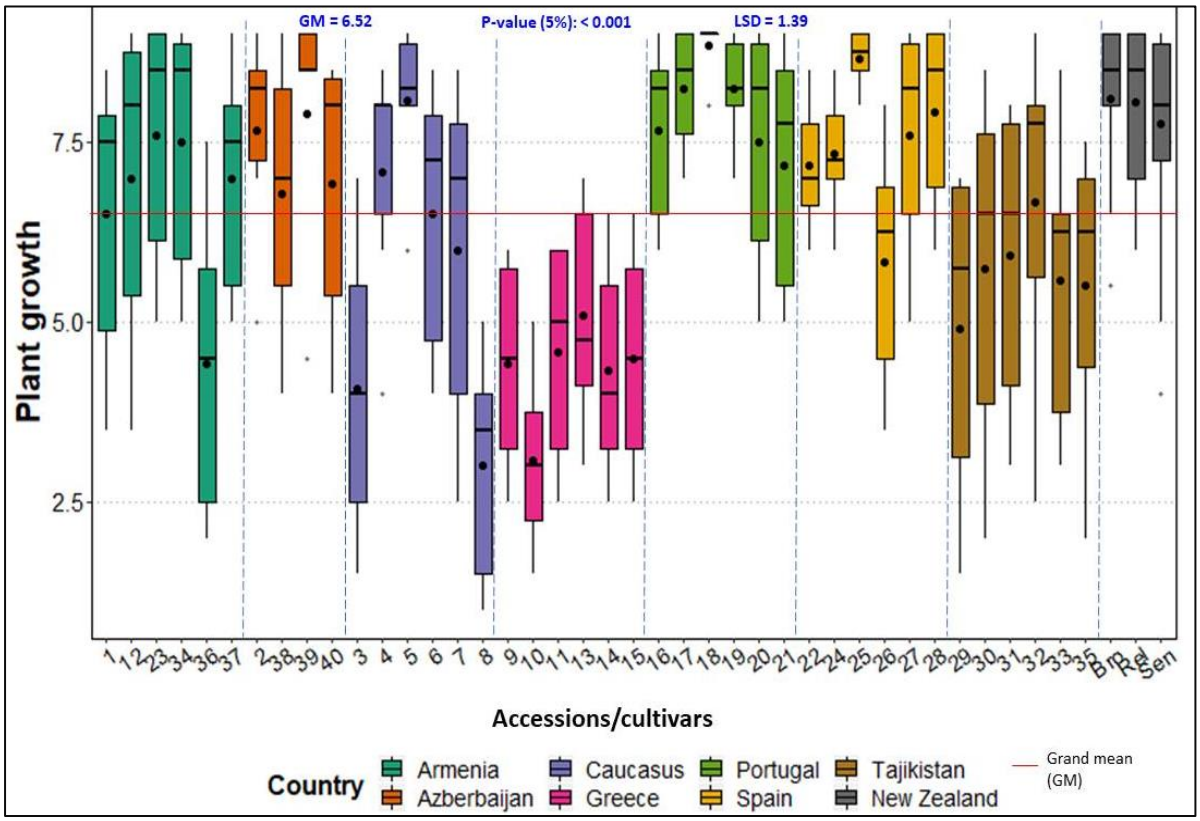


Figure 3.8 Boxplot showing variation in the mean plant growth (PLG), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars combined across seasons.

growth in winter as indicated by their low PLG means. Similarly, for spring, highly significant (P≤0.05) variation in PLG exists among the tested accessions/cultivars.

Accessions 17, 18, 19, 25 and Broadway made a significant come back in spring while accessions 27, 2, 39, 5, 16 picked up well with significantly high (P≤0.05) PLG (Figure 3.8). More than a half of accessions evaluated were rated above the accession grand mean for PLG including the three commercial cultivars. As was the case in winter, almost all the accessions from Greece and Tajikistan are still struggling to pick up with others even in spring for growth with PLG score range recorded below the average PLG (Figure 3.8).

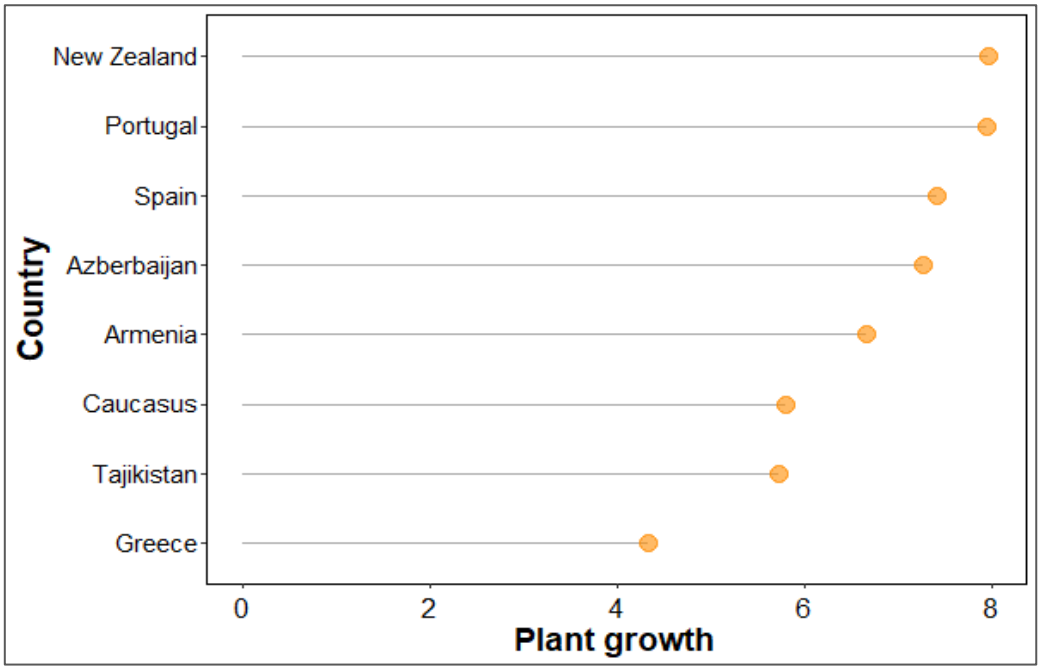


Figure 3.9 Representation of mean plant growth (PLG) on the Lollipop chart for 43 accessions against respective countries of origin.

On the other hand, most accessions from Armenia, Azberbaijan and Spain show substantial growth (PLG) increase from winter to spring. During summer, majority of the accessions/cultivars reached maximum plant growth except for all accessions originating from Greece and a couple of them from Caucasus with PLG scores well below average. Highest PLG was recorded in six accessions *viz.* 12, 23, 34, 39, 18, 28 in summer compared to adapted test cultivars and the rest of the accessions. Except for accessions 18 and 28, the other four are among the accessions with poor growth and plant establishment in cold winter but eventually picked up in spring to reach their maximum growth under summer condition. Accessions 18, 25 and 19 obviously stands out among others including the three commercial checks for their consistently high PLG observed in each season as well as across all seasons (Figure 3.8). Accessions with highest values for plant growth had their origin from Portugal and New Zealand followed by population from Spain while Greece had the least plant growth values amongst others (Figure 3.9).

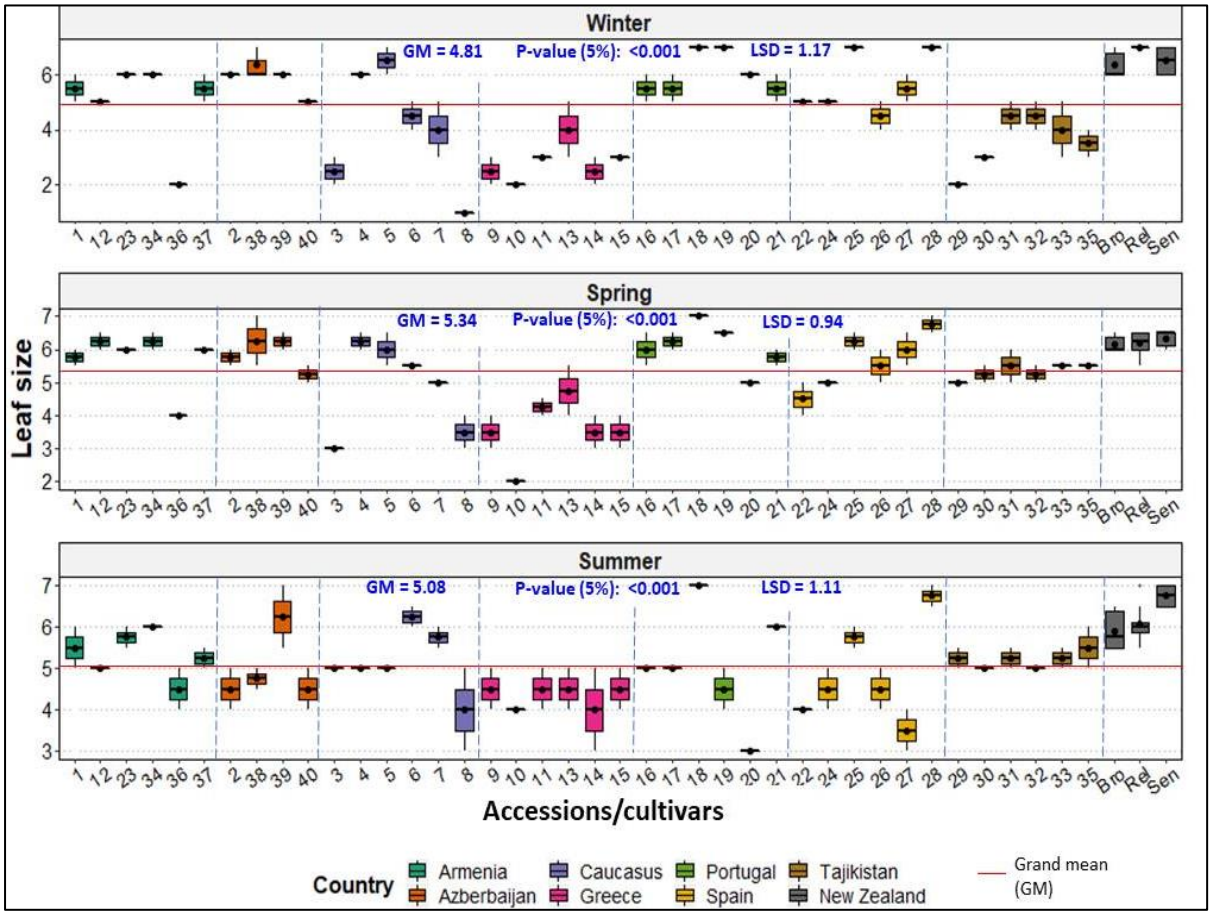


Figure 3.10 Boxplot showing variation in the mean leaf size (LS), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars recorded in winter, spring and summer.

3.4.1.2 Leaf size (LS)

Significant variation (P≤0.05) among accessions for leaf size (LS) was observed in each season and across seasons (Figure 3.10). Mean LS among accessions was greater in spring observation (5.3) compared to summer (5.1) and winter (4.7). Accessions 19 and 25 are at par with Relish for having large leaf sizes (LS 7.0-7.1) under winter condition followed closely by 5 and 18.

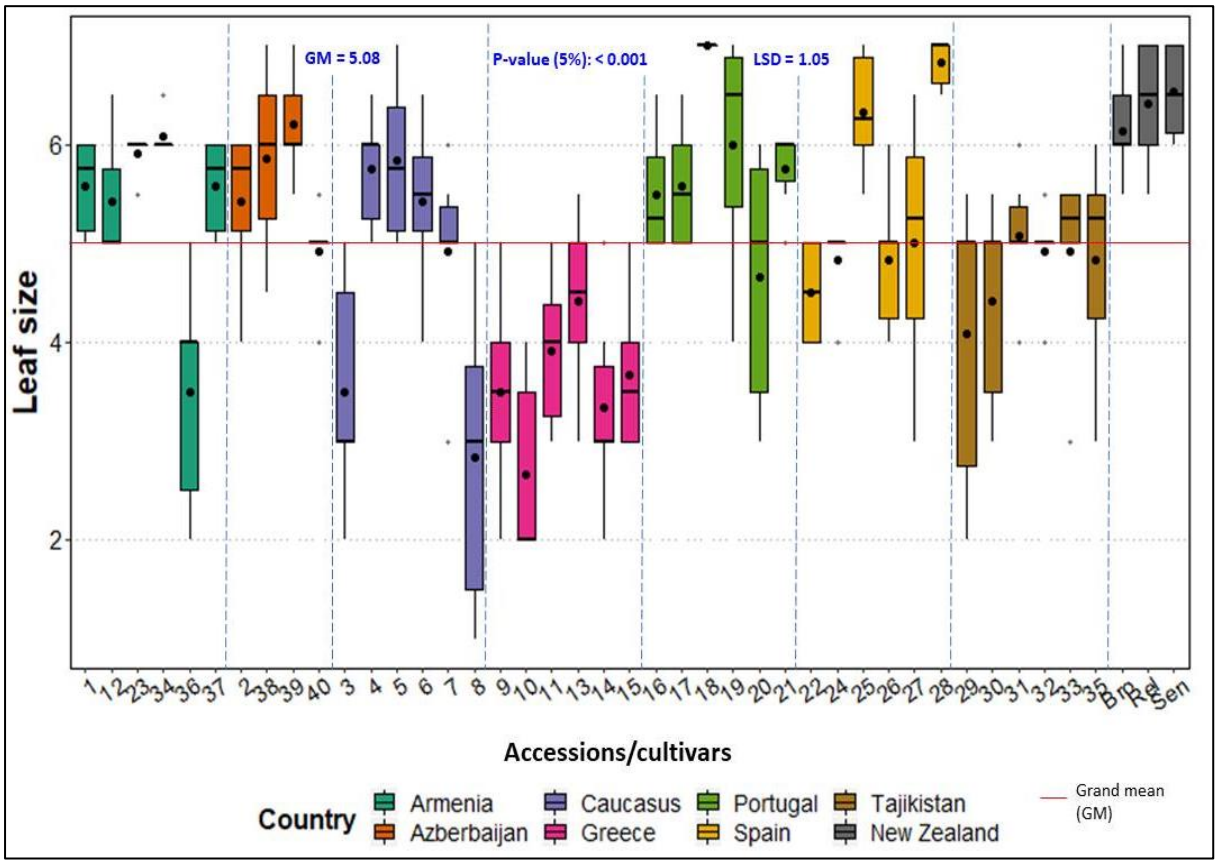


Figure 3.11 Boxplot showing variation in the mean leaf size (LS), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars combined across seasons.

On the contrary, accessions 8, 36, 10 and 29 are among the ones with significantly (P≤0.05) small LS with scores ranging from 1.1 to 2.1. Slight increase in LS was observed in the test accessions from winter to spring (Figure 3.10). Accessions with significantly (P≤0.05) large leaf size are 18, 28, and 19. Majority of accessions including the three cultivars have medium to large leaf size with scores between 4.5 to 6.5 while accession 10 maintained the least LS score together with 3 in spring. Likewise, for winter and spring, leaf sizes among the accessions/cultivars are significantly (P≤0.05) different in summer (Figure 3.10). It is obvious that mean LS generally reduce under summer condition for most of the accessions as red clover plants reached their floral and reproductive phase. Nevertheless, accessions 18, 28 and Sensation have the highest score for LS in summer as compared to others.

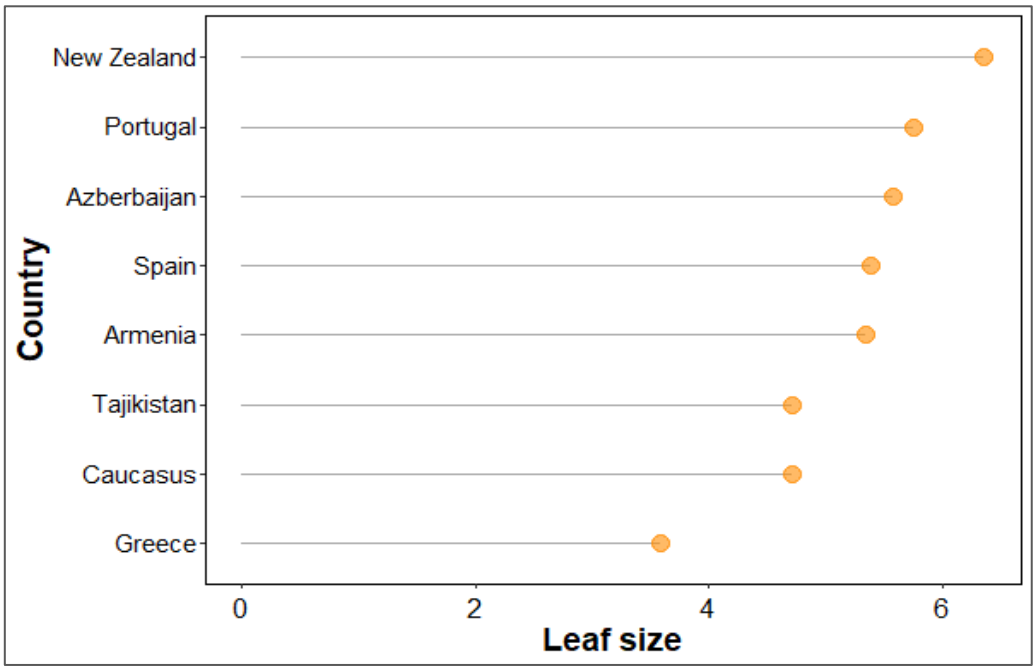


Figure 3.12 Representation of mean leaf size (LS) on Lollipop chart for 43 accessions against respective countries of origin.

Overall, accessions 18 and 28 have the highest combined mean across the seasons for LS followed by cultivars Sensation and Relish and accessions 25 and 39 (Figure 3.11). It is also noticed that accessions originated from Greece consistently produce smaller leaf sizes across

all seasons. On the contrary, the commercial cultivars from New Zealand and accessions from Portugal have larger leaf size (Figure 3.12).

3.4.1.3 Stem number (STN)

The number of red clover stems relates well with herbage yield. Highly significant (P≤0.05) differences were noticed in stem number (STN) for the accessions under observation for the respective seasons (winter, spring and summer) and across seasons (Figure 3.13).

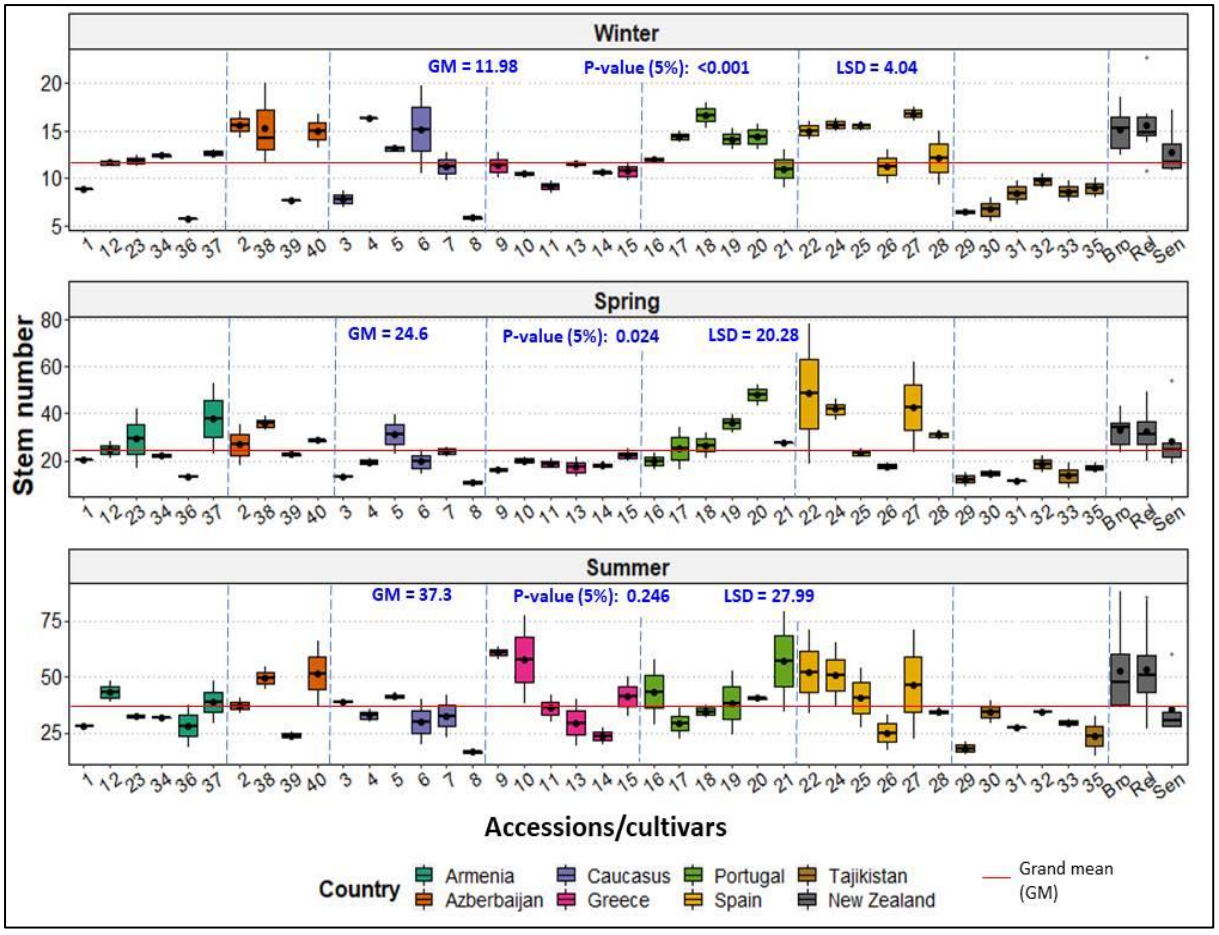


Figure 3.13 Boxplot showing variation in the mean stem number (STN), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars recorded in winter, spring and summer.

The highest stem count per plant (16.6 stems) was recorded in accession 27 and 18 with origin from Spain and Portugal respectively in winter. Number of stem count (STN) per plant

differs between accessions based on the country of origin. Apparently, accessions mainly from Portugal, Spain and Azerbaijan have high STN that are almost similar to the check cultivars except the Tajikistan's accessions. Number of stem count among accessions increase by more than 50% from winter to spring. Similarly, the error coefficient of variation percentage for accessions (ECV %) increased by two-fold from winter to spring which indicate less precision in spring data. Accessions with the most STN in spring are 22 and 20 then followed closely by 27 and 24 compared to others including the check cultivars. Majority of the accessions from Tajikistan maintained the least STN even in spring.

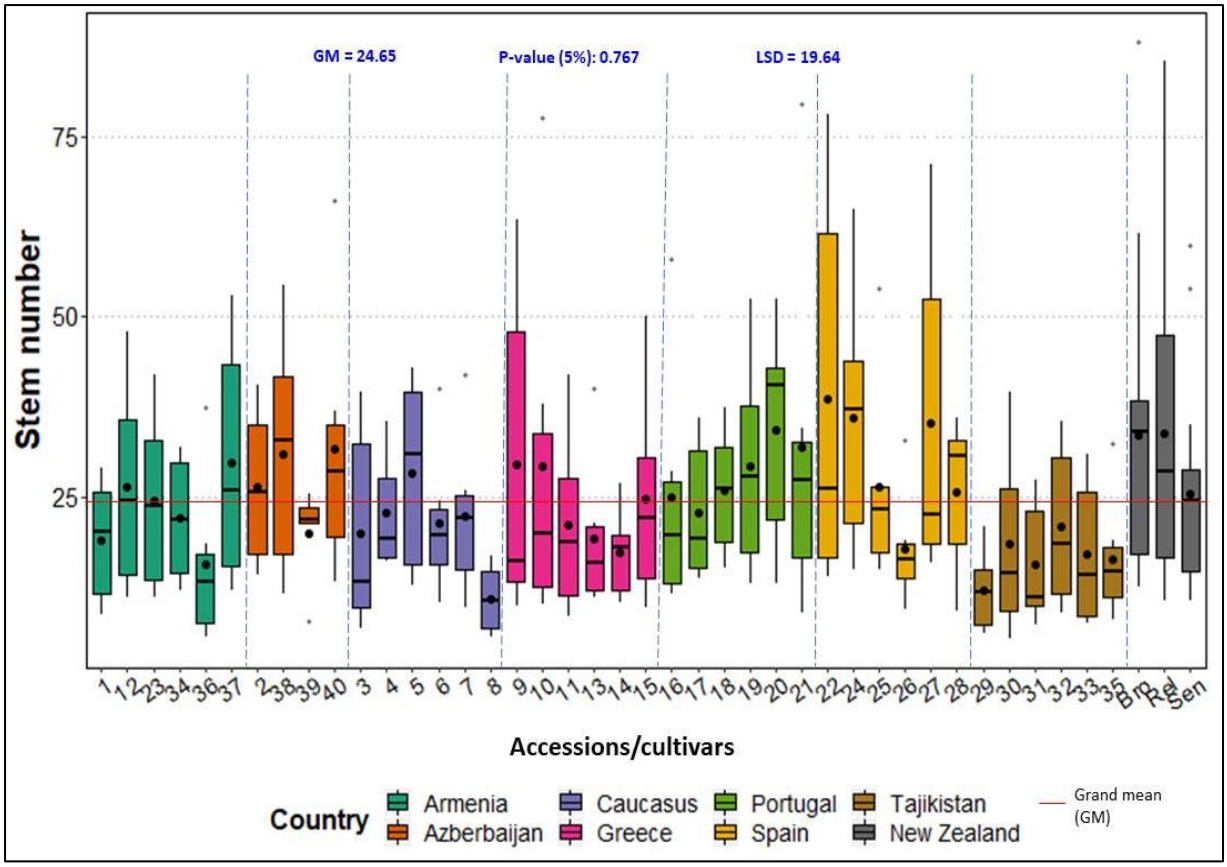


Figure 3.14 Boxplot showing variation in the mean stem number (STN), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars combined across seasons.

In the summer assessment, accessions 9, 10 and 21 were identified to have the significantly ($P \leq 0.05$) highest mean of STN per plant that range between 57 to 61 in comparison to others

including the three commercial cultivars. Accessions 22 and 24 both from Spain recorded the highest mean of stem number compared to others including the checks across seasons (Figure 3.14). Overall, cultivars from New Zealand and accessions from Spain produced more stems compared to the ones originating from Tajikistan and Caucasus (Figure 3.15).

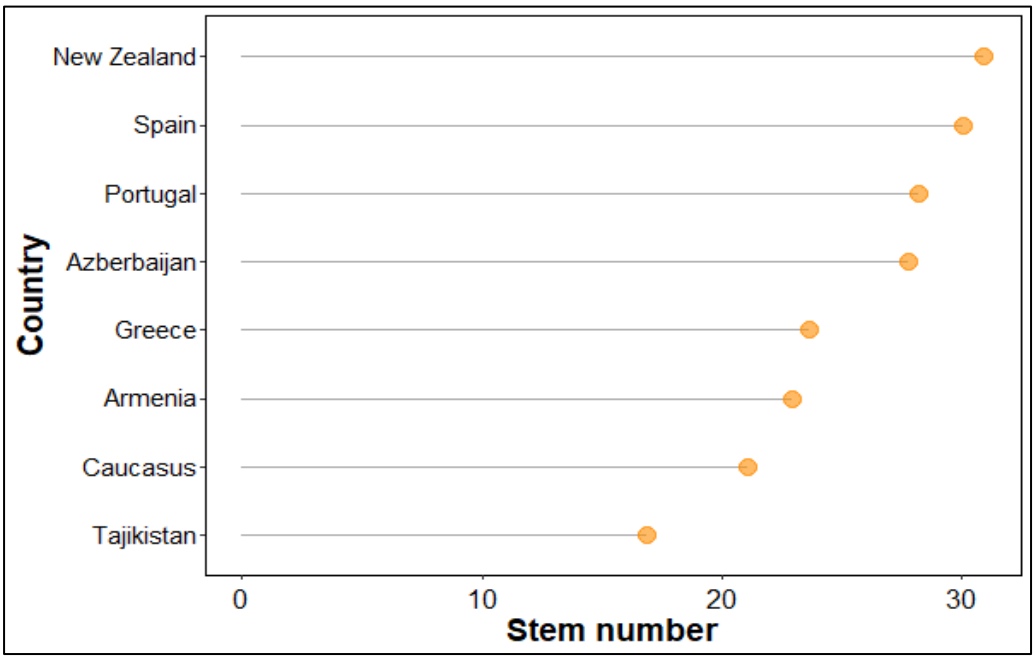


Figure 3.15 Representation of mean stem number (STN) on Lollipop chart for 43 accessions against respective countries of origin.

3.4.1.4 Plant height (PLH)

Plant height is one of the most important yield components and is often used in breeding as a criterion in the selection of superior genotypes to increase yield. Plant height (PLH) in each season differs ($P \leq 0.05$) significantly between the tested accessions/cultivars with mean PLH range from 0.5-6.8, 3.9-16.6, 23.6-57.6 cm for winter, spring and summer respectively (Figure 3.16). Accessions 18 had the highest PLH of 6.8 cm in winter followed by 25, Sensation and 28. As indicated by the PLH grand mean, there is a three-fold increase in PLH from winter to spring and further increase by four-fold from spring to summer. The average height of plants of all accessions/cultivars across seasons was 17.1 cm (Figure 3.17).

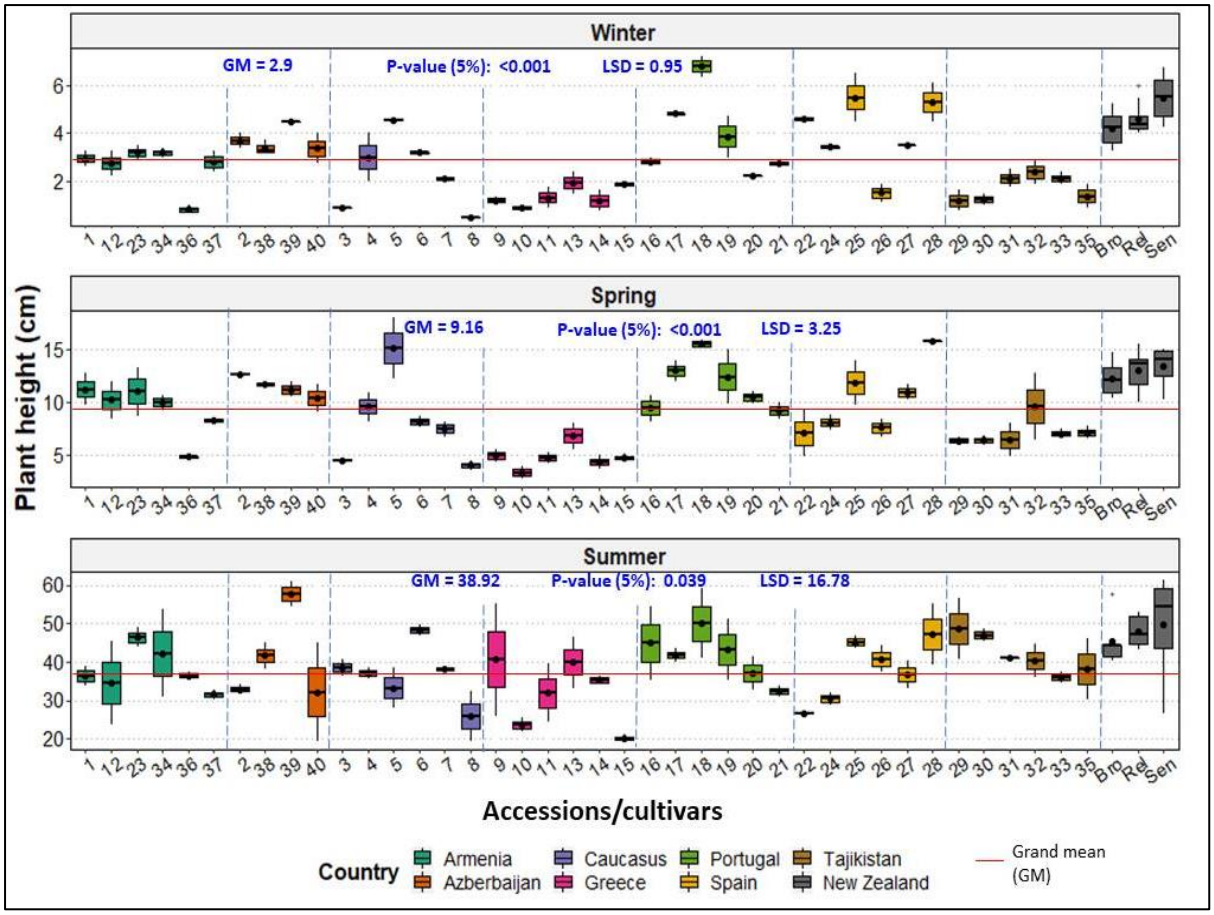


Figure 3.16 Boxplot showing variation in mean plant height (PLH) (cm), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars recorded in winter, spring and summer.

Accession 28 moved up the rank to secure the first position in spring in terms of PLH with 16.6 cm followed by both 5 and 18 with mean PLH of 15.5 cm. In summer, accessions 39, 18 and Sensation recorded the highest PLH mean of 50 cm and above. The same three accessions/cultivars maintained the highest PLH across the seasons (Figure 3.17). On the other hand, accessions 8 and10 have consistently recorded the lowest PLH for each season whilst combined PLH means across seasons tagged accessions 15 to be the shortest among others. The New Zealand commercial cultivars lead in terms of plant height followed by accessions from Portugal and Azerbaijan (Figure 3.18).

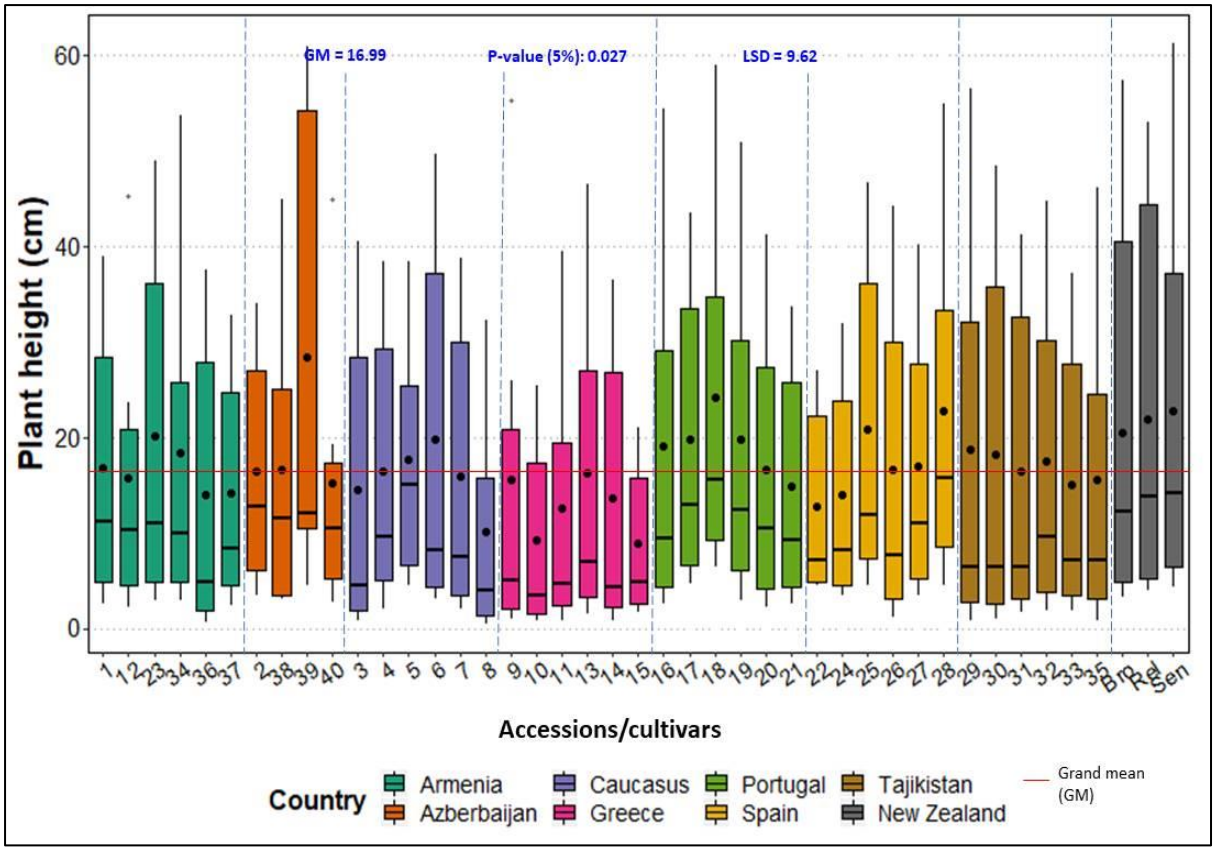


Figure 3.17 Boxplot showing variation in the mean plant height (PLH) (cm), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 accessions/cultivars combined across seasons.

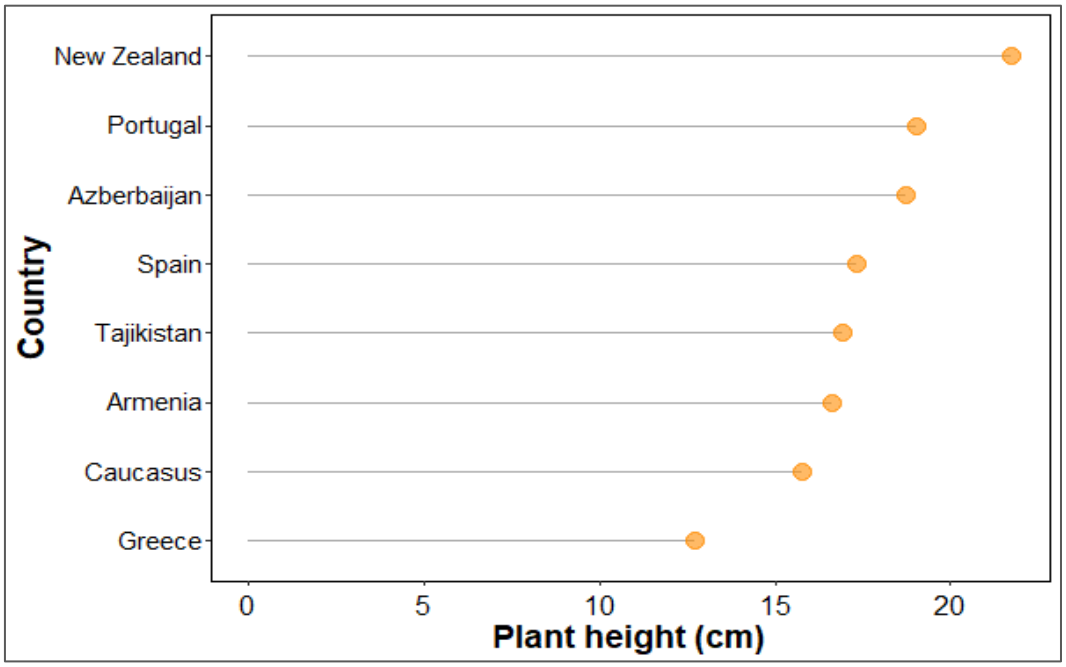


Figure 3.18 Representation of mean plant height (PLH) on Lollipop chart for 43 accessions against respective countries of origin.

3.4.1.5 Area of plant spread (APLS)

Unlike white clover, red clover typically has a semi-prostrate to erect growth habit with short stolons/stems that does not readily spread or creep out from its growing point. This type of growth pattern in white clover is desirable due to the fact that it facilitates excellent grazing tolerance and improves plant's tolerance to drought. Thus, assessment for plant spread in this study was undertaken to identify accessions with prostrate growth habit with stoloniferous type spread to be used in further red clover breeding for improved persistence and drought tolerance. Variation in the mean area of plant spread (APLS) (m^2), P-values (5%) and least

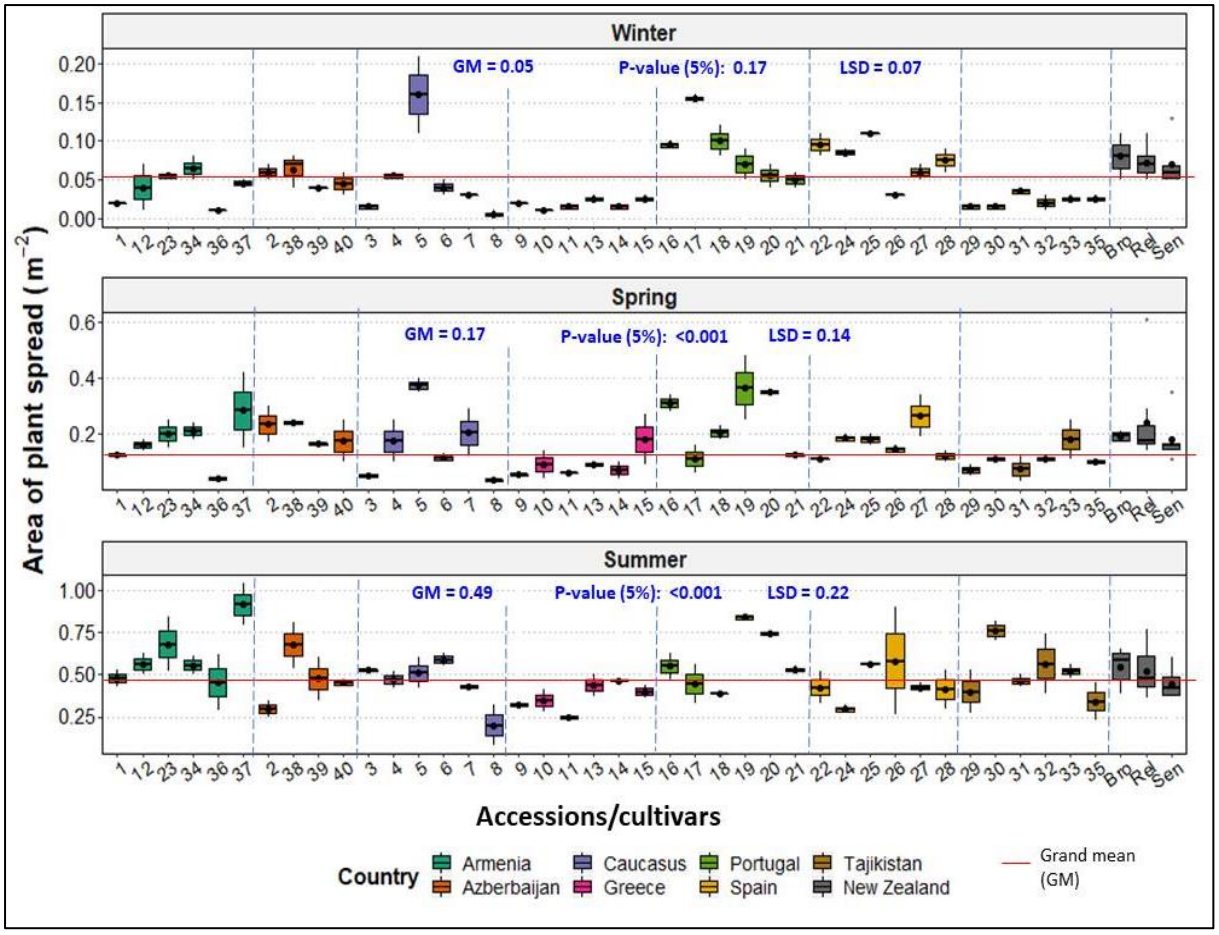


Figure 3.19 Boxplot showing variation in the mean area of plant spread (APLS) (m^2), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 red clover accessions evaluated in winter, spring and summer.

significant differences (LSD) for the 43 accessions/cultivars recorded under each season and combined across seasons were summarized as boxplots in Figure 3.19 and 3.20 respectively.

The overall APLS mean of the 43 accessions/cultivars in winter is 0.051 m^2 while it ranged between 0.0096 to 0.1163 m^2 (Figure 3.19). There was no clear difference ($P<0.05$) in the mean spread of plants (APLS) among the entire red clover accessions in winter as indicated by the $LDS_{0.05}$ value. However, differences in means of the accessions evaluated show that 21 accessions including the three cultivars (Relish, Broadway and Sensation) had mean APLS slightly above the overall grand mean.

On the other hand, significantly ($P<0.001$) large differences were detected among the accessions tested in spring compared to winter assessment with APLS range from 0.038 m^2 (08) to 0.375 m^2 (05) resulted in 0.136 m^2 overall mean. Although 22 accessions including the 3 cultivars had above average APLS, only four accessions (05, 19, 16 and 20) were significantly ($P<0.05$) higher compared to the rest including the 3 cultivars. Accession 5 represented by accession code 05 with the highest APLS originate from Caucasus while three others are from Portugal. Overall, APLS among the accessions doubled from winter to spring and further into summer.

Summer assessment show a four-fold increase in the APLS as indicated by the overall mean from 0.165 to 0.494 m^2 (Figure 3.19). Mean of APLS among the test accessions also increase with values range from 0.201 m^2 in 08 to 0.913 m^2 in 37. In addition, highly significant ($P<0.001$) differences in APLS were evident among the test accessions in summer. Accessions with significantly ($P<0.05$) highest APLS in summer are 37, 19, 30 and 20. The other 15 accessions including Relish and Broadway are among the 15 accessions with above average APLS values but not significantly high. The rest of the accessions with below average APLS values between 0.480 to 0.201 m^2 could be the non-spreading plant type with

erect branching. Accessions 20 and 19 had an increased APLS value steadily across spring and summer seasons.

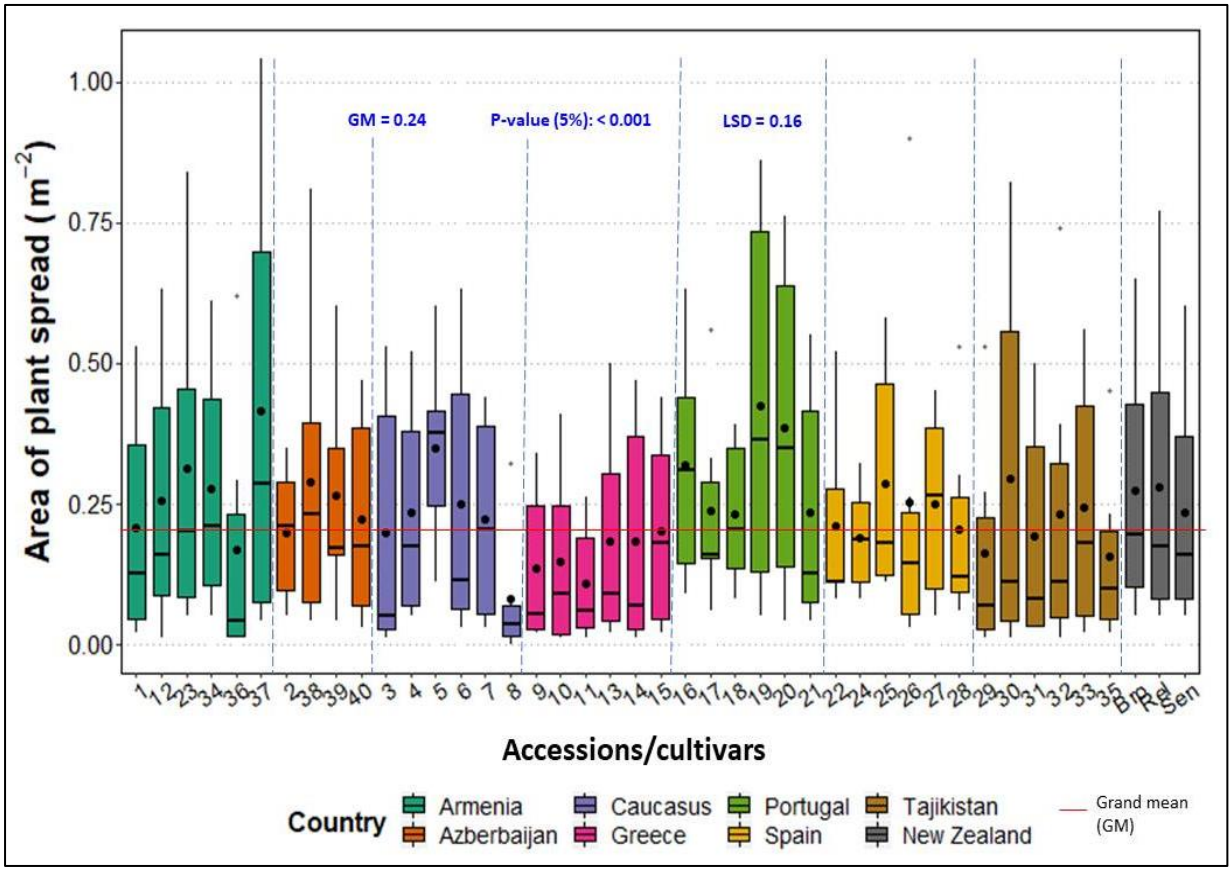


Figure 3.20 Boxplot showing variation in the mean area of plant spread (APLS) (m^2), grand mean (GM), P-value (5%) and least significant difference (LSD) for the 43 tested red clover accessions (during the summer period) from pooled data analysis across the three seasons.

Combined analysis across the three seasons detected significant ($P<0.05$) variation in the APLS among the 43 red clover accessions/cultivars (Figure 3.20). It is also evident that the accessions responded differently under different seasons (accession-by-season interaction effect) as presented in Figure 3.17-18. Accessions 37 (0.412 m^2) from Armenia and 19 (0.410 m^2) from Portugal had comparably higher APLS across seasons while closely followed by 20 (0.367 m^2) from Portugal. Overall result of plant spread show that 17 accessions including

cultivar Relish and Broadway had moderate to high spreading habit. However, accessions with below average values together with cultivar Sensation do not spread out easily.

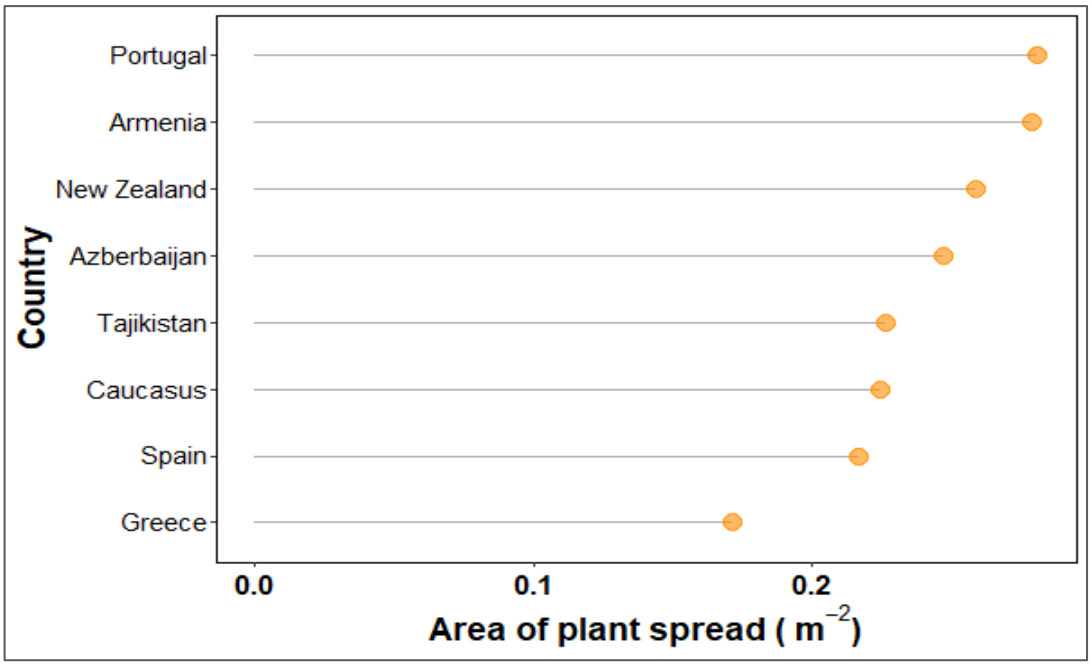


Figure 3.21 Representation of mean area plant spread (APLS) on Lollipop chart for 43 accessions against respective countries of origin.

It is evident in this study that accessions originating from certain geographical regions have some characters in common. Although not statistically different, accessions from Portugal, Spain and New Zealand (three cultivars) had high APLS means in winter compared to accessions from other origins (Figure 3.19). For spring assessment, accessions mostly from Armenia, Azerbaijan, Portugal and Spain including the NZ cultivars were found to have high APSL values. Under summer condition, high plant spread occurred in accessions mostly from Armenia and Portugal. Overall, accessions with the highest value of plant spread across seasons are from Portugal and Armenia followed by three New Zealand commercial cultivars (Figure 3.21). Conversely, accessions from Greece and Spain have relatively low APLS values.

3.4.2 Analysis of variance components and accession mean repeatability estimates

3.4.2.1 Seasonal variation

Variance component and repeatability estimates were computed for each season separately and then followed by combined analysis across the three seasons (winter, spring and summer). Genotypic variation among the 40 red clover germplasm accessions and 3 commercial checks for the twelve traits indicated by BLUP means, range, associated standard errors, P-values and associated repeatability estimates, measured during winter, spring and summer were presented in Table 3.3 and 3.4.

Analysis of variance showed highly significant ($P < 0.05$) genotypic differences among the 43 accessions/cultivars for all ten traits in both winter and spring sets of measurements. Estimates of repeatability ranged from 0.45 to 0.95 in winter and 0.53 to 0.95 in spring, which was considerably high for all the traits. Significant genotypic variation ($P < 0.05$) between accessions/cultivars was estimated for the traits in summer except for STN, PLH and SML. Repeatability was used an estimation of broad-sense heritability for the upper limit of the genetic source of variation (Falconer 1989). Although the repeatability was generally high for all the traits in summer, the range was low compared to winter and spring. The traits TFL, PLG and LS had high repeatability (> 0.80) in summer. Traits PLG and LS steadily maintained the high repeatability across all three seasons. There is substantial increase in the magnitude of means and range for most of the quantitative traits measured in summer and spring in comparison to winter. However, it is evident that leaf size (LS) and medial leaf width (MLW) show a decreasing trend from spring to summer while medial leaf length (MLL) increased exponentially from winter to summer.

Table 3.3 Results from within individual season REML analyses of data for 12 traits from the 40 red clover germplasm accessions and 3 commercial checks presented as BLUP means, range, variance components and associated standard errors (± SE), and repeatability (R_1^2) on an accession mean basis.

Traits	Seasons											
	Winter				Spring				Summer			
	Mean	Range	$\sigma^2_g \pm$ SE	R_1^2	Mean	Range	$\sigma^2_g \pm$ SE	R_1^2	Mean	Range	$\sigma^2_g \pm$ SE	R_1^2
PLG	4.50	1.0-8.5	3.26 ± 0.78***	0.91	7.24	3.0-9.0	2.68 ± 0.62***	0.95	7.66	4.5-9.0	1.46 ± 0.40***	0.82
PGH	8.16	6.0-9.0	0.26 ± 0.15*	0.45	7.78	5.1-9.9	0.81 ± 0.25**	0.77	5.67	3.0-7.9	1.16 ± 0.36***	0.73
LS	4.72	1.1-7.1	2.47 ± 0.57***	0.95	5.30	2.0-7.0	1.14 ± 0.27**	0.91	5.08	3.3-6.8	0.57 ± 0.16***	0.81
STN	11.86	5.5-16.6	6.97 ± 2.16***	0.74	24.28	11.2-47.8	45.45 ± 22.50*	0.53	37.36	15.7-61.0	0.00 ± 0.00ns	0.00
PLH	2.81	0.5-6.8	1.91 ± 0.45***	0.92	8.98	3.9-16.6	9.45 ± 2.35**	0.88	38.92	23.6-57.6	23.55 ± 15.53ns	0.41
MLW	12.93	6.8-18.8	11.40 ± 2.80***	0.90	21.14	11.1-31.8	15.58 ± 3.73**	0.91	20.14	14.9-26.0	4.20 ± 1.71**	0.60
MLL	13.82	5.9-24.2	19.52 ± 4.82***	0.89	26.80	12.6-43.1	47.95 ± 11.24**	0.93	36.54	23.2-47.6	18.59 ± 7.70**	0.68
IAN	1.69	0.9-2.9	0.25 ± 0.07***	0.82	1.77	0.9-3.1	0.18 ± 0.07*	0.66	1.99	0.7-3.3	0.16 ± 0.07**	0.65
APLS	0.05	0.005-0.157	0.001 ± 0.0003***	0.89	0.16	0.04-0.37	0.004 ± 0.002**	0.57	0.49	0.21-0.93	0.02 ± 0.005***	0.72
SML	2.62	1.5-3.1	0.12 ± 0.05**	0.63	2.31	1.2-2.9	0.18 ± 0.02**	0.85	1.36	0.8-2.6	0.06 ± 0.04ns	0.42
TFL	-	-	-	-	-	-	-	-	5.13	3.1-7.1	1.37 ± 0.34***	0.89
FLC	-	-	-	-	-	-	-	-	2.70	2.0-4.5	0.14 ± 0.07*	0.52

Levels of significant differences: Very highly significant at P<0.001=***; highly significant at P<0.01=**; significant at P<0.05 = *; and not significant P>0.05. Abbreviation for the traits: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf; TFL–tendency to flower; and FLC–flower colour.

Table 3.4 Results from across season REML analyses of data for the 10 traits from the 40 red clover germplasm accessions and 3 commercial checks presented as BLUP means, range, error coefficient of variance (ECV %), variance components and associated standard errors (± SE), and repeatability (R_2^2) on a accession mean basis.

Traits	Mean	Range	ECV (%)	σ^2_g ± SE	σ^2_S ± SE	σ^2_{gs} ± SE	σ^2_{gb} ± SE	σ^2_ε ± SE	R_2^2 ± SE
PLG	6.52	3.5-8.4	9.21	1.97 ± 0.50***	0.11 ± 0.06ns	0.51 ± 0.11***	0.16 ± 0.07**	0.36 ± 0.05	0.87 ± 0.04
PGH	7.16	5.3-8.3	11.25	0.53 ± 0.17***	1.39 ± 0.29ns	0.20 ± 0.09**	0.03 ± 0.07ns	0.65 ± 0.09	0.74 ± 0.09
LS	5.08	3.2-6.5	8.15	0.83 ± 0.24***	0.0001 ± 0.002ns	0.56 ± 0.10***	0.07 ± 0.03***	0.18 ± 0.03	0.77 ± 0.06
STN	24.80	16.7-32.8	40.46	23.71 ± 10.33**	106.42 ± 15.64ns	1.90 ± 4.67ns	4.29 ± 6.62ns	98.5 ± 11.79	0.55 ± 0.13
PLH	17.07	13.3-20.4	27.4	5.38 ± 2.91*	0.00 ± 0.00ns	6.87 ± 2.79***	0.99 ± 1.60ns	22.7 ± 2.58	0.45 ± 0.16
MLW	18.21	12.3-24.7	10.70	7.38 ± 2.00***	2.95 ± 0.93ns	3.01 ± 0.82ns	0.19 ± 0.34ns	3.58 ± 0.54	0.81 ± 0.05
MLL	25.91	17.2-36.2	10.69	20.31 ± 5.69***	10.66 ± 2.91ns	8.16 ± 2.08***	2.56 ± 1.36*	7.67 ± 1.24	0.79 ± 0.06
IAN	1.81	1.5-2.3	20.09	0.07 ± 0.04*	0.006 ± 0.008ns	0.11 ± 0.03***	0.03 ± 0.02ns	0.16 ± 0.02	0.48 ± 0.16
APLS	0.24	0.15-0.34	34.22	0.003 ± 0.001***	0.009 ± 0.003ns	0.004 ± 0.001***	0.0001 ± 0.0004ns	0.007 ± 0.001	0.57 ± 0.12
SML	2.09	1.7-2.4	16.40	0.06 ± 0.03**	0.05 ± 0.03ns	0.11 ± 0.03***	0.00 ± 0.00ns	0.12 ± 0.02	0.49 ± 0.14

Levels of significant differences: Very highly significant at P<0.001=***; highly significant at P<0.01=**; significant at P<0.05 = *; and not significant P>0.05. σ^2_g –genotypic variance; σ^2_s–seasonal variance; σ^2_{gs}–genotype-by-season interaction; σ^2_{gb}– genotype-by-replicate interaction; σ^2_ε–experimental error variance; ECV (%)–error coefficient of variation. Abbreviation for the traits: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

3.4.2.2 Across season variation

Variation in the performance of plants is due to both genetic and environment factors. Genotypic variance components in this study involved accessions/cultivars as genotypes, genotype-by-season interaction, and genotype-by-block interaction. Environmental variances include seasons and blocks, whereas in other experiments, sites and years are considered as macro-environmental variances. The means, range, ECV %, estimations of genotypic components (σ^2_g), genotype-by-block interaction (σ^2_{gb}), genotype-by-season interaction (σ^2_{gs}), experimental errors (σ^2_ε) and seasonal (σ^2_s) variances with their corresponding SEs (±) and repeatability (R^2_2) are also presented in Table 3.4.

Examination of the estimates revealed significant components for each effect, except for seasonal variance effects of all traits: PLG, PGH, LS, STN, PLH, MLW, MLL, IAN, APLS and SML (Table 3.4). Season effects were not significant (P>0.05) for all the traits. Experimental error variance or residuals accounted for the next biggest source of variation in STN with variance of 98.5 recorded. Interaction between season and block have significant effects only on area of plant spread (APLS), shape of medial leaf (SML) and plant growth habit (PGH).

The analysis showed significant (P < 0.05) genotypic variation among the accessions and cultivars for all the studied traits which is indicated by higher genotypic variance than their standard errors (Table 3.4). Except for STN and MLW, interaction effect between genotype-by-season was very highly significant (P<0.001) for all other traits. Genotype-by-block interaction effects was very highly significant (P<0.001) for leaf size (LS) whereas highly to moderately high significant for plant growth (PLG) and medial leaf length (MLL) respectively. Among the studied traits, high error coefficient of variation (ECV

%) for genotypes was recorded with STN, APLS and PLH. Variance components analysis also confirmed that variations due to experimental errors are quite high for these traits. Generally, it is obvious in this study that genotypic variance accounted for most of the variations for majority of the traits except for variations in PLH, IAN and SML in which their effects are mainly due to the genotype-by-season interaction.

Estimates for repeatability and their corresponding standard errors are shown in Table 3.3 and 3.4. Repeatability (R_2^2) for the traits across seasons varied from 0.87 for PLG to 0.45 for PLH (Table 3.4). Generally, repeatability estimates was significantly high ($R_2^2 \geq 0.50$) for most of the traits except PLH, IAN and SML with intermediate repeatability ($R_2^2 < 0.50$). The traits PLG, PLH, LS, MLW and MLL had relatively high repeatability (0.74-0.87). Traits with significant repeatability (R_2^2) were indicated by their lower standard error values.

3.4.3 Multivariate analysis

3.4.3.1 Cluster analysis

Cluster analysis was conducted to determine the underlying grouping structure among the accessions/cultivars for each season individually and combined across seasons. Cluster analysis for winter, spring and across seasons were done based on ten traits (PLG, PGH, LS, STN, PLH, MLW, MLL, IAN, APLS and SML) while two additional traits (TFL and FLC) were included in the analysis for summer. The accession clusters and their respective means and standard deviations for each season and across all seasons are summarized in Table 3.5-3.8.

Winter clustering: Cluster analysis based on the ten morphological and yield related traits helped identify eight clusters in winter. Cluster I contained six accessions (Appendix 2)

characterized by poor plant growth (PLG), smaller leaf size (LS), least number of stem, shorter plant height, with smallest medial leaf width and length and less spreading (APLS), which is significantly different from other clusters (Table 3.5). Cluster II comprised the largest number of accessions (10) categorized by semi-prostrate growth habit, round medial leaflet shapes, shorter in height and low stem number with poor plant growth. This cluster comprised of accessions mainly from Tajikistan and Greece. The third (III) cluster is made up of only two accessions with prostrate growth habit characterized by small and elongated leaf shape with low plant growth. Seven accessions with moderate plant growth are grouped in cluster IV. Cluster V was composed by two accessions, with remarkably highest number of stem and intensity of anthocyanin pigmentation on stems and leaves. Cluster VI comprised the second highest number of accessions (9) including cultivar Broadway, which is characterized uniquely for its ovate leaf shape then accompanied by high stem number mostly growing in upright direction (prostrate) with high anthocyanin pigmentation. The seventh (VII) cluster is made up of three accessions with tall and vigorously growing plants, large leaf size, and increased number of stems with the tendency to spread out from their growing points and occupied large area. Cluster VIII is made up of cultivar Relish and Sensation with two other germplasm accessions which mainly corresponds to taller plants and larger leaf size with increased medial leaf length and width.

Table 3.5 Summary of cluster analysis with means and standard deviation values for traits measured from the 43 red clover accessions, from BLUPs generated based on the winter measurements.

Cluster	No. of accessions	Mean values ± St. Dev.									
		PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
I	6	1.9 ± 0.6	8.0 ± 0.6	2.0 ± 0.5	8.0 ± 2.6	0.9 ± 0.3	7.6 ± 0.7	7.2 ± 0.7	1.1 ± 0.3	0.01 ± 0.005	3.0 ± 0.05
II	10	3.2 ± 0.5	8.4 ± 0.5	3.9 ± 0.9	9.4 ± 1.6	1.8 ± 0.6	11.1 ± 1.4	10.9 ± 1.9	1.4 ± 0.4	0.02 ± 0.008	2.9 ± 0.22
III	2	2.7 ± 0.1	9.0 ± 0.0	3.5 ± 0.4	11.1 ± 0.6	2.0 ± 0.2	9.4 ± 0.2	10.7 ± 1.3	1.5 ± 0.8	0.02 ± 0.004	1.9 ± 0.03
IV	7	4.7 ± 0.5	7.4 ± 0.5	5.5 ± 0.6	12.8 ± 1.6	3.2 ± 0.4	14.2 ± 2.2	14.8 ± 1.8	1.7 ± 0.5	0.05 ± 0.009	2.6 ± 0.48
V	2	6.8 ± 0.2	9.0 ± 0.0	4.8 ± 0.1	15.2 ± 0.4	4.0 ± 0.8	12.4 ± 0.7	12.8 ± 0.5	2.7 ± 0.3	0.09 ± 0.014	3.0 ± 0.01
VI	9	5.8 ± 1.0	8.5 ± 0.5	6.0 ± 0.6	14.7 ± 1.8	3.3 ± 0.6	15.5 ± 1.9	17.2 ± 1.8	2.1 ± 0.5	0.07 ± 0.015	2.2 ± 0.37
VII	3	7.6 ± 0.8	8.7 ± 0.6	6.4 ± 0.8	14.2 ± 1.1	5.0 ± 0.5	17.3 ± 1.3	20.0 ± 1.8	1.9 ± 0.1	0.14 ± 0.023	2.5 ± 0.49
VIII	4	6.8 ± 1.0	6.8 ± 0.6	6.8 ± 0.2	14.3 ± 2.2	5.6 ± 0.9	18.6 ± 1.5	22.2 ± 2.3	1.8 ± 0.2	0.08 ± 0.013	2.4 ± 0.15

Legend: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

Table 3.6 Summary of cluster analysis with means and standard deviation values for traits measured from the 43 red clover accessions, from BLUPs generated based on the spring measurements.

Cluster	No. of accessions	Mean values ± St. Dev.									
		PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
I	22	8.5 ± 0.3	7.1 ± 1.1	6.1 ± 0.5	28.5 ± 7.1	11.8 ± 2.1	24.1 ± 2.5	32.7 ± 3.6	1.9 ± 0.5	0.21 ± 0.08	1.9 ± 0.3
II	13	6.9 ± 0.8	8.2 ± 0.6	5.3 ± 0.4	23.0 ± 11.6	7.6 ± 1.3	20.4 ± 2.2	23.8 ± 3.0	1.7 ± 0.8	0.14 ± 0.06	2.6 ± 0.4
III	8	4.5 ± 1.1	8.5 ± 1.1	3.4 ± 0.7	16.6 ± 4.4	4.6 ± 0.6	15.1 ± 2.1	16.7 ± 2.8	1.6 ± 0.4	0.07 ± 0.05	2.8 ± 0.2

Legend: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

Table 3.7 Summary of cluster analysis with means and standard deviation values for traits measured from the 43 red clover accessions, from BLUPs generated based on the summer measurements

Cluster	No. of accessions	Mean values ± St. Dev.											
		PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML	TFL	FLC
I	10	5.8 ± 1.2	7.0 ± 0.9	4.3 ± 0.3	40.3 ± 15.2	30.7 ± 6.8	17.4 ± 2.0	30.1 ± 4.3	2.4 ± 0.6	0.34 ± 0.1	1.4 ± 0.5	4.2 ± 1.1	2.8 ± 0.7
II	12	8.4 ± 0.7	5.3 ± 0.8	4.8 ± 0.8	39.3 ± 7.3	38.8 ± 4.8	20.0 ± 2.4	37.0 ± 5.4	2.3 ± 0.2	0.57 ± 0.1	1.0 ± 0.1	6.3 ± 0.7	2.8 ± 0.3
III	21	8.2 ± 0.7	5.2 ± 1.3	5.6 ± 0.8	34.9 ± 10.2	42.9 ± 6.5	21.5 ± 2.2	39.4 ± 3.8	1.6 ± 0.5	0.52 ± 0.1	1.5 ± 0.4	4.9 ± 1.0	2.6 ± 0.5

Legend: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf; TFL–tendency to flower; and FLC–flower colour.

Table 3.8 Summary of cluster analysis with means and standard deviation values for traits measured from the 43 red clover accessions, from BLUPs generated from the across seasons analysis

Cluster	No. of accessions	Means ± St. Dev.									
		PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
I	2	7.1 ± 0.08	8.1 ± 0.21	4.8 ± 0.19	32.1 ± 1.00	15.4 ± 0.38	15.6 ± 0.04	20.6 ± 0.26	2.2 ± 0.11	0.22 ± 0.010	2.4 ± 0.10
II	9	4.5 ± 0.62	7.6 ± 0.45	3.8 ± 0.41	22.5 ± 3.66	15.1 ± 1.28	14.7 ± 1.16	20.5 ± 1.75	1.8 ± 0.15	0.19 ± 0.024	2.2 ± 0.09
III	7	5.8 ± 0.30	7.4 ± 0.36	4.8 ± 0.28	20.4 ± 1.74	16.9 ± 0.63	17.5 ± 0.86	24.7 ± 1.49	1.6 ± 0.07	0.23 ± 0.029	2.2 ± 0.12
IV	7	7.5 ± 0.38	7.5 ± 0.26	5.4 ± 0.38	28.4 ± 2.17	17.3 ± 0.89	19.2 ± 1.48	28.0 ± 1.63	1.9 ± 0.12	0.30 ± 0.034	1.9 ± 0.15
V	10	7.0 ± 0.47	7.0 ± 0.32	5.4 ± 0.27	24.7 ± 2.49	17.1 ± 0.83	19.2 ± 0.99	27.0 ± 1.57	1.9 ± 0.13	0.24 ± 0.012	2.1 ± 0.06
VI	8	7.8 ± 0.43	6.1 ± 0.64	6.1 ± 0.26	26.4 ± 3.70	19.7 ± 0.75	21.3 ± 1.69	31.2 ± 2.76	1.7 ± 0.11	0.25 ± 0.021	2.0 ± 0.03

Legend: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

Spring clustering: The 43 accessions/cultivars grown under spring conditions were separated into three distinct clusters of germplasm, mainly characterized by the same ten traits as in winter (Table 3.6). The first cluster presented the highest number (22) of accessions/cultivars mainly from Portugal, Azerbaijan, Spain and the three commercial cultivars (Appendix 3). This cluster grouped together the accessions that expressed the highest level of desirable traits: robust plants growth with larger leaf size, high stem number, increased plant height, high anthocyanin pigmentation, semi-prostrate growth habit and increased area of plant spread. In contrast, cluster III comprised of eight accessions with poor growth and low level of expression for the traits mentioned above. Accessions in cluster II comprised of 13 accessions and were moderately characterized for all the traits.

Summer clustering: Cluster analysis of the summer data was based on twelve morphological, yield related, and flowering traits which identified three distinct clusters (Table 3.7). Clusters I, II, III comprised of 10, 12, 21 accessions respectively (Appendix 4). The firs cluster were composed of accession germplasm mainly from Greece with relatively poor plant growth with semi-prostrate growth habit, smaller leaves, high stem number, high intensity of anthocyanin pigmentation and low tendency to flower. Cluster II characterized accessions mainly with superior plant growth, intermediate growth habit, elongated leaf shape, increased area of plant spread with profuse flowering habit. Cluster III comprised of accessions mostly from Portugal, Tajikistan, Spain and Caucasus including the three cultivars. This cluster comprised of accessions typically with high plant growth, large leaf size mostly elongated-ovate shaped, increased plant height and low intensity of anthocyanin pigmentation.

Cluster analysis across seasons: Cluster analysis based on the accession-by-BLUP matrix generated from the across season REML analysis included ten morphological and yield related traits (PLG, PGH, LS, STN, PLH, MLW, MLL, IAN, APLS and SML). Results of this analysis identified two major groups and six distinct clusters as summarized in Table 3.8 and Figure 3.22. Group A was sub-divided into 3 clusters (I, II, III) that contained 18 accessions whilst group B, the largest group with 25 accessions also had 3 clusters (IV, V, VI) that comprised of the three cultivars (Broadway, Relish and Sensation) together with other accession germplasm (Figure 3.22). Accessions in Group A showed characteristics of prostrate growth habit, shorter plant heights, and small to medium plant types with poor plant growth/adaptation. This group was composed by accessions mainly from the Western Mediterranean region (Greece, Tijikistan, and Caucasus) and Spain. Group B, on the other hand, clustered the accessions with generally maximum plant growth and moderate to high level of expression for the yield related traits (LS, STN, PLH and APLS).

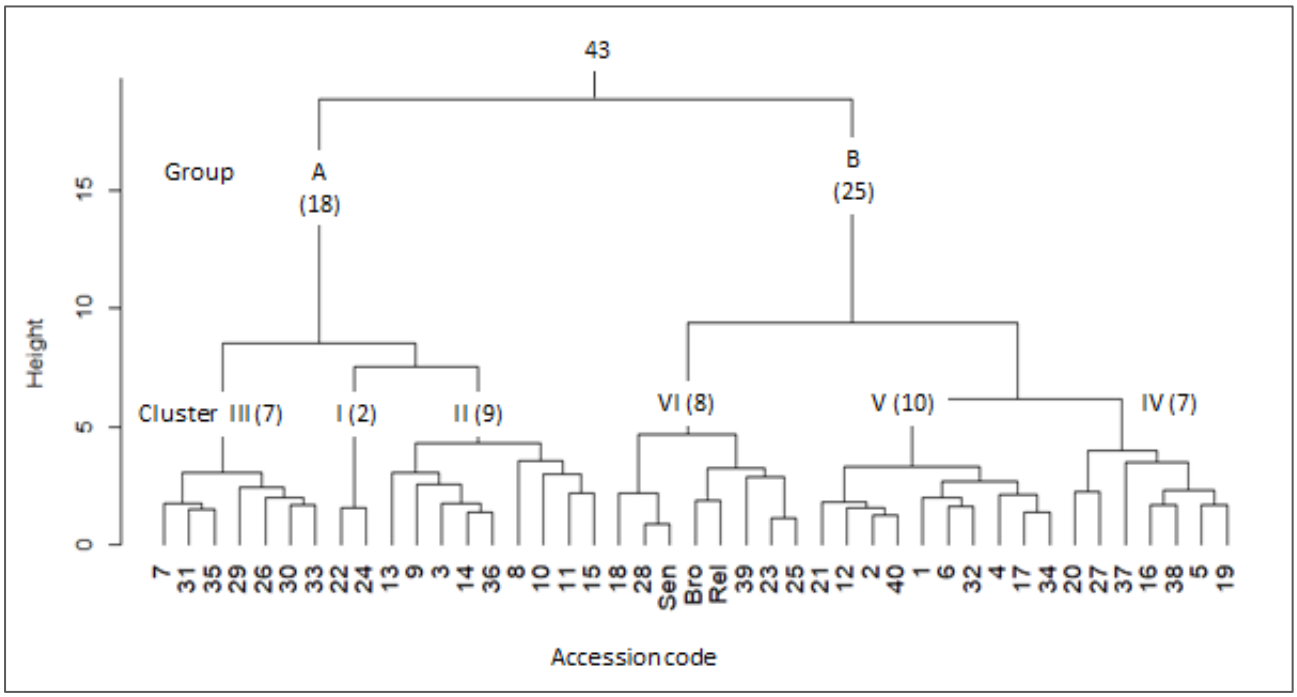


Figure 3.22 Dendrogram of the 43 (represented with accession/cultivar code) red clover entries generated from clustering of standardised BLUP values based on 10 morphological traits estimated from the combined analysis across winter, spring and summer. Numbers in parentheses are the total number of accessions in a cluster at a specific grouping level.

In cluster I, only two plants with semi-prostrate to prostrate growth habit, ovate to round leaf shape, high stem number and strong pigmentation for anthocyanin were characterized. Clusters II and III composed of sixteen germplasm accessions with generally poor plant growth and low to average level of expression for the yield related traits. These clusters composed of accessions with origin mainly from Greece and Tajikistan.

Clusters IV and V have shown some similarities in most of the traits except for high stem number and increased area of plant spread which was obvious in cluster IV compared to cluster V. Cluster VI comprised of five accessions and three cultivars which were characterized by intermediate to semi-prostrate growth habit, superior plant growth, the largest leaflet size and increased plant height.

3.4.3.2 Principal component analysis

Principal component analysis (PCA) is a multivariate statistical method often used in breeding programmes to summarise large genotype-by-trait and genotype-by-environment interaction data matrices (Jahufer *et al*. 2002; Cooper & DeLacy 1994). This multivariate analysis tool provides a graphical summary of the relationship among traits and also genotype and trait association on a multidimensional scale. Combining PCA with cluster analysis as presented in this thesis as pattern analysis, provides the possibility of identifying genotype groups associated with specific trait that is important for identifying useful plant types for breeding programs. (Jolliffe, 1985; Hossein, 1993; Jahufer *et al*. 1997).

Breeding trials most often deal with large number of variables which at times becomes difficult when trying to summarise the information available. PCA can enhance identification of relationships among traits or individuals that are not apparent in genotype-by-trait mean

data matrices. The methods of PCA greatly reduce the dimensionality of multivariate dataset by further combining the variables in such a manner so that the first PC explains the maximum variation (Hossein, 1993). The second PC explains the majority of the remaining variance and so on. The first and second PC in most cases could elucidate the existing variation.

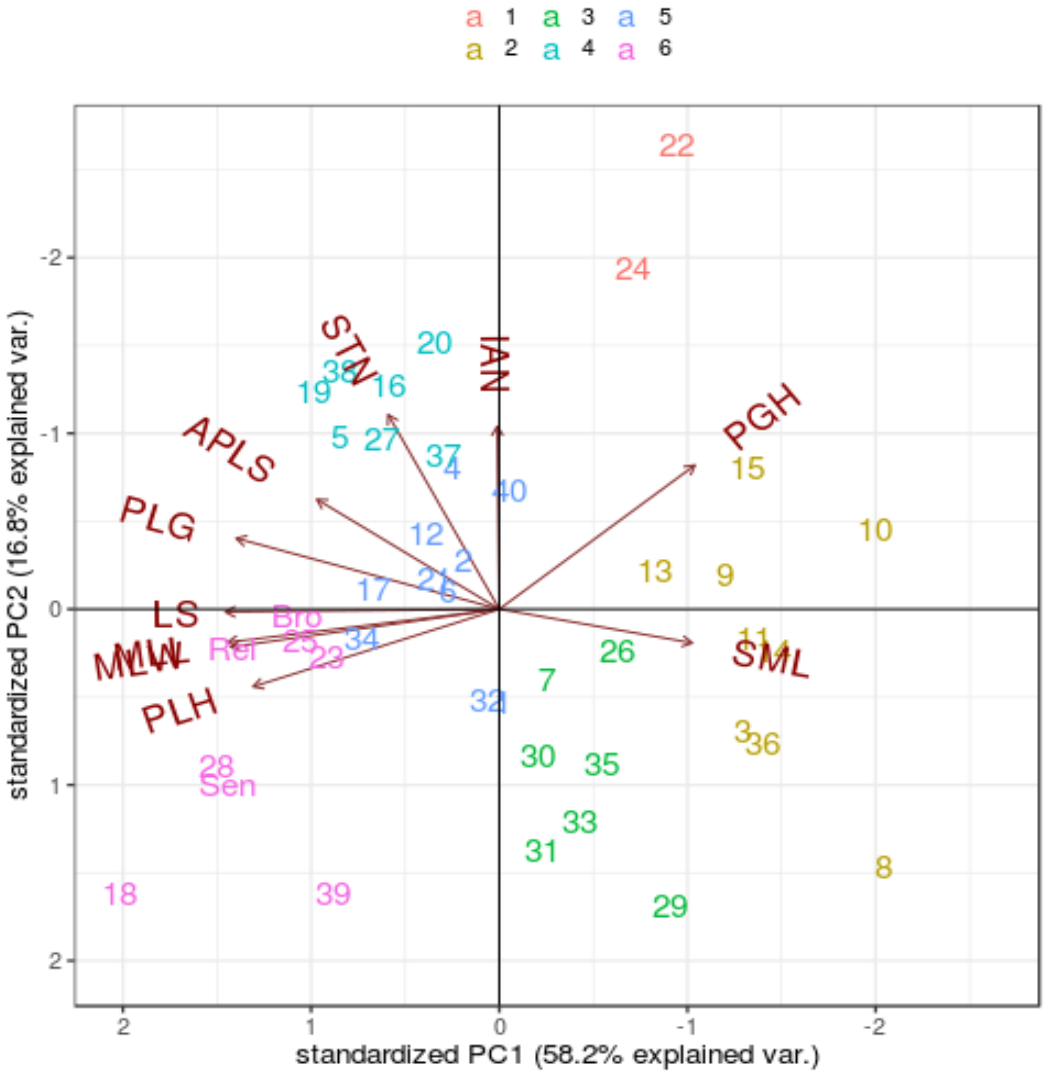


Figure 3.23 Two dimensional biplot from principal component analysis showing the graphical representation of the relationships among 43 accessions based on the 10 traits, generated from pattern analysis based on standardized BLUP values estimated from analysis combined across winter, spring and summer. The accession codes with different colours represent clusters and the vectors represent red clover traits.

Legend: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

Principal component analysis (PCA) in this study was performed to explore the importance of different traits in explaining genotypic variation at a multivariate level among the 43 entries. This type of multivariate analysis is useful for screening germplasm accessions and serves as a guide for selecting parent material for hybridization (Chozin, 2007). Two dimensional biplot of the first two principal components (PC1 and PC2) presented in Figure 3.23 was used to evaluate the patterns of relations in the studied traits, accessions/cultivars and their interactions.

The accessions in the biplot are denoted by number coding, and the directional vectors from the point of origin (average) denoted by alphabetical initials signify the ten traits. The principal component analysis performed on 43 accessions and ten morphological traits across all three seasons accounted for 75 % of the total variation, the first (PC 1) and the second (PC 2) axes accounted for 58.2 and 16.8% respectively which are sufficient to draw meaningful judgements using biplot method (Figure 3.23). According to the PCA biplot the red clover accessions scattered in all the quarterlies which indicated high level of genetic diversity in the population.

Accessions that are close to each other on the biplot and presented with same colour are from the same cluster group. PCA further demonstrate entries and clusters with higher expression of the studied traits on the left side of the mid-point of the axis than that on the right side. Accessions on the same parallel line, relative to the ordinate have similar expression of traits. On the same note, accessions and traits overlap between axis and PCs indicate some degree

of similarities. Therefore, the PCA biplot indicated the presence of six distinct clusters which is similar to clustering method by dendrogram (Figure 3.22). PCA also grouped accessions comprised of clusters I to III with below average expression of all morphological and yield related traits towards the right axis of PC1 and PC2. However, accessions with higher expression of the studied traits were presented towards the left axis. The alignment and clustering of the entries with PCA correspond with clustering by dendrogram in Figure 3.22.

The length of vectors that represent the traits at the origin of PCs (average) and their projected values on each PC indicate how much weight they have on that PC. Thus, the variation of measured traits studied through PCA revealed that PLG, APLS, STN, IAN and PGH contributed more positively to PC1. From this analysis, it is clear that PLG, STN and PGH have more influence on PC1 than the other two traits (APLS and IAN). In contrast, the traits PLH, MLW and MLL strongly influence (or have more say) PC2 than SML. Similar trend was reported by Ahsyee (2013) in a diverse population of red clover. Leaf size (LS) was found to be overlapped between PC1 and PC2 which indicate that LS had influence on both PCs. Accessions or clusters lying in close proximity to a specific trait (vector) indicate strong expression to that trait. For example, accessions 01, 04 and 12 from cluster V and accessions 37, 27, 16 in cluster I were related more to STN. Likewise, accessions that are close to each other tend to have similar response to specific traits just like the majority of accessions closely congregating around STN.

Moreover, the angles between directional vectors on the PC biplot indicate whether the traits/variables correlate to each other. When two vectors are close forming a small angle (<90˚), the two traits they represent are positively correlated. If two vectors meet each other at 90˚ angle, they are not likely to be correlated. Traits that are negatively correlated diverge and form a large angle up to 180˚. In view of that, the PC biplot in Figure 3.23 demonstrate

positively strong to intermediate correlation between PLG and yield related traits like LS, APLS, MLW, MLL, PLH, and STN. In contrast, PLG was negatively correlated to PGH and SML. Generally, the PC biplot (Figure 3.23) had shown similar correlation to that projected in Table 3.12.

3.4.3.3 Phenotypic correlation between plant growth and other morphological traits

Pearson's correlation coefficient (r) is a measure of the strength of linear association between two variables. Phenotypic correlation (r_p) is an indirect method of selection based on the association between two characters that can be directly observed. It is widely accepted that phenotypic correlation could change due to differences in seasons, years, environments and populations (Falconer, 1989). Consequently, phenotypic correlations in this study were

Table 3.9 Phenotypic Pearson correlation for pairs of traits among the 43 red clover germplasm under winter condition.

Trait	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
PLG										
PGH	-0.15									
LS	0.89***	-0.27								
STN	0.80***	-0.21	0.75***							
PLH	0.91***	-0.36*	0.87***	0.73***						
MLW	0.89***	-0.28	0.94***	0.68***	0.87***					
MLL	0.89***	-0.27	0.93***	0.68***	0.90***	0.97***				
IAN	0.56***	0.13	0.46**	0.57***	0.44**	0.42**	0.42**			
APLS	0.89***	0.02	0.74***	0.67***	0.81***	0.78***	0.78***	0.51***		
SML	-0.33*	0.03	-0.44**	-0.40**	-0.36*	-0.35*	-0.43**	-0.27	-0.32*	

Phenotypic correlation coefficients (r_p) significantly different from zero at P<0.05=*, at P<0.01=**, and at P<0.001=***. Abbreviation for the traits: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

analysed separately for each season and combined across seasons. Phenotypic correlations estimated between all possible sets of the traits assessed in this study are presented on the

Table 3.9-3.12. Since plant growth (PLG) assessment which represents visual forage yield is the most important trait in this study and has direct association with forage yield, correlations of all other traits in this experiment were paired and compared with PLG.

Phenotypic correlation of ten traits for 43 accessions of red clover evaluated during winter is presented in Table 3.9. Winter performance of these accessions show significantly (P<0.001) positive correlations between PLG and all other traits (LS, STN PLH, MLW, MLL, IAN and APLS). Significantly high positive correlation was apparent between LS and all other traits. Likewise, there is existence of strong positive correlation for all other traits except for PGH and SML. PGH and SML evidently did not correlate well with the rest of the other traits. Although PLG is negatively correlated with SML, it shows significant negative correlation (rp=-0.33). The PGH was not well correlated with all the traits except a significant negative

Table 3.10 Phenotypic Pearson correlation among 43 red clover germplasm under spring growing condition

Trait	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
PLG										
PGH	-0.40**									
LS	0.89***	-0.54***								
STN	0.62***	-0.14	0.35*							
PLH	0.85***	-0.66***	0.83***	0.50***						
MLW	0.82***	-0.55***	0.92***	0.29	0.88***					
MLL	0.87***	-0.55***	0.91***	0.38*	0.90***	0.93***				
IAN	0.29*	0.14	0.15	0.26	0.20	0.11	0.11			
APLS	0.68***	-0.16	0.54***	0.64***	0.81***	0.60***	0.54***	0.60		
SML	-0.67***	0.39**	-0.58***	-0.48**	-0.63***	-0.53***	-0.71***	-0.02	-0.61***	

Phenotypic correlation coefficients (r_p) significantly different from zero at P<0.05=*, at P<0.01=**, and at P<0.001=***. Abbreviation for the traits: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

correlation with PLH. The trait SML, on the other hand, had significant (P<0.05) but are negatively correlated to PLG, LS, STN, PLH, MLW, MLL and APLS whilst no relationship established for PGH and IAN.

There was a significant (P < 0.05) strong and positive phenotypic correlation between PLG and all yield related traits (LS, STN PLH, MLW, MLL and APLS) in spring as shown in Table 3.10. The trait IAN show weak positive association with PLG whereas significant (P < 0.05) and negative correlation between PLG and PGH and PLG and SML exist. The trait PLH shown strong negative correlation with LS, PLH, MLW and MLL while moderate for SML. The correlation between LS and traits PLH, MLL, MLW and APLS was positive and

Table 3.11 Phenotypic Pearson correlation among 43 red clover germplasm under summer growing condition

Trait	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML	TFL	FLC
PLG												
PGH	-0.62***											
LS	0.56***	-0.7***										
STN	0.06	0.27	-0.12									
PLH	0.63***	-0.76***	0.62***	-0.25								
MLW	0.58***	-0.62***	0.72***	-0.18	0.53***							
MLL	0.60***	-0.64***	0.71***	-0.20	0.59***	0.81***						
IAN	-0.35***	0.20	-0.42**	0.18	-0.36*	-0.15	-0.27					
APLS	0.49***	-0.10	0.09	0.07	0.29	0.04	0.26	-0.20				
SML	-0.08	0.14	0.06	-0.05	-0.05	0.03	-0.12	-0.08	-0.14			
TFL	0.49***	-0.30*	-0.09	0.09	0.13	0.09	0.07	0.07	0.13	-0.39*		
FLC	0.11	0.01	-0.08	0.22	-0.15	-0.32*	-0.34*	0.03	-0.1	0.06	0.27	

Phenotypic correlation coefficients (r_p) significantly different from zero at P<0.05=*, at P<0.01=**, and at P<0.001=***. Abbreviation for the traits: PLG–plant growth; PGH–plant growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf; TFL–tendency to flower; and FLC–flower colour.

significantly (P < 0.05) very high whereas LS and SML was negative and high. STN was highly and positively correlated with the attributes PLH and APLS, while PLH was greatly correlated to MLL, MLW and APLS. A positive and significantly strong correlation was noticed for MLL and MLW while moderate to high correlation observed between MLW and APLS and MLL and APLS.

Phenotypic Pearson correlation under summer condition was done for twelve traits *viz*. PLG, PGH, LS, STN, PLH, MLW, MLL, IAN, APLS, SML, TFL and FLC as presented in Table 3.11. PLG was highly and positively correlated with LS, PLH, MLW, MLL, APLS and TFL while it was negatively but significantly (P < 0.05) associated with PGH and IAN. PGH was negatively correlated to LS, PLH, MLW, MLL, IAN, APLS and TFL but were not significant with APLS and only moderately significant with TFL. Traits that have significantly high correlation with LS are PLH, MLW and MLL while PLH is highly related to MLW and MLL. The traits MLW and MLL maintained strong positive relationship between each other across all the seasons.

Table 3.12 Phenotypic Pearson correlations among traits for 43 red clover germplasm across seasons (winter, spring and summer)

Traits	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
PLG										
PGH	-0.47***									
LS	0.92***	-0.64***								
STN	0.54***	0.01	0.34*							
PLH	0.73***	-0.79***	0.82***	0.12						
MLW	0.85***	-0.68***	0.95***	0.21	0.81***					
MLL	0.83***	-0.68***	0.92***	0.24	0.80***	0.95***				
IAN	0.20	0.23	0.04	0.32*	-0.09	-0.04	-0.06			
APLS	0.66***	-0.11	0.57***	0.44**	0.41**	0.52***	0.53***	0.07		
SML	-0.57***	0.35*	-0.55***	-0.36*	-0.48**	-0.52***	-0.66***	0.06	-0.48**	

Phenotypic correlation coefficients (rp) significantly different from zero at P<0.05=*, at P<0.01=**, and at P<0.001=***. Abbreviation for the traits: PLG–plant growth; PGH–plant

growth habit; LS–leaf size; STN–stem number; PLH–plant height; MLW–medial leaf width; MLL–medial leaf length; IAN–intensity of anthocyanin on leaf/stem; APLS–area of plant spread; SML–shape of medial leaf.

Analyses for phenotypic correlation across the three seasons for the ten traits were summarized in Table 3.12. Evidently PLG continue to show positive and highly significant phenotypic correlation values with LS, STN, PLH, MLW, MLL and APLS. As also observed in each season, PLG was negatively correlated with PGH and SML, but overall data indicate strong negative correlation. PGH has significantly strong negative relationship with LS, PLH, MLW and MLL. Very high positive correlation was observed between the following traits: PLH, MLW, MLL and APLS. STN was in lowest and moderate correlation with IAN and APLS respectively. Highly significant ($P < 0.05$) positive relationship was estimated between PLH and MLW, PLH and MLL, MLW and MLL while moderate to high correlation exist between PLH and APLS, MLW and APLS as well as MLL and APLS. However, significant ($P < 0.05$) negative correlation was noticed between SML and most of the traits except for PGH and IAN.

3.4 Discussion

3.5.1 Introduction

Efficient breeding and selection of red clover cultivars with improved performance largely depends on sufficient genetic diversity. Use of germplasm collections in breeding are made possible only after prior exploitation of their genetic potential and characterization based on specific traits of interest. Therefore, the discussions on this experiment on assessing genotypic variation among a world collection of red clover germplasm were focused on phenotypic variance partitioned in to genotypic and environmental components. The possibility of improving the traits under investigation through heritability studies was also

discussed. Mean performance of yield related traits and clustering according to corresponding traits provided insights into selection of suitable germplasm accessions to be used in future breeding. This chapter also discussed the possibility for indirect selection for certain traits with low genotypic variation and repeatability estimates.

3.5.2 Genotypic performance for yield related traits

Significant ($P \leq 0.05$) differences among accessions were detected for the plant growth (PLG) in winter, spring and summer. The highest average PLG value (8.5) in winter was obtained for the Spanish and Portuguese accessions 25 and 18 respectively, and were 46% higher compared with average yields for all materials (Figure 3.7). Eight other accessions were also ranked with the three commercial cultivars of New Zealand for their vigorous plant growth in winter with high values of PLG (6.0-7.5). However, the above two accessions also outperformed cultivars Broadway, Relish and Sensation in terms of PLG by 18, 27 and 30 % respectively. High PLG vigour indicates increased forage yield as visual assessment of plant growth (visual forage yield) is highly associated with actual forage yield (Riday, 2009). Thus, accessions with high PLG in winter are potentially high yielding materials that can adapt and survive through cold winter condition. The thirteen accessions not only have high PLG, but generally recorded higher mean values for leaf size (LS), plant height (PLH) and stem number (STN) as indicated in Figures 3.8-3.10 and also well correlated to each other (Table 3.9-3.12). Since winter survival is an important consideration for red clover forage and seed production (Tucak *et al*., 2009), materials showing high level expression of these positive traits together with vigorous PLG are potential parents to select for breeding for winter hardiness and persistence under New Zealand condition.

It is worth mentioning here that materials from Greece, Tajikistan and Caucasus were struggling with poor plant growth under New Zealand's winter condition while materials from Portugal are at per with locally (NZ) adapted cultivars followed by Spain. Materials from Portugal and Spain had high growth quite similar to NZ cultivars because they originate from regions characterized by warm temperate, Mediterranean climate (located between 30° and 45° latitude north and south of the Equator) with distinct wet season in winter, which is quite similar to New Zealand's climatic condition (Peel *et al*., 2007). However, materials from Greece, Tajikistan, Caucasus, Azerbaijan and Armenia were previously exposed to continental climate typically with less rainfall: hot dry summer and mild rainy winters. It is expected that germplasm with origin from any of these regions will face difficulty adapting to NZ's cold and wet winter condition due to genotype-by-environment interaction effect. Without the consideration for other experimental and genetic factors, these could be one possible reason for such differences seen in growth performance of the introduced germplasm. Nevertheless, this situation is likely to vary with changes in seasons which will be explored in spring and summer observations.

Although there was significant ($P<0.05$) variation among the test accessions in spring, substantial increase by 37% was recorded for plant growth from winter to spring. Further 5% increase in plant growth was achieved from early spring to mid-summer. It is obvious from this study that majority of the accessions tested have reached their maximum plant growth between late spring and early summer. From mid-summer onwards, plants were already into their flowering stage which indicates that these entries are summer active. Accessions with significantly ($P<0.05$) highest plant growth in spring are 5, 18 and 17 all with the maximum PLG value of 9.0 which is higher than the values obtained by the local checks (Table 3.3). Since forage yield is an important criterion for selection of red clover for grazing and hey

production, these accessions are undoubtedly the most preferred candidates to be considered for use in breeding for improved forage yield or any further research.

Huge variation in plant growth was observed in summer with high visual forage yield (PLG) was recorded in accessions 12, 23, 34, 39, 18, 28 compared to adapted test cultivars and the rest of the accessions. Except for accessions 18 and 28, the other four are among the accessions with poor growth and plant establishment in cold winter but eventually picked up in spring to reach their maximum growth under summer condition. Since the four high yielding accessions originate from Armenia and Azerbaijan which experience mostly continental climatic condition characterized by low precipitation, cold winter and warm summer, the spring and summer condition in NZ is ideal for enhanced growth and productivity in these materials. Hence, these materials are suitable for temporary cultivation under spring and summer particularly for hay production or short-term grazing in NZ, while prospect for further breeding is at large feasible.

The accessions of red clover under evaluation responded differently to differences in seasons that resulted in significant genotype-by-season (σ^2_{gs}) interaction effects in all the traits (Table 3.4). Despite the differences in seasons experienced by the test materials in field, six accession germplasm 18, 25, 19, 17, 5 and 28 and two cultivars (Broadway and Relish) clearly show high and stable PLG values (visual forage yield) across all the seasons as well as for each season. Moreover, there is enough evidence also to proof that these six germplasm accessions have higher values of other positive traits related to yield assessed in this study such as large leaf size (LS), increased number of stems (STN), tall plant height (PLH) and high spreading ability (APLS) among other desirable morphological attributes (Figure 3.10-3.11, 3.13-3.14, 3.16-3.17, 3.19-3.20). This result suggests that the ability of

these materials to survive under New Zealand condition looks promising regardless of their origin. Therefore, it is recommended that these accessions be given due consideration in the selection of next germplasm pool as new source of diversity to be crossed with high yielding locally adapted cultivars like Broadway and Relish for development of more superior breeding population.

3.5.3 Variance components analysis

The analyses of variance components revealed highly significant effects ($P<0.001$) of genotype (σ^2_g) for plant growth (PLG), plant growth habit (PGH), leaf size (LS), stem number (STN), plant height (PLH), medial leaf length (MLL), medial leaf width (MLW), intensity of anthocyanin (IAN), area of plant spread (APLS) and shape of medial leaflet (SML) under each season except for STN, PLH and SML in summer (Table 3.3). The two additional traits *viz*. tendency to flower (TFL) and flower colour (FLC) observed in summer were also significantly ($P<0.05$) different. This clearly indicates that there is presence of sufficient genetic variation among the entries examined for most of the observed traits and improving these traits through breeding seems possible. Similarly, there are significant ($P<0.05$) genotypic variation (σ^2_g) detected with combined analysis across seasons for all ten traits (Table 3.4). Significantly ($P<0.05$) high accession-by-season variances (σ^2_{gs}) were noticed for most of the traits across seasons whereas non-significant differences in STN and MLW were detected. The genotypic variances (σ^2_g) exceeded the seasonal variances for PLG, LS, MLW and MLL which indicate that variation in these traits were more influenced by additive genetic effect. Traits controlled by additive genetic variance are basically stable in performance regardless of environmental variances due to inheritance of a particular allele and allele's relative effect on phenotype (Byers, 2008) and can be easily improved through breeding. On the other hand, PGH, STN and APLS had high seasonal variances (σ^2_s) which

signify that these traits are highly controlled by environmental variances and are likely to change with variability in environmental conditions. Genotype-by-season variance (σ^2_{gs}) took the biggest part in total variability for the traits PLH, IAN and SML. This suggests that red clover accessions under investigation differ in their values (highly heterogeneous) for these traits across different seasons. Effect of the interaction of genotype and environment such as; genotype-by-season or genotype-by-year often complicates selection for improving genotype performance across multiple environments. Traits with high genotype-by-environment interaction variances are the traits that often complicate selection as well as difficult to improve through breeding. As for the traits PLH, IAN and SML, high significant accession-by-season variance (σ^2_{gs}) was estimated and this suggests that the red clover accessions evaluated in this study differed in their relative performance for these traits across different seasons, suggesting the need to evaluate genotypes for several seasons over years. Variance component analyses indicate large experimental error variance component (σ^2_{ε}) for the traits STN, PLH, IAN, APLS and SML. Although sufficient variance were captured by the estimates of different variance components (σ^2_g, σ^2_s, $\sigma^2_{gs,}$ $\sigma^2_{gb,}$ σ^2_{sb}), experimental error variance component (σ^2_{ε}) for STN, PLH, IAN, APLS and SML still accounted for large portion of the total variance. This clearly indicates the existence of random noise in the data perhaps due to inconsistency in assessment for these traits. Higher error coefficient of variation (ECV %) recorded in these traits also confirms that there is greater dispersion (deviation) of data away from the means. This is not surprising because traits such as STN, PLH, APLS are practically tedious and often difficult to measure in field under mixed pasture and in competition with weeds. Measurement of such quantitative traits needs a more systematic approach to better quantify them in order to minimise errors in future. Season effects were not significant ($P<0.05$) for all the traits measured, indicating the relative

ranking of accessions over seasons has been constant, which could also suggest that seasonal influence along had no major effect on traits' variability in these accessions.

Broad-sense heritability or repeatability (R^2) was used in estimating heritability due to red clover being the cross pollinated species. Thus, estimation of genotypic variance components in this study combined the additive and dominance variance to acquire traits' repeatability. Repeatability (R_1^2) for the traits varied from 0.45 for PGH to 0.95 for LS, 0.53 for STN to 0.95 for PLG and 0.00 for STN to 0.89 for TFL in winter, spring and summer respectively (Table 3.3). Repeatability (R_2^2) for across seasons ranged from 0.87 for PLG to 0.45 for PLH (Table 3.4). Generally, repeatability estimates were significantly high (R_2^2 0.74-0.87) for most of the traits particularly PLG, PLH, LS, MLW and MLL suggesting that selecting for these traits would be easily achieved without much difficulty. A major objective in red clover breeding is to improve forage yield and since these five quantitative traits are directly related to yield, we are very optimistic that using these materials as parent would result quickly in more superior cultivars. Higher genotypic variance components (σ^2_g) achieved for these traits is also a positive sign that greater amounts of heritable genetic variation are present in this population. However, total variances due to season (σ^2_s), accession-by-season (σ^2_{gs}), replication-by-accession (σ^2_{gb}), replication-by-season (σ^2_{sb}) and experimental error (σ^2_ε) exceeds that of genotypic variance (σ^2_g) for the traits STN, PLH, IAN, APLS and SML, thus, leading to moderate repeatabilities (R^2 0.45 to 0.55).

3.5.4 Correlations between traits

In any plant breeding program, it is equally vital to understand the associations between the various traits, since intense selection on any one trait can affect others in both positive (by improving desirable characteristics) and negative direction (Tucak *et al*., 2013). Thus, the

understanding of the strength and direction of the relationships among traits is especially important for characters with low genetic variation, in order to allow for indirect selection to achieve genetic progress. The analysis of phenotypic correlation coefficient for the studied traits and the level of significance for each of the season and across seasons were presented in Table 3.9-12.

The examination of investigated red clover accessions under each of the season and across seasons show significant ($P<0.001$) positive correlations between PLG and all other quantitative traits (LS, STN PLH, MLW, MLL, IAN, APLS) including TFL in summer. This result leads to the conclusion that the possibility of selecting materials with high forage yield can be done even in any one of the seasons or in the first year of the evaluation. Similarly, majority of the aforementioned quantitative traits are interrelated positively in each of the season as well as across seasons. Very low positive to negative correlations were noticed for qualitative morphological (PGH, IAN and SML) with quantitatively measured traits (PLG, LS, STN PLH, MLW, MLL, APLS). This indicates that selection for these qualitative traits can only be possible through direct selection on trait-by-trait basis, which is also indicated by moderate to high repeatabilities.

Although STN, PLH and APLS value differences were noted within the tested accessions, low repeatability values were observed. However, this study shows that these important quantitative traits (STN, PLH and APLS) in red clover ultimately depend on high plant growth (PLG) or visually observed yield (Table 3.12). Likewise, PLH and APLS positively correlate with LS while APLS correlates well with PLH and MLW across seasons. Having said that, visually observed yield *via* PLG with high repeatability also has very strong ($P<0.001$) positive correlation with LS, STN, PLH, MLW, MLL and APLS. This clearly

suggests that indirect selection for increased expression of these traits using highly correlated traits is possible.

On the contrary, traits with both non-significant negative or positive correlations such as PLH and IAN, PLH and SML and IAN and SML (Table 3.12) and low-moderate repeatability (Table 3.4) can be quite difficult to improve through either direct or indirect selection methods. The effort to improve such difficult traits in red clover should consider exploring diversity in different sets of red clover population or possibilities for using other breeding methods, for example, molecular techniques for effective selection. As increasing forage yield, measured as PLG in this study, is one of the most important objectives of red clover breeding in New Zealand, its correlations with the other yield related characters should undergo further investigation particularly across multi-locations and years in order to generate more information to be used in the breeding programs.

3.5.5 Cluster analysis

Since red clover accessions evaluated in this study had their own particular response to seasonal variation, (vary significantly in their performance due to season effect and accession-by-season interaction effect), cluster analysis was done on season-by-season basis to allow hierarchical classification of materials with similarities and differences in traits. This is particularly important to provide breeders with information on accessions' specific adaptation and variation in trait expression to select suitable materials for specific seasons. Apparently, cluster analysis for the tested accessions was based on morphological features and to some extent in accordance to their geographical origin.

Winter clustering based on ten traits identified eight clusters (Table 3.5). Clusters I-IV were accessions with generally poor plant growth (visually observed yield) and other yield related traits (smaller leaf size, least number of stems, shorter plant height, smallest medial leaf width and length and less spreading. This cluster comprised of 25 accessions mainly from Tajikistan, Greece and Caucasus showing poor to moderate adaptation to winter and could relate to low genetic potential. Since materials from Greece, Tajikistan and Caucasus originally were used to thrive in continental climate which is quite different from New Zealand (Peel *et al*., 2007), adaptation to new environment may seem impossible. These accession germplasms may not be considered immediately in breeding for forage yield improvement but could contribute as important source of diversity for other traits.

Cluster V comprised of only two accessions has abundant number of stem and high intensity of anthocyanin pigmentation on stems and leaves. Accessions 24 and 22 both from Spain indicate phenotypic dissimilarity from the others that could form valuable sources of genetic variability in improvement programs for red clover. The nine accessions including cultivar Broadway in cluster VI is characterized uniquely for its ovate leaf shape and prostrate growth. The remaining five accessions and cultivars Relish and Sensation made up clusters VII and VIII. These materials show superior winter growth and display high level expression of desirable yield related traits such as tall and vigorously growing plants, large leaf size, increased number of stems and tendency to spread out from their growing points and occupied large area. The five accessions categorized in these clusters had excellent winter forage (PLG) with other yield traits which is superior or at par with locally tested cultivars (Relish, Sensation and Broadway). As these materials very much corresponds to high forage yield and winter survival, which can be a very promising material for breeding programmes.

Spring clustering: Evaluation of red clover entries under spring condition were separated into three distinct clusters of germplasm, mainly characterized by the same ten traits as in winter (Table 3.6). The first (I) cluster comprised of 19 accessions mainly from Portugal, Azerbaijan, Spain and the three commercial cultivars. This cluster grouped together the entries that expressed the highest level of desirable traits: robust plants growth with larger leaf size, high stem number, increased plant height, high anthocyanin pigmentation, semi-prostrate growth habit and increased area of plant spread. Materials in this cluster show high forage yield potential that are perhaps suitable for temporary grazing in spring and summer or hay production. The existence of high-level expression of desirable traits in this population also indicates high genetic potential for improving forage yield. In contrast, cluster III comprised of eight accessions with poor growth and below average performance for the above-mentioned traits while cluster II comprised of 13 accessions moderately characterized for all the traits.

Summer clustering: Unlike winter and spring, cluster analysis for summer season was based on ten previously assessed traits and two flowering traits which identified three distinct clusters (Table 3.7). The first cluster (I) was composed of accession germplasm mainly from Greece with relatively poor plant growth, semi-prostrate growth habit, smaller leaves, high stem number, high intensity of anthocyanin pigmentation and low tendency to flower. It is clear now that Greek germplasm accessions consistently show very low performance for all studied traits across all the seasons. This clearly suggests that these materials have very poor adaptation and are not suitable for growing in New Zealand. Cluster II characterized accessions mainly with superior plant growth, intermediate growth habit, elongated leaf shape, increased area of plant spread with profuse flowering habit. Accessions in this cluster have combination of superior plant growth and profuse flowering habit which is highly

desirable in red clover especially for forage and seeds production. The third (III) cluster comprised of entries typically with high plant growth, large leaf size mostly elongated-ovate shaped, increased plant height, low intensity of anthocyanin pigmentation and moderate flowering. Entries in this cluster also possess some of the highly desirable features in red clover that could form the genetic base for subsequent breeding specifically for forage and seeds production.

Cluster analysis across seasons: Cluster analysis combined across seasons characterized plants based on ten morphological and yield related traits (PLG, PGH, LS, STN, PLH, MLW, MLL, IAN, APLS and SML). Results of this analysis identified two major groups and six distinct clusters as summarized in Table 3.8 and Figure 3.22. Group A was sub-divided into 3 clusters (I, II, III) that contained 18 accessions whilst group B, the largest group with 25 entries also had 3 clusters (IV, V, VI) that comprised of the three cultivars (Broadway, Relish and Sensation) together with other accession germplasm. Accessions in Group A highly corresponds to prostrate growth habit, shorter plant heights, and small to medium plant types with poor plant growth indicating poor adaptation to New Zealand (NZ) condition. This group was composed by entries mainly from the Western Mediterranean region (Greece, Tajikistan, and Caucasus) and Spain. From this study, it is obvious that majority of the materials from these four geographical regions may not adapt well to NZ. However, two accessions with semi-prostrate to prostrate growth habit, ovate to round leaf shape, high stem number and strong pigmentation for anthocyanin were characterized in cluster I. Since short and spreading type cultivars with abundant stems are related to drought tolerance and persistency, these materials may be considered by breeders as potentially valuable source of persistency for breeding purposes in NZ. This relationship between prostrate growth habit and persistency was also reported by Smith and Bishop (1993). As suggested by

Mirzaie-Nodoushan *et al*. (1999), more persistent types may be useful in systematic crossings in order to increase the persistence of erect to semi-erect commercial cultivars. Group B, on the other hand, clustered the entries with generally maximum plant growth and moderate to high level of expression for the yield related traits (LS, STN, PLH and APLS).

Clusters IV and V have shown some similarities in most of the traits while cluster VI with five germplasm accessions and three cultivars which were characterized by intermediate to semi-prostrate growth habit, superior plant growth, the largest leaflet size and increased plant height. Combined cluster analysis across seasons confirms that accessions from Spain, Portugal, Armenia and Azerbaijan generally adapt well in NZ. The high PLG presented by the accessions of cluster VI may be used in top-cross program with existing commercial cultivars to develop future cultivars with enhanced forage yield and persistency in NZ or can be utilized in polycross to derive new breeding population.

Generally, this study reveals that positive traits required for breeding were identified in all clusters in combination with some undesirable traits. Although red clover with prostrate growth habit, shorter plant height, finer stem and small leaf size are negatively correlated with yield, they tend to correlate well with vegetative persistency. Hence, the effort in breeding for improved forage yield and persistency could possibly consider combining some of the traits related to persistency as well as favourable yield related traits. Incorporation of yield and persistence related traits in promising entries could be utilized in red clover breeding program to combine productivity and persistency for NZ condition.

3.5.6 Principal component analysis

Analysis of variance indicated significant differences among the tested red clover accessions for most of the traits which prompted us to do further analysis using principal component analysis (PCA). The use of PCA enabled us to examine the variation in the germplasm and to estimate the relative contribution of various traits to total variability. Furthermore, PCA facilitates the identification of the most important traits related to forage yield. Widespread distribution of accessions/cultivars across all quarterlies of the PCA biplot indicates high level of genetic diversity in the accessions (Figure 3.23). Variance components analysis done in this study also confirms with result from the PCA.

PCA biplot indicated the presence of six distinct clusters in which clusters I to III comprised of entries with below average expression of all morphological and yield related traits towards the right axis of PC1 and PC2. However, accessions with higher expression of the studied traits were presents towards the left axis. The alignment and clustering of the accessions with PCA (Figure 3.23) corresponds well with clustering by dendrogram in Figure 3.22. Although there were no definite boundaries in the PCA biplot, there were some overlaps between accessions from different clusters showing similarities in traits.

The principal component analysis performed on 43 accessions/cultivars and ten morphological traits pooled across all three seasons accounted for 75 % of the total variation. The first (PC1) and the second (PC2) axes accounted for 58.2 and 16.8% respectively (Figure 3.23). The variation of measured traits studied through PCA revealed that plant growth (PLG), area of plant spread (APLS) and stem number (STN) contributed more positively to PC1 followed by intensity of anthocyanin (IAN) and plant growth habit (PGH). In contrast, the traits plant height (PLH), medial leaf width (MLW) and medial leaf length (MLL)

strongly influence PC2 than shape of medial leaf (SML). Similar trend was reported by Ahsyee (2013) in a diverse population of red clover. Accessions 01, 04 and 12 from cluster V and accessions 37, 27, 16 in cluster I were related more to STN. Likewise, entries that are close to each other tend to have similar response to specific traits just like the majority of entries closely congregating around STN. The PC biplot demonstrate positively strong to intermediate correlation between PLG and yield related traits like LS, APLS, MLW, MLL, PLH, and STN. This implies that LS, APLS, MLW, MLL, PLH and STN are the most important traits related to forage yield. In contrast, PLG was negatively correlated to PGH and SML. Generally, the PC biplot (Figure 3.23) had shown similar correlation to that projected in Table 3.12.

Results of the PCA revealed that there is a large quantity of variability in the introduced red clover germplasm and three local cultivars that is required for additional genetic enhancement. PCA identified only some characters that plays prominent role in classifying the variation existing in the germplasm. Yield related traits identified in this study would be used by breeders as preliminary evaluation tool to fast-track the selection process for forage yield improvement.

3.6 Conclusion

Significant differences were estimated among the red clover germplasm accessions for all the traits measured across all seasons except for stem number (STN) and plant height (PLH) in summer. The relative contribution of genotypic variance to PLG, PGH, LS, MLW, MLL, STN and APLS was larger than the total contribution of non-heritable components that resulted in high accession mean based repeatability (R^2_2 >0.50) except for the traits PLH, IAN and SML. Higher non-genotypic variances due to season (σ^2_s), accession-by-season

(σ^2_{gs}), accession-by-replicate (σ^2_{gb}), and experimental error (σ^2_{ε}) for the traits IAN, APLS and SML indicate the potential genetic variation available among the accessions to be used in breeding. The effect of season (σ^2_s) was highly significant for the traits PLH, IAN and SML, suggesting the need to evaluate germplasm for several seasons over several years. The general performance of the observed germplasm varies significantly in each season. Accessions 25 and 18 from Spain and Portugal respectively had significantly higher visual forage yield (PLG) in winter compared to the other entries and local cultivars of red clover. High yielding accessions for spring are 5, 18 and 17 while 6 accessions *viz.* 12, 23, 34, 39, 18, 28 look promising.

The red clover accessions responded differently to differences in seasons that resulted in significant genotype-by-season (σ^2_{gs}) interaction effects in all the traits. Despite the differences in seasons, six accession germplasm *viz.* 18, 5, 19, 17, 5 and 28 and the two cultivars, Broadway and Relish, clearly show high and stable visual forage yield across all the seasons as well as for each season. High plant growth and forage yield measured in the introduced germplasm accessions indicates their high genetic potential for adaptation to New Zealand conditions. Besides high forage yield, those accessions also exhibited favourable values of other desirable traits such as large leaf size (LS), increased number of stems (STN), tall plant height (PLH) and high spreading ability (APLS), representing valuable source of genetic diversity for further development of superior red clover cultivars for New Zealand conditions.

Indirect selection for forage yield seems possible for each of the seasons and across seasons due to strong positive correlations found between plant growth (PLG) and other yield related quantitative traits such as leaf size (LS), stem number (STN), plant height (PLH), plant

spread (APLS) and medial leaf length (MLL) and width (MLW). It is also clear that indirect selection is also feasible for other pairs of traits apart from PLG which show positive interrelationship. Qualitative morphological traits such as plant growth habit (PGH), intensity of anthocyanin pigmentation (IAN) and shape of medial leaflet (SML) with quantitatively measured traits (PLG, LS, STN PLH, MLW, MLL and APLS) are not well correlated or negatively correlated and selection can only be done directly on trait-by-trait basis. It is obvious that IAN and SML will potentially be difficult to improve through breeding and indirect selection due to low repeatability and weak correlation, thus would require additional genetic diversity and other appropriate breeding techniques to improve expression of these traits.

The red clover accessions evaluated in this study show great variation in response to seasonal differences, thus resulted in distinctness among clusters which provides information on germplasm specific adaptation. Accessions with very poor to moderate adaptation are mainly from Tajikistan, Greece and Caucasus, whilst accessions with high adaptation and high level of expression for all desirable yield related traits that are at par with cultivars Relish, Sensation and Broadway, are mostly from Spain, Portugal, Armenia and Azerbaijan. Consequently, there is a need to select germplasm with specific adaptation to winter, spring and summer which is suitable for short-term grazing and conservation feeds. Since red clover is a perennial crop, selection for specific adaptation may not always be economically beneficial. Thus, there is need to select germplasm with wide adaptation across seasons. The combined cluster analysis across seasons characterized the germplasm into six diverse clusters in which cluster VI containing five accessions showing excellent adaptation to New Zealand conditions in terms of visual forage yield and other favourable traits. Genetic potential and diversity in these five accessions should be further explored in red clover

breeding programs for development of highly adapted cultivars with improved forage yield and vegetative persistence for general use in New Zealand. Cluster analysis reveals that positive traits required for breeding were identified in all clusters in combination with some undesirable traits and require careful selection of parent material for any breeding activities in order to accumulate favourable alleles while minimizing linkage drag.

Principal component analysis (PCA) performed on ten morphological traits pooled across all three seasons accounted for 75 % of the total variation. The first (PC1) accounted for 58.2% of the total variability, which was highly influenced by plant growth (PLG), area of plant spread (APLS) and stem number (STN). The PC2 accounted for 16.8% of the variation and is strongly influenced by plant height (PLH), medial leaf width (MLW) and medial leaf length (MLL). The use of PCA shows considerable amounts of genotypic variability in the studied accessions to be used in breeding and relative contribution of various traits to total variability. PCA further helped identify STN, PLH, LS, MLW, MLL and APLS as important traits with positive correlation to visually observed forage yield or plant growth (PLG). The multivariate analysis using PC in this study corresponds well with analysis of variance components, cluster analysis and analysis for phenotypic correlation coefficients.

3.6.1 Limitations and recommendations for future studies

1. Assessment of these introduced germplasm was initially planned to be studied under mixed sward of perennial ryegrass and red clover so that persistence under mixed swards and grazing could be assessed. However, due to poor germination and establishment of perennial ryegrass after sowing, assessment for persistence of red clover under perennial ryegrass and red clover sward with grazing pressure was not possible. In order to get

information on persistence, it is recommended that future studies using these germplasm accessions should be conducted using already established perennial ryegrass swards.

2. Forage yield was visually assessed from plant growth vigour scores in this experiment. Although plant growth assessment or visually observed yield was reported to be highly correlated with forage yield, it is highly recommended to take actual yield dry matter measurements to generate reliable information from future studies.

3. Due to the time limitation of my study, information on reproductive and seed yield traits was not generated. Since seed yield is an important trait in red clover, it is suggested to consider investigating these traits in future trials.

4. To acquire more accurate and dependable estimates of variance components, repeatability, genotypic correlation among traits and genotypic performance, these germplasm accessions need to be evaluated over successive seasons, years and across multiple locations.

Chapter 4.0 SCREENING FOR CLOVER ROT (SCLEROTINIA) RESISTANCE IN SELECTED RED CLOVER COMMERCIAL CULTIVARS OF NEW ZEALAND

4.1 Abstract

Clover rot (*Sclerotinia trifoliorum* Erikss.) is an important disease of red clover in temperate regions where the crop has been intensively cultivated for livestock feed. Although red clover is not intensively cultivated in New Zealand (NZ), there is evidence of the presence of clover rot - affecting both red and white clovers - in certain parts of the country. Its prevalence across the country is at variance, but at times turn out to be devastating when climatic condition is favourable, particularly under pure swards red clover pastoral system. At present, no completely resistant cultivars are available worldwide and also, little is known about the status of current commercial cultivars of red clover for susceptibility to clover rot in NZ. Although developed locally, some NZ red clover cultivars are also used in European and Mediterranean agricultural systems where prevalence of clover rot disease is very high. Previous observations done on clover rot in NZ indicated that incidence of this disease is not cultivar specific. Even white clover is an alternate host for clover rot disease, and its incidence in NZ is well documented. Importantly, effects of climate change on local weather conditions could potentially induce the incidence and spread of Sclerotinia pathogen further. This could be considered as a potential threat to NZ pastoral industry to prepare for any possible future outbreak of clover rot disease. In this experiment, we proposed to investigate the response of eleven selected NZ commercial cultivars to clover rot disease through artificial inoculation under high disease pressure in glasshouse to identify source of resistance for further breeding purposes. In this experiment, two isolates of *Sclerotinia trifoliorum* Erikss were cultured in the laboratory with an attempt to produce *Sclerotinia* ascospores that will be used to inoculate the red clover plants with set concentration of ascospores under

controlled glasshouse condition. The species *S. trifoliorum* generally undergoes series of developmental stages including production of mycelium, sclerotia, stipes and apothecia under right condition before it produces ascospores to infect plants. *In-vitro* production of sclerotia has been effectively achieved on PDA media. Our attempt to produce ascospores was not successful, leading to delay in artificial inoculation of plants in glasshouse. Once ascospores are collected, inoculum concentration will be prepared and young plants inoculated under controlled glasshouse condition. Disease incidence (DI) on inoculated plants will be assessed to identify cultivars with low DI to form the source of resistance for further breeding purposes. This thesis only presents the background of the experiment, materials and methods and expected results with some recommendations. This result suggests the need to improve on *in-vitro* techniques for ascospore production, understand appropriate culture condition and determine right choice of *S. trifoliorum* isolate to facilitate fertilization.

4.2 Introduction

Red clover is infected by several microorganisms during their development at different stages. Some can easily be characterized as casual organisms of specific diseases producing distinct signs while others may be pathogenic (harmful) and non-pathogenic (beneficial) in which their effects on red clover plant frequently depends on the interaction between other organisms and abiotic factors (Skipp & Watson, 1996). Among the various biotic and abiotic stresses, red clover disease is highly interactive and complex, which restricts red clover growth and persistence that eventually determines herbage yield and animal productivity.

A wide range of pathogenic diseases influence red and white clover in New Zealand apart from the economically important white clover mosaic virus (WCMV). The significant fungal diseases being root rot (*Fusarium solani* (Mart.) Sacc.) and *Fusarium oxysporum* Schlccht.

(Nan *et al*., 1991), pseudopeziza leaf spot *(Pseudopeziza trifolii)*, sooty blotch (*Mycosphaerella killiani*), pepper spot *(Leptosphaerulina trifoii),* sclerotinia clover rot *(Sclerotinia trifoliorum),* and rust *(Uromyces trifolii)* (Woodfield & Caradus, 1996). These fungal pathogens - given their prevalence - are perhaps a vital addition to other biotic stresses. The use of fungicide to pasture as a control measure generally responded poorly (Watson *et al*., 1985). Red clover plants usually die as a result of deterioration of their primary axis, i.e. the tap root and crown, due to direct effect of fungal diseases or infection by stem nematode *Ditylenchus dipsaci* (Kuhn) Filipjev (Tylenchidae) (Skipp & Christensen, 1990) and treading damage. Tap root injury by invertebrates is likely to contribute to the establishment of fungal pathogens. All these factors have contributed to poor persistence of sown red clover under New Zealand farms.

Clover rot is induced by *Sclerotinia trifoliorum* Erikks. or *S. sclerotiorum* de Bary., although *S. trifoliorum* causes severe damage on red clover (Vleugels *et al*., 2012). Clover rot is most common and its prevalence is high in European red clover where snow cover is heavy with mild winters. The species *S. sclerotiorum* is a pervasive pathogen with a broad range of host including vegetables, oilseed and ornamental crops whilst *S. trifoliorum* has host limited particularly to Leguminous crops. However, several studies (Saharan & Mehta, 2008; Pratt & Rowe, 1995; Boland & Hall, 1994) have confirmed that *S. sclerotiorum* is also capable of causing clover rot and infects red clover mostly under favourable conditions.

Sclerotinia species particularly *S. trifoliorum* in the form of sclerotia, dark resting bodies, exists in contaminated fields and develops apothecia in autumn. When fully matured, the apothecia release huge quantities of ascospores in to the air. The ascospores then infect red clover leaves and disease slowly develops during winter when plants undergo winter stress.

In contrast, *S. sclerotiorum* frequently develops apothecia in spring and disease progresses in early summer (Öhberg, 2008; Taylor & Quesenberry, 1996). Both Sclerotinia pathogens have similar symptoms (Saharan & Mehta, 2008; Pratt & Rowe, 1995). The transmission of *S. trifoliorum* and outbreak of clover rot is somewhat determined by gastopods like *Deroceras panormitanum*, *D. reticulatum* and *A. hortensis* that usually feeds on sclerotia and the apothecia (Shakeel & Mowat, 1992). Since clover rot development relies heavily on weather conditions, efforts to identify resistant plants through natural infection are not always reliable. Sclerotia are able to survive in the soil for up to seven years and give rise to ascospores the following year given the favourable condition. Measures to control clover rot are quite difficult given the fact that fungicide is inefficient, uneconomical and its use in numerous countries is not registered for red clover (Raynal *et al.*, 1991). Efforts to improve level of resistance in red clover in the last decades were done through natural infection; however advancement is slow due to high variation in disease severity. Selection of resistant genotypes can efficiently be done using artificial inoculation where other factors likely to affect disease severity can be ruled out. Thus, artificial inoculation or bio-test method is recommended in resistance breeding for clover rot (Saharan & Mehta, 2008; Öhberg, 2008; Delclos & Duc, 1996). Until now, resistance breeding is hindered by availability of few sources of resistance and not much information available on plant specific mechanisms that affect clover rot resistance.

Plants react differently when in contact with pathogens and rate of infection is determined by the interaction of pathogens, host plants and environment condition, which is known as the disease triangle. A disease resistant plant is completely immune from the effects of pathogens while maintaining production level at economic threshold levels, whereas disease tolerance refers to plants that exhibit disease damage to some extent although being exposed to

considerable pathogen levels. There are several plant specific mechanisms that determine the type of resistance in plants. Plants with basal resistance safeguard it from non-host pathogens using preformed structures (Poland *et al*., 2009). Qualitative or host specific resistance is a gene-for-gene effect mediating resistance allele that protects host plants from attack by adapted pathogens (Gururani *et al*., 2012; Kou & Wang, 2010). Resistance conferred by single gene can easily be overcome by disease within few years. Nevertheless, there are certain levels of quantitative disease resistance (QDR) typically observed in plants usually controlled by several genes with partial effects that are referred to as quantitative resistance loci. QDR is the form of resistance generally preferred by breeders due to its durability and effectiveness against a wider range of pathogen races. Conventional breeding methods can be used to improve QDR via several phenotypic backcross selections even without knowing the resistance loci (Vleugels, 2013). QDR in red clover can be further improved using molecular breeding such as marker assisted selection (MAS). Resistance breeding can easily be achieved using specific gene or tightly linked markers with MAS in future provided that genes for resistance loci are identified. Actually, breeding for QDR by utilizing MAS has been successfully used to develop resistance against several pathogens (Vleugels, 2013) such as white mould resistance in common beans (Kou & Wang, 2010; Miklas, 2007).

Contrary to the situation in Europe and parts of the Mediterranean region, the prevalence of clover rot affecting red clover in New Zealand (NZ) at present is generally low and is considered as moderately important. Hence, the effect of clover rot has not been well studied under NZ condition. Apart from unfavourable local weather condition, low incidence of clover rot could be related to low intensity of red clover production, popularity of white clover over red clover and mixed swards perennial ryegrass clover based pastoral system.

In spite of that, several studies have reported the incidence of Sclerotinia clover rot under NZ condition. A study on the incidence of clover rot among the available white clover cultivars in NZ has shown differences in susceptibility (Watson, 1988). According to a personal communication with John Ford (June 03, 2020), a forage plant breeder with Agricom & PGG Wrightson Seeds/DFL), clover rot was an issue in red clover pastures in certain parts of NZ in the 80's, which prompted a greenhouse screening for the pathogen at that time. Significant reductions in red clover plant numbers due to clover rot after field establishment were noted by Ledgard *et al*. (1988) at Waikato dairy pasture. Up until recently, incidence of clover rot was reported in several pure swards red clover fields and evaluation trials in NZ from time to time, but is very random and its effect is not line specific (J. Ford, personal communication, June 03, 2020).

This indicates that certain parts of NZ, especially, on the South Island that experiences mild winters and snow at high altitudes with high precipitation and humidity could favour the spread of *S. trifoliorum* and reduce persistence and productivity of red clover. There is also a possibility that incidence of clover rot could increase as red clover production further intensifies in NZ farms. Besides, effects of climate change in NZ are highly uncertain (Nottage *et al*., 2010), although emergence and incidence of existing pests and diseases are likely to increase, and enable the emergence of new diseases (Hennessy *et al*., 2007). It is predicted that changes in temperature and rainfall patterns may also alter the spread and distribution of existing pests and diseases (Nottage *et al*., 2010). According to the National Institute of Water and Atmosphere (NIWA) model, NZ will receive more rain and frequent drought than usual in the next decades and onwards Nottage *et al*. (2010). Since Sclerotinia clover rot epidemiology favours temperate climate with high precipitation and humidity

(Taylor *et al*., 1996), this condition could possibly promote the spread and distribution of clover rot disease in red clover plants in future.

There are no clover rot resistant cultivars of red clover available at the moment, and even the status of NZ commercial cultivars to clover rot disease is not known. New Zealand red clover cultivars although developed locally, are also used in European agricultural systems where the disease is prevalent. Currently NZ exports seeds of cultivar Sensation in to UK market while other varieties including Relish are in evaluation in different parts of Europe prior to approval for formal variety release (J. Ford, Personal communication, May 10, 2021).

Control with fungicide can be possible with a number of products on the market for Sclerotinia like Rovral, Carbendazim and Benomyl if incidence is high enough. Management practices like mixed swards system can be also effective in controlling the devastating effects of clover rot, but even so a sustainable and most economical control method would be to breed and make available resistant cultivars. Given this scenario, there is need to screen commercial cultivars currently on the NZ market for susceptibility to clover rot and further development of resistant plants in preparation for any future outbreak. Similarly, screening for clover rot resistance cultivars locally is important globally particularly for cultivars intended for European seed markets.

4.2.1 Objectives

This experiment was aimed at investigating the variation in clover rot susceptibility among eleven commercial cultivars from New Zealand, with the objective of identifying sources of resistance.

In this study, we proposed to infect the selected commercial red clover cultivars with cultured sclerotinia ascospores via artificial inoculation under glasshouse condition with locally sourced sclerotinia isolates. Incidence of clover rot is expected to be assessed under controlled glasshouse condition and possible sources of resistance for breeding purpose are expected to be identified. To our knowledge, no previous studies have screened such collections of red clover for clover rot resistance using artificial inoculation under glasshouse condition in New Zealand. The information generated will set the basis for future research on clover rot resistance breeding.

4.3 Materials and Methods

4.3.1 Red clover germplasm

The original proposed plan for this experiment was to use the same population of 43 red clover germplasm under investigation from the first experiment to screen in glasshouse for resistance to sclerotinia. This was not possible due to limited and uneven quantity of seeds stock available for the experiment, particularly for the introduced germplasm. Alternatively, eleven red clover commercial cultivars of New Zealand were selected to be tested for resistance to sclerotinia clover rot disease. These cultivars include Aber Claret, Amigain, Astred, Broadway, Ceibo, Colenso, FF9615, Rajah, Relish, Sensation and Tuscan. Although there is limited information on the status of these cultivars' susceptibility to sclerotinia under New Zealand condition, cultivar Rajah was observed to be the most affected amongst others in a red clover field trial in Christchurch (J. Ford, personal communication, January 17, 2020). Hence, cultivar Rajah was selected as a susceptible check to compare against performances of other cultivars in this experiment.

4.3.1.1 Growing of red clover seedlings

The germination and growing of seedlings were done according to procedures suggested by Jahufer *et al*., (1997). Fully developed seeds from 11 red clover cultivars were scarified with sandpaper then placed in moist petri dishes. Seeds in petri dishes were incubated in dark at 25˚C for three days in order to break seed dormancy and induce germination. The germinated seeds were planted on plastic seedling trays with potting mix in glasshouse and left for two weeks and then transplanted again into nursery pots (9 cm diameter x 10 cm depth) containing sterile potting mix (short-term mix). The pot plants were placed on ebb and flow table in glasshouse with temperature maintained between 15 and 20 ˚C under relative humidity of about 70 to 80 % for three months.

4.3.2 Sclerotinia strain

Initially, we planned to use locally available strain of sclerotinia from infected red clover plants around Palmerston North. Searching for sclerotinia in the form of mycelium and sclerotia was undertaken from May to June 2019 in two separate fields of red clover grown in pure sward at Massey University farm in Palmerston North. However, during the time of field collection, we could not find any infected plant with visible symptoms of sclerotinia clover rot disease. Thus, we obtained *Sclerotinia trifoliorum* Erikks strain ICMP 7318 in the form of freeze-dried sclerotia from the Manaaki Whenua – Landcare Research, New Zealand. The first attempt to produce hypothecia and ascospores from this strain was unsuccessful due to difficulty in breaking the dormancy of sclerotia. The procedures for production of hypothecia and ascospores were reassessed and the process repeated with additional strain of sclerotinia ICMP 7319 obtained again from Manaaki Whenua – Landcare Research collected from different location. Additional strain of pathogen was needed to facilitate fertilization among the two strains in order to produce apothecia.

4.3.2.1 Production of Sclerotinia ascospores

In order to perform artificial inoculation on red clover plants in glasshouse, it requires the following procedures to produce sufficient sclerotinia ascospores: *in-vitro* production of sclerocia, breaking of sclerotia dormancy, induction of apothecia and collection of ascospores. Each of the method was explained in detail below. However, due to the unsuccessful first attempt in producing hypothecia and ascospores, time became my limiting factor in completing all the stages in the experiment. I was only able to complete *in-vitro* production of sclerotia, breaking sclerotia dormancy, and initiated the production of apothecia. If apothecia are successfully developed into full maturity, then ascospores is expected to be released so they will be collected to perform artificial inoculation on red clover plants in glasshouse. Details of these procedures will be reported later once these stages of the experiment are completed.

4.3.2.2 *In-vitro* methods for sclerocia production

Sclerotinia ascospores can be obtained if there is successful production of quality sclerotia in sufficient quantity. This can be easily achieved using *in-vitro* techniques suggested by Vleugels (2011) and Marum *et al*. (1994). In this experiment, potato dextrose agar (PDA) media was prepared according to instructions provided by the manufacturer (39 g of medium in 1000 ml of distilled water). The media suspension was boiled in microwave to completely dissolve the media and then pH adjusted to 5.6 ± 0.2 at 25 ˚C. After that, the media suspension was sterilized by autoclaving at 121 ˚C for 15 minutes following established laboratory procedures. About 20-25 ml of autoclaved medium was thoroughly dispensed into 9 cm diameter sterile petri plates in the laminar flow. Sclerotinia in the form of freeze-dried sclerotia was dissected in to smaller pieces. Then single piece of dissected sclerotia was added on the PDA media in each petri dish. The petri dishes containing the cultures were

sealed with parafilm and incubated in the dark at room temperature for at least 4 weeks to initiate production of sclerotia.

Mycelium started to develop on PDA from the sub-cultured sclerotia after the first week in dark and further differentiated in to newly developed sclerotia on the third week. From this stage onwards, we used the methods by Delclos and Raynal (1995). The fully developed sclerotia was harvested after 5 weeks then thoroughly washed to remove agar from the media. After this process, the sclerotia were left to air dry for three days at room temperature. Dried sclerotia at this stage can produce apothecia if given the right condition and could even be stored at 5 ˚C for prolonged periods (Raynal, 1990).

4.3.2.3 Breaking of dormancy

The newly formed sclerotia are dormant until this dormancy is overcome and placed in appropriate condition to form apothecia. In order to facilitate that, dried sclerotia were incubated at 30°C for 28 days in petri dishes to mimic the summer period and break the dormancy (Delclos & Raynal, 1995).

4.3.2.4 Apothecia production

Apothecia can be induced after breaking the existence of dormancy in the sclerotia (Figure 4.8 A and B). It is reported that in order to produce numerous apothecia with good quality, temperature should be maintained between 15°C and 17°C under high humidity (80-100 %) with adequate quantity of white light (Vleugels *et al.*, 2013).

Sclerotia were buried at the depth of about 1-2 cm in wet vermiculite in air-tight transparent containers and incubated at 15°C in 80-100% relative humidity for 6-8 weeks. To maintain

moisture and humidity in the container, distilled water was filled up to at least 1 cm. Photoperiods of 12 hours of 100 μmol/m² light per day was maintained in the incubator for the duration of apothecia induction which is required for the differentiation and maturation of apothecia. Once apothecia in produced, it is expected to live for at least three weeks while simultaneously producing ascospores. Previous studies done by several researchers reported successful production of apothecia and release of ascospores after 4-5 weeks (Vleugels, 2013; Delclos & Raynal, 1995; Marum *et al*., 1994). However, for this experiment, only first stipeses were successfully initiated after three weeks while differentiation into apothecia was prolonged. Details of these procedures will be reported later once these stages of the experiment are completed.

4.3.2.5 Collection of ascospores

Upon the release of ascospores, collection of ascospores ejected by apothecia will be done on daily basis using 5 μm membrane filter connected to a vacuum pump and stored at 4°C in a Petri dish with silica gel. Since ascospores are readily released into the air, it is suggested that the membrane filter must be connected to vacuum pump and the funnel immediately placed above the container when opening the lid in order to capture the ascospores on the filter (Vleugels, 2013). As reported by Delclos and Raynal (1995), ascospores released by apothecia into the air can be collected up to three times a day, at least a second after opening the container lids.

For inoculation of plants to take place, ascospores collected on membrane filter will be brought into suspension in sterile water with 5 g/l glucose and 50 μl/l Tween 20 in test tubes (Vleugels, 2013). To collect as much spores as possible, test tubes with ascospores suspension must be swirled for approximately 3 minutes (Marum *et al*., 1994). Ascospore

concentration will be determined under microscope with the help of haemocytometer to ensure that sufficient spores (between 8,000-10,000 spores/ml) are collected to infect plants (Jovita *et al*., 2015; Vleugels, 2013).

4.3.3 Experiment design and glasshouse condition

Artificial inoculation of 11 red clover cultivars is to be carried out in a glasshouse under controlled environment at the Massey University Plant Growth Unit. Growing conditions (temperature, humidity and irrigation) in glasshouse is controlled during the inoculation of pathogen spores. Experimental plants will be laid on ebb and flow tables that will be flooded daily and tables covered with transparent plastic cap to create a moist environment preferably maintained at 100 % humidity with temperature to be set between 15°C and 25°C.

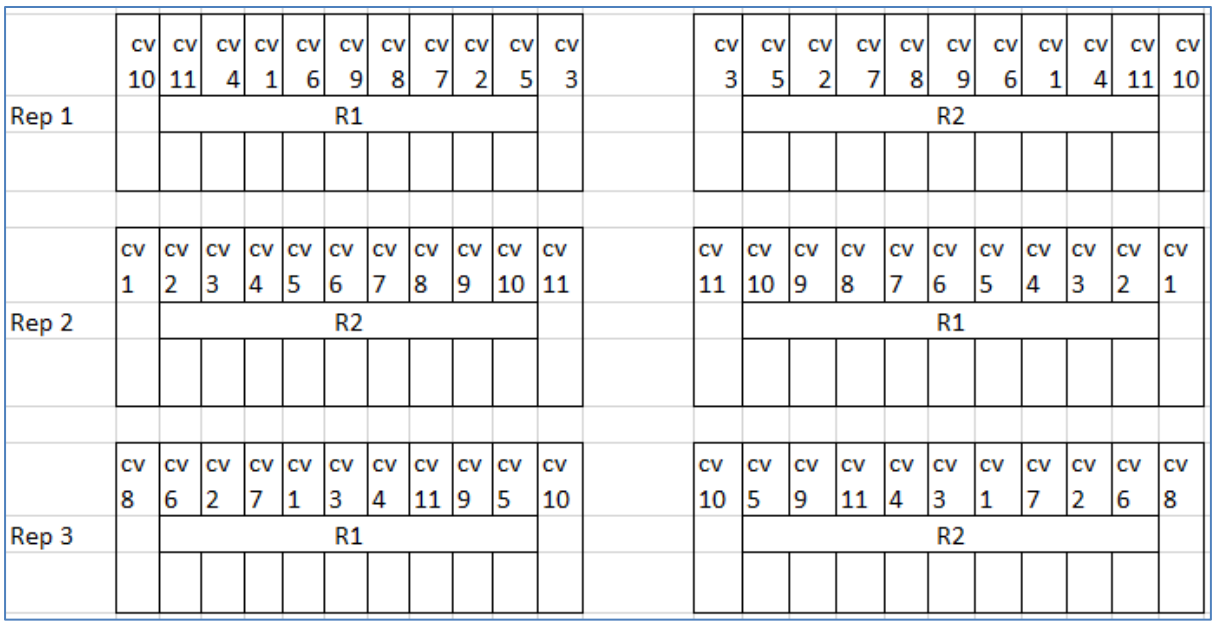


Figure 4.1 Schematic diagram of proposed glasshouse experiment for 11 cultivars of red clovers and two application rates of sclerotinia inoculum laid out in a split plot design.

Design of the glasshouse pot experiment is laid out using a split plot arrangement in a completely randomized design (CRD) with three replicates (Figure 4.1). Treatments in each replicate are divided into two main plots, and with each plot further sub-divided into 11 subplots. The main plot treatments consist of two rates (R1 = 8000-10,000 spores /ml and

R2 = 0 spores/ml of distilled water as a control) of *Sclerotinia Trifoliorum* Erikks. ascospores. The subplot treatments consist of 11 red clover cultivars (cv): Aber Claret (cv 1), Amigain (cv 2), Astred (cv 3), Broadway (cv 4), Ceibo (cv 5), Colenso (cv 6), FF9615 (cv 7), Rajah (cv 8) Relish (cv 9), Sensation (cv 10), Tuscan (cv 11). Cultivar Rajah is regarded as the susceptible check based on observation in a red clover field trial in Christchurch (J. Ford, personal communication, January 17, 2020). Each cultivar per treatment is represented five times in each of the replicate. A total of thirty plants were required per cultivar in the trial.

4.3.4 Artificial inoculation of plants in glasshouse

Once concentration of ascospores is determined, artificial inoculation of plants in glasshouse is to be done according to Vleugels *et al.* (2011). It is important to expose each plant to same amount of inoculum, so we propose to spray about 1.0 to 1.5 ml of ascospores suspension per plant only once on two-month-old plants until plants are completely wet. In order to avoid variation in degree of infection between plants as a result of "escape" or differences in density of inoculum, the inoculum will be thoroughly mixed before spraying to ensure more evenly spread of inoculum on the plants.

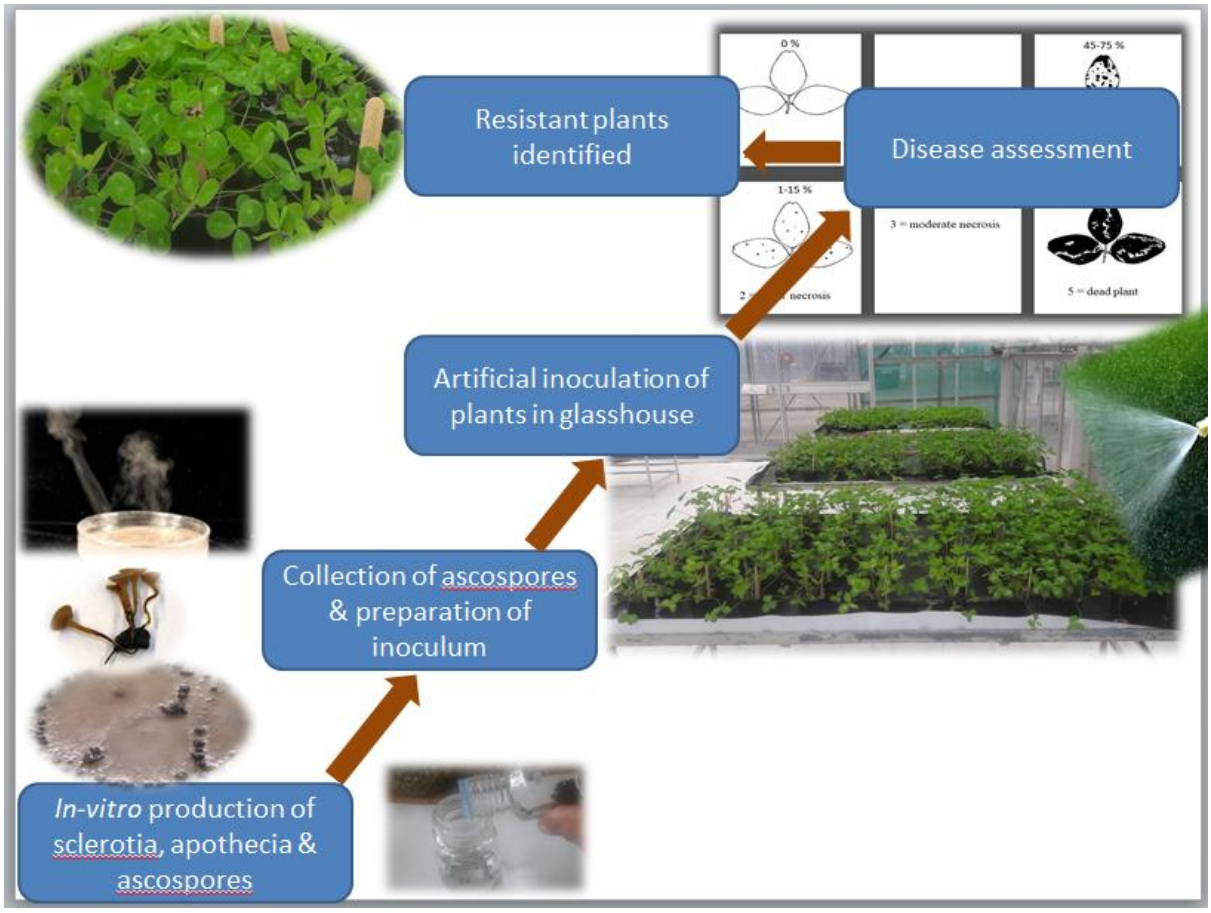


Figure 4.2 Diagram of the screening procedure: *in-vitro* production of sclerotinia, collection of ascospores, plant seedlings, inoculum spray, clover rot assessment and selection of resistant plants.

We initially proposed to perform artificial inoculation on two months old plants. However, this will not be possible due to failure in production of ascospores in our first attempt. By now, the plants are already six months old but are continuously trimmed every two months to maintain their young vegetative growth. Ascospores inoculum is proposed to be sprayed on regenerated leaves and biomass two to three weeks after their last trim.

4.3.5 Disease assessment

Two weeks after the inoculation under the abovementioned condition, plants will be scored for incidence of sclerotinia clover rot disease. Plants with visible lesions or necrosis on leaves

will be scores on a 5-point disease severity score: 1 = healthy plant, 2 = slight necrosis, 3 = moderate necrosis, 4 severe necrosis, and 5 = dead plant (Figure 4.2).

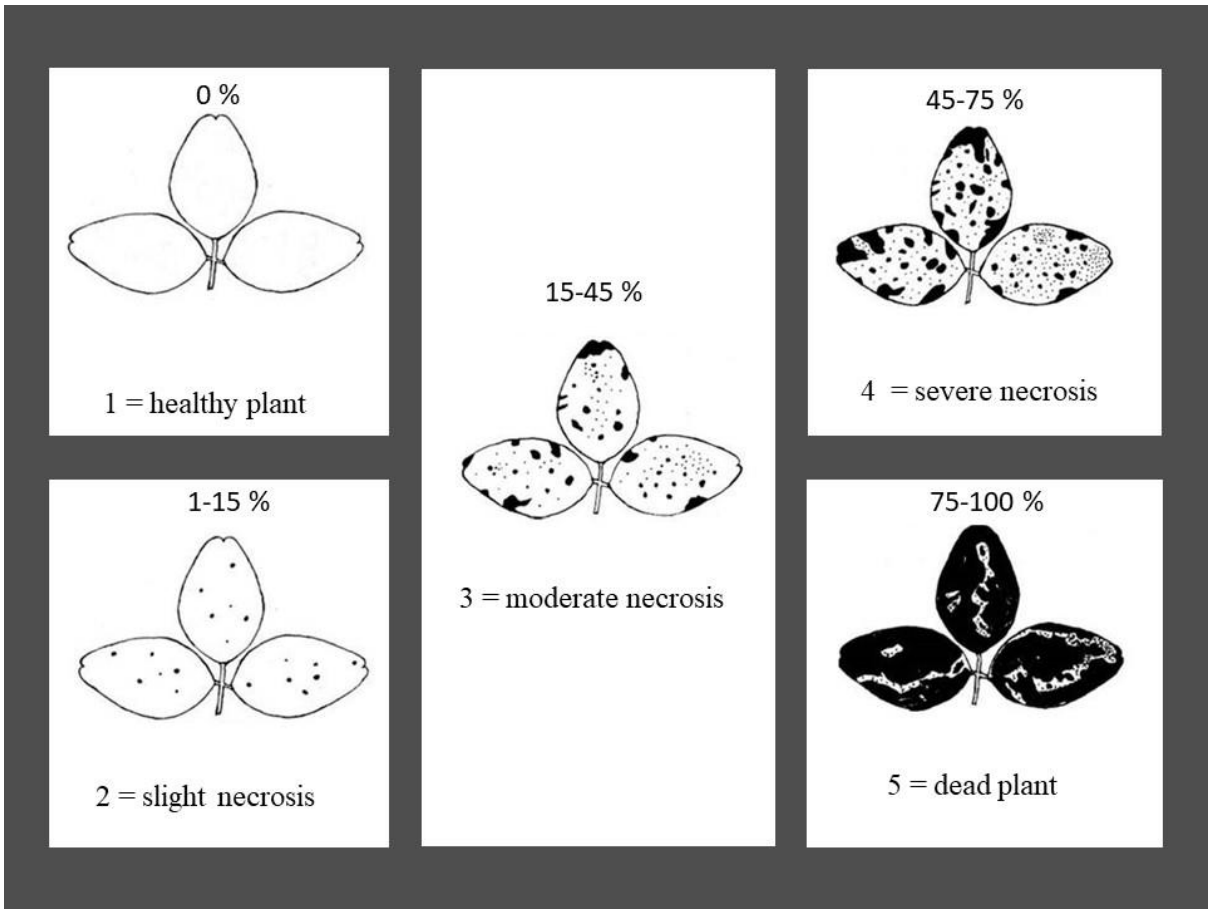


Figure 4.3 Disease assessment key for red clover caused by *Sclerotinia trifoliorum* Erikss. Pictorial diagrams of red clover leaf showing percentage of leaf area damage on 5-point disease scale.

The number of plants survives the inoculum after 28 days will be recorded to determine survival percentage. Disease incidence (DI) and disease severity index (DSI) will be calculated according to Dixon and Doodson (1974) as expressed in Equations 4.1 and 4.2 respectively. Cultivars with disease score of 1 will be considered highly resistant while score of 5 highly susceptible to sclerotinia clover rot. Likewise, low DI values of cultivars will be related to greater cultivar's resistance to sclerotinia.

$$\mathbf{DI} = \frac{\textbf{No. of infected plants}}{\textbf{Total no. of plants assessed}} \ \mathbf{x\ 100} \qquad (4.1)$$

$$\mathbf{DSI}\ (\%) = \frac{\textbf{}\sum\textbf{ of all disease rating}}{\textbf{Total no. of rating x maximum disease grade}} \ \mathbf{x\ 100} \qquad (4.2)$$

Disease scoring will be done at one week's interval over a period of four weeks after inoculation to assess disease progress and severity over time. Repeated observation over time will enable us to determine the area under the disease progress curve (AUDPC), which is useful in examining the progress of disease over time. Formula to determine AUDPC was adopted from Simko and Piepho (2012) (Equation 4.3).

$$\mathbf{AUDPC} = \sum_{i=1}^{n-1} \frac{y_i + y_{i+1}}{2} \mathbf{x}\ (t_{i+1} - t_i) \qquad (4.3)$$

where y_i is an assessment of a disease (score, proportion, percentage, etc.) at the *ith* observation, t_i is time at the *ith* observation, and *n* is the total number of observations.

Incidences of other diseases will also be recorded in order to avoid confusion between other foliar diseases and sclerotinia clover rot.

4.3.6 Foliage assessment

Fresh foliage (g) and aerial biomass (g) weights will be recorded and oven dried at 60°C for 48 hours to obtain dry matter weights from the same number of plant samples assessed for clover rot disease. Foliage and aerial biomass of plant will be considered in this study in

order to determine if there is a positive relationship between clover rot incidence and biomass yield.

4.3.7 Statistical analysis

Data for disease severity assessment and weight of foliage and aerial biomass will be subjected to analysis of variance (ANOVA) for test of significance at 95% confidence interval. A split plot ANOVA will be used with inoculum rates as factor one (main plot effect) and cultivars as second factor (sub-plot effect). Since the sub-plot treatments (cultivars) are nested in the main plot, the interaction between the two factors will be determined for test of significance ($P<0.05$). Analysis for correlation between clover rot and foliage weight and aerial biomass will be conducted. DeltaGen (v0.02) statistical software (Jahufer & Luo, 2018) will be used for all data analysis. Area under the disease progress curve (AUDPC) will be calculated as per Equation 4.3 and graph plotted using Microsoft Excel.

4.4 Expected results

Our main expectation after completion of this study is to identify cultivars with high level of resistance to sclerotinia clover rot as sources of resistance for breeding purposes. It is unfortunate that the result of this experiment was not presented in this thesis due to time limitation for my study, however, the experiment is still in progress. The experiment stages already completed were; growing of red clover seedlings in glasshouse, *in-vitro* production of sclerotia, breaking of sclerotia dormancy and production of apothecia. Once apothecia are successfully initiated, then we expect to collect ascospores that will be used for artificial inoculation of plants in glasshouse. Arrangements were made for the continuation of the experiment until ascospores are collected so that plants are inoculated for final data to be

collected. While conclusion about the findings in this experiment will be presented later after obtaining the final results; this thesis only presented the preliminary results for the experimental stages completed, limitations and suggestions for further studies.

4.4.1 Preliminary results

Although this experiment was still in progress and final results are not yet available, this thesis under sub-headings 4.4.1.1 to 4.4.1.4 only presents the early stages of experiment that were accomplished.

4.4.1.1 Growing of red clover seedlings in glasshouse

The eleven cultivars of red clover were successfully scarified, germinated (Figure 4.4) and grown in nursery in glasshouse for artificial inoculation to take place (Figure 4.5).

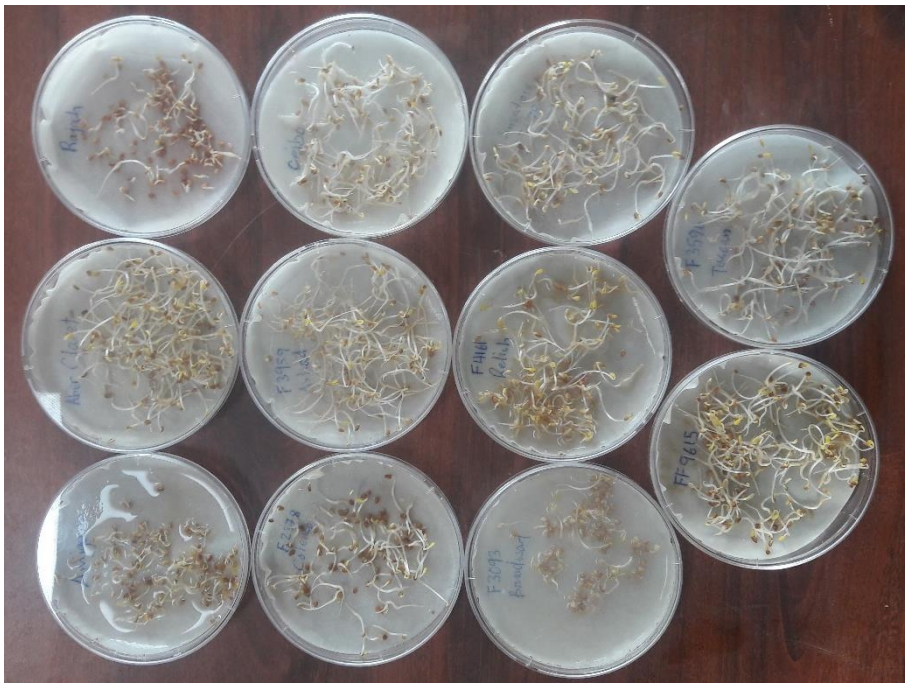


Figure 4.4 Three-days-old germinated seeds of eleven red clover cultivars in petri dishes after seed scarification and incubation at 25 ºC in the incubator.

Figure 4.5 Three months old red clover seedlings raised in nursery pots and laid on the ebb and flow tables in glasshouse in preparation for artificial inoculation with sclerotinia ascospores

4.4.1.2 *In-vitro* production of sclerotia

The *in-vitro* production of sclerotia in potato dextrose agar (PDA) media was achieved without much difficulty. After PDA media was prepared, sclerotia for the two isolates of *Sclerotinia trifoliorum* was cultured in PDA media, new sclerotia were successfully produced after 28 days (Figure 4.6).

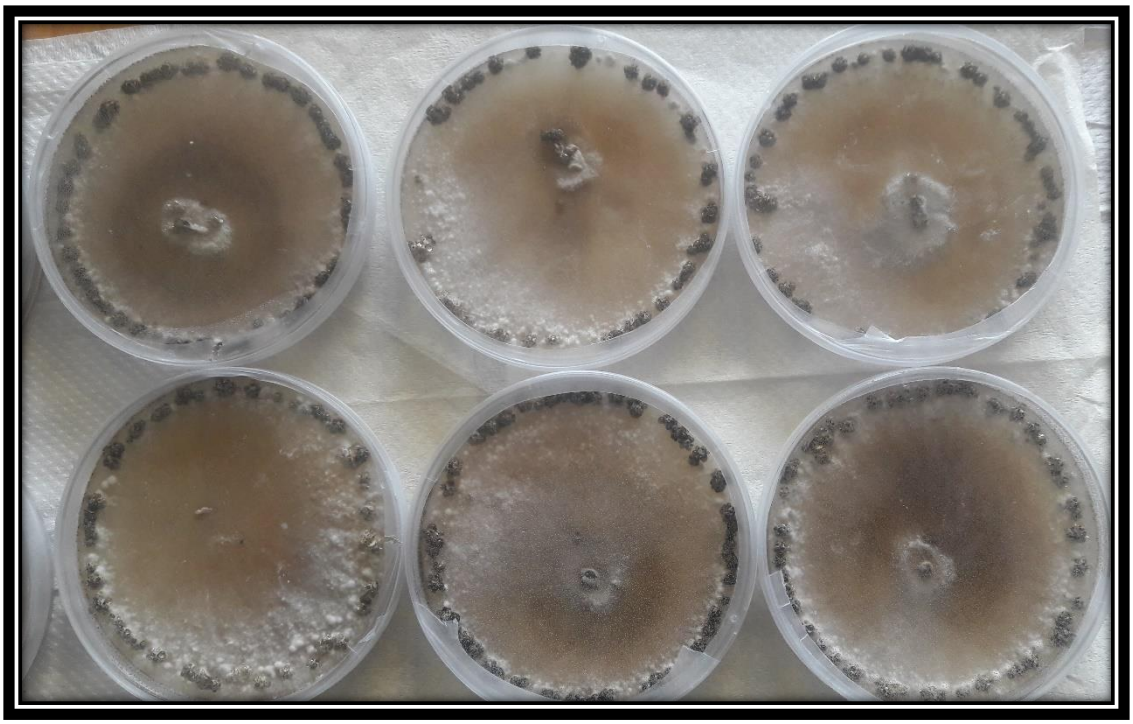


Figure 4.6 Isolates of *Sclerotinia trifoliorum* Erriks. formed sclerotia on PDA media in petri dishes after 28 days incubation in dark at room temperature.

4.4.1.3 Breaking of sclerotia dormancy

After harvesting of sclerotia from the PDA media, the black resting bodies of sclerotia (Figure 4.7) were thoroughly washed with running water and then incubated at 30 ºC for 30 days in the incubator to break their dormancy.

Figure 4.7 Incubation of black resting bodies of sclerotia in the incubator for 30 days at 30 ºC to break their dormancy.

4.4.1.4 Production of apothecia

If sclerotia dormancy is successfully overcome, it is expected that apothecia will be produced and eventually produce ascospores within 30 days given the right condition. In this experiment, the apothecia were successfully produced in the form of stipes after the second

Figure 4.8 A. Apothecia initiated in glass jar filled with wet vermiculite after 30 days in growth chamber at 15 ºC with humidity between 90-100 %. **B.** Stipes, thread-like structures produced after germination of sclerotia. Stipes initiation is the early stage of apothecia before they fully develop to produce ascospores.

attempt at 30 days in the growth chamber (Figure 4.8 A and B). We are expecting apothecia to reach its full growth stage and start to produce ascospores that will be eventually collected for artificial inoculation. However, slow differentiation of stipes leading to the formation of apothecia was the main cause for the delay in this experiment.

4.5 Discussions

4.5.1 Introduction

Although the second experiment on screening for resistance to clover rot among eleven commercial red clover cultivars of New Zealand using artificial disease inoculation in glasshouse was not completed, discussion in this chapter is based on the preliminary results on the experimental stages that were already completed while presenting the proposed methodologies, expected results and some recommendations. The possibility of incorporating source of resistance to sclerotinia in improving existing cultivars and new germplasm understudied in the first experiment is also discussed.

4.5.2 Discussion of preliminary and expected results

Our main expectation after completion of this study is to identify cultivars with high level of resistance to sclerotinia clover rot as sources of resistance for breeding purposes. In order to achieve this expectation, there is need to understand variation in clover rot susceptibility for the selected 11 cultivars under investigation. Moreover, it is important to make sure that resistant cultivars identified through the artificial inoculation technique from this study are indeed resistant so as to improve selection efficiency for clover rot resistance. Although not much is known about the number of genes responsible for sclerotinia resistance, it is thought to be polygenic (Scott, 1984). A recent study by Vleugels and Bockstaele (2013) on a segregated population derived from a cross between resistant and susceptible parents

indicated that there are three major effect genes and perhaps several minor genes controlling resistance to clover rot, thus resistance to clover rot is not a highly quantitative trait. There is no evidence of race specific resistance, but there are reports of differences in the aggressiveness of specific isolates (Vleugels, 2013; Scott, 1984). The multiple gene effect on sclerotinia suggests that it is possible to develop resistant cultivars with horizontal resistance. A study by Vleugels and Bockstaele (2013) concludes that resistance to sclerotinia clover rot was not inherited maternally. This implies that resistant cultivars identified from this study will be used as male parent in crossing program to transfer resistant genes to best susceptible cultivars or germplasm. Selection for clover rot resistant can be achieved with less difficulties at least after three generations as narrow-sense heritability (h^2) for sclerotinia resistance is low (h^2 0.20) to moderate (h^2 0.70) (Delclos & Duc, 1996; Vleugels & Bockstaele, 2013). Heritability depends on red clover ploidy levels, genetically diverse population from different backgrounds and selection pressure.

Apart from selecting the source of resistance to sclerotinia for breeding purpose, we also expect to generate information on susceptibility status of the 11 commercial cultivars. The information generated will set the basis for future research on clover rot resistance breeding in New Zealand. Improved resistant cultivars derived from this selection will at large benefit the New Zealand's pastoral industry and even other countries around the world.

4.5.3 Limitations

The most important limiting factor in this study was the failure in production of apothecia in the first attempt of the experiment. The failure in that stage of the experiment was due to combination of several factors. Unsuccessful fertilization between sclerotia, breaking of

dormancy and conducive culture conditions required to stimulate growth are important considerations in production of apothecia and ascospores.

1. Firstly, induction of apothecia needs to be fertilized depending on the type of sclerotia, which we unknowingly disregarded in our first attempt. There are two mating types in *Sclerotinia trifoliorum*. The + mating type can fertilize itself (self-fertile) and other sclerotia strains called homothallism. The – mating type cannot fertilize itself (self-sterile): sclerotia of this mating type need to be fertilized by a + mating type or interact with other compatible individual for fertilization to take place, which is known as heterothallism. Therefore, it is possible that some sclerotia (when they are the – type and have not been in contact with the + type) simply do not form apothecia. Unfortunately, the source of the Sclerotinia strain that we were dealing with in this experiment lack the information regarding mating compatibility.

2. Secondly, apothecia will only develop if dormancy of the sclerotia is successfully overcome with heat treatment. Although dried sclerotia were incubated at 30°C for 4 weeks, the attempt to produce apothecia was not successful. Previous studies reported that if dormancy is successfully overcome but other conditions are still missing or not sufficient, then the chance of apothecia development is very limited.

3. Even though we managed to solve the problem by successfully breaking the sclerotia dormancy and producing apothecia, another issue we faced was the slow growth of apothecia. Mushroom was not formed after 4 weeks, yet apothecia elongated from the sclerotia and narrow branches (called stipes) appeared (Figure 4.8 A and B). Delay in the differentiation of apothecia indicates that the light conditions during apothecia induction are not optimal. Stipes need sufficient light of the correct spectrum to differentiate into the

mushroom form to eventually produce ascospores. When sclerotia with stipes are brought in suitable light conditions, they can still form mushrooms.

4. To further complicate the issues mentioned above, different methods of producing apothecia and ascospores were reported by different researchers in which reproducibility of each experimental method is uncertain.

4.5.4 Recommendations

1. The problem of unsuccessful fertilization between sclerotia isolates can be overcome by including additional isolate in the experiment. In our second attempt to produce apothecia, we ordered an additional *Sclerotinia trifoliorum* isolate and repeated the entire process of producing sclerotia from two different *Sclerotinia trifoliorum* isolates. The sclerotia from different isolates were brought together to allow them to have contact with each other to facilitate fertilization. This was achieved by keeping the harvested sclerotia for two days in glass containers under water (1 container per isolate) at room temperature and water was changed daily. In order to facilitate fertilization of sclerotia, the old water removed from the two isolates was mixed together in one beaker and re-distributed it over all sclerotia.

2. In order to break the dormancy of sclerotia, we increase the incubation period by additional 7 days at 30°C. Breaking the dormancy works better when sclerotia are in moist conditions, and soil+vermiculite media mix works better than pure vermiculate during the incubation period (T. Vleugels, personal communication, October 08, 2019). In view of that in our second attempt, we followed exactly the above advice and managed to produce

apothecia after exposing the sclerotia in the growth chamber at 15°C for 4 weeks, 12 hours light and 80-100% relative humidity. Eventually, we managed to solve the problem by successfully breaking the sclerotia dormancy and producing apothecia.

3. Apothecia differentiation and maturation can be fast tract by increasing the intensity of light with correct spectrum in growth chamber. Combination of white TL light as well as enough red and blue light especially in Grolux or Fluora TL lights to stimulate growth is required. It is recommended that white TL tubes in the growth chamber needs to be replaced with some TL tubes used to stimulate plant growth, such as Grolux or Fluora.

4. It should be noted that methods of producing apothecia used in this study only seems to work for some isolates (T. Vleugels, personal communication, October 08, 2019). Thus, it is recommended to use a method that works for most isolates or use alternate isolates when one isolate does not form apothecia.

Chapter 5.0 GENERAL DISCUSSIONS

5.1 Introduction

Success in plant breeding depends on several major factors including availability of sufficient genetic diversity, understanding the genetics and inheritance of the species and traits of interest and using of appropriate breeding and selection methods. The initial step in the development of a breeding program is to identify appropriate germplasm that will provide genetic diversity to enhance genetic gain. Characterization of the genotypic diversity among germplasm accessions is important for identification of material to be integrated into breeding programs. The two experiments in this study are related to identification of appropriate germplasm sources with primary traits of interest from local and international collections for breeding purpose. The first experiment was on assessing genotypic variation among introduced red clover germplasm, whilst the second study was on developing *in-vitro* procedures for screening of local cultivars for resistance to *S. trifoliorum.*

5.2 Genotypic variation among red clover germplasm

The first experiment was conducted in field in order to estimate genotypic variation for key morphological traits among 40 red clover germplasm accessions, and identify valuable sources with potential genetic diversity for future breeding programs. Assessments were done on ten morphological traits and subsequently analysed using various univariate and multivariate techniques.

Significant genotypic differences were estimated among the red clover germplasm accessions for all the traits measured across all seasons except for stem number (STN) and plant height (PLH) in summer. This result indicates the magnitude of potential genetic diversity among the germplasm accessions characterized. High genotypic variation and repeatability (R_2^2)

estimated for plant growth (PLG), leaf size (LS), plant height (PLH), shape of medial leaf (SML), medial leaf width (MLW) and medial leaf length (MLL) indicate a high potential additive genetic variation for these traits among the accessions. Higher non-genotypic variances due to season (σ^2_s), accession-by-season (σ^2_{gs}), accession-by-replicate (σ^2_{gb}), and experimental error (σ^2_ε) for intensity of anthocyanin (IAN), area of plant spread (APLS) and shape of medial leaf (SML) indicate potentially large variation due to environmental factors. The effect of season (σ^2_s) was highly significant for the traits PLH, IAN and SML, suggesting the need to evaluate germplasm for several seasons over several years. Positive correlations among some traits in this study could be important for breeders when using methods associated with correlated response to selection or indirect selection methods, particularly for traits with low heritability and difficult to select.

The accessions responded differently to seasonal variation leading to significant genotype-by-season (σ^2_{gs}) interaction effects in all the traits and distinctness among clusters which provides information on germplasm specific adaptation. Despite the differences in seasons, six accession germplasm *viz.* 18, 5, 19, 17, 5 and 28 and the two cultivars, Broadway and Relish, clearly show high and stable visual forage yield across all seasons. High plant growth and forage yield measured in the introduced germplasm accessions indicates their high genetic potential for adaptation to New Zealand conditions. Besides, the six promising entries also exhibit favourable values of other desirable yield related traits which are at par or better than the three commercial cultivars. Further investigations are required in to other aspects of these promising entries including vegetative persistence under grazed and mixed pasture swards, resistance to major pests and diseases, seed production ability and plant secondary metabolites such as the phytoestrogenic compounds and polyphenol oxidase. Actually, these are important issues currently surrounding the red clover production in New

Zealand (Ford and Barrett, 2011). Utilization of international germplasm has so far made positive improvement in some of the recent cultivars. Identification of these entries represents the valuable source of genetic diversity for further development of superior red clover cultivars for New Zealand conditions.

5.3 Screening for *S. trifoliorum* resistance

Vegetative persistence in red clover grown in New Zealand is affected by several biotic and abiotic factors including clover rot disease caused by *S. trifoliorum*. Clover rot is among the many known diseases of red clover in NZ, but its incidence is moderately low and varies from time to time depending on weather condition. Since there is evidence of clover rot incidence reported in several red clover fields across NZ, there is possibility of disease epidemic as red clover production further intensifies under changing and favourable climatic conditions. Hence, the second experiment was focused on assessing the susceptibility level of clover rot caused by *S. trifoliorum* among eleven selected NZ commercial cultivars with the aim of identifying sources of resistance for further breeding. Progress on breeding for resistance to clover rot worldwide is slow due to lack of resistant germplasm and screening under variable field condition. This experiment used *in-vitro* procedures to culture *S. trifoliorum* in producing sclerotinia ascospores for artificial inoculation treatment in selected red clover cultivars.

Two isolates of *S. trifoliorum* were sourced locally and cultured *in-vitro* on PDA media in the laboratory. Sclerotia were successfully produced without any difficulty, but the effort to produce apothecia and ascospores failed due to failure in overcoming dormancy in sclerotia. Thus, certain laboratory procedures were revised particularly on culture condition and process of breaking sclerotia dormancy. The repeat of these procedures was successful in

resolving dormancy issue in sclerotia which gave rise to numerous thread-like structures called stipes. Stipe is the immature stage of apothecia that later matures and produce ascospores, especially under increased light intensity and right culture condition. Even though sclerotia had germinated into several stipes, slow differentiation in its development to form apothecia was experienced, likely due to inadequate light quality in growth chamber. As a result of delay experienced in these procedures, artificial inoculation and screening of plants in glasshouse was not possible.

The difficulty in *in-vitro* culturing of *S. trifoliorum* to successfully produce ascospores for plant inoculation depends very much on culture condition as *S. trifoliorum* is highly sensitivity to environmental condition (Vleugels, 2013). Due to high pathogen-environment interaction, it is obvious that production of ascospores can only be possible if suitable culture condition is created at various stages of *S. trifoliorum* life cycle. However, differences in protocols for *in-vitro* production of ascospores were also reported by various researchers in which assurance for reproducibility of this experiment is not always guaranteed.

If all went well with this experiment, we expect to collect *S. trifoliorum* ascospores and inoculate red clover for screening under controlled glasshouse condition. Cultivars with low disease incidence will be considered as resistant to clover rot disease to form the source of resistance in further breeding purposes. Similar studies to identify possible sources of resistance in cultivars, landraces and wild ecotypes were done by several researchers including Jovita *et al*., (2015); Vleugels *et al*., (2013); Öhberg *et al*., (2008); Öhberg *et al*., (2005); Dabkevičienė & Dabkevičius, (2005); and Ortega *et al*., (1997). However, red clover germplasm under these studies significantly differed in susceptibility level without completely resistant to clover rot. Sources of resistance using accessions and wild ecotypes

may not be easily achieved due to several backcrosses required in eliminating the unwanted characters associated with resistance. Consequently, breeding for clover rot resistance incorporating sources of resistance identified in this experiment can effectively be done by inter-crossing resistant plants and superior cultivars with low susceptibility using top-cross breeding method. This limitation can further be addressed by using other methods of breeding such as ploidy manipulation and various molecular techniques.

5.4 Conclusions

Generally, it can be concluded that identification of diverse, adapted and superior red clover germplasm from the international collection in the first experiment is vital for red clover breeding in New Zealand. In this study, identifying distinct germplasm accessions with the genetic potential to contribute towards developing new and novel breeding populations to be used in the improvement of red clover seasonal growth and vegetative persistence, particularly for New Zealand conditions, is a significant outcome. Although the second experiment was inconclusive, it is equally important to identify resistance source to *S. trifoliorum* from adapted or introduced germplasm for development of resistance cultivars to prevent potential future disease outbreaks in New Zealand as red clover further intensifies.

5.5 Recommendations

The first experiment successfully identified six promising germplasm accessions with diverse background for future breeding. However, there is more to investigate about these germplasm accessions particularly on important issues affecting red clover production in New Zealand such as vegetative persistence, biotic and abiotic stresses, quality and nutrition. Likewise, there is need also for repeating this experiment to generate additional information on actual forage yield, dry matter and seed yield. It is recommended to further evaluate the six adapted

accessions across multiple locations in New Zealand over successive seasons and years to allow for more reliable estimates of variance components, repeatability, genotypic correlation among traits and genotypic performance.

The *in-vitro* culturing of *S. trifoliorum* for production of ascospores in the second experiment depends very much on culture condition as well as pathogen-environment interaction at various stages of pathogen's life cycle. Therefore, there is a need to fully understand the *S. trifoliorum* life cycle and its interaction with environment and reproductive systems of the pathogen to minimize similar issues in future. It is also important that future screening to determine red clover resistance to *S. trifoliorum* be done under both glasshouse and field condition since plant-pathogen interaction and disease incidence varies under these two different conditions. As in greenhouses plants tend to appear more susceptible than in the field, the results from the greenhouse tests may not always be representative for what may be expected in the field. Therefore, field assessment alongside glasshouse screening complements each other in identifying reliable sources of resistance.

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ABBREVIATIONS

2n	diploid
μmol/m²	micro moles per square meter
μm	micro meter
μL	microliter
σ^2_g	genotypic variance
σ^2_{gs}	genotype-by-season interaction
σ^2_{gb}	genotype-by-replication interaction
σ^2_{sb}	season-by-replication interaction
σ^2_{ε}	experimental error variance
σ^2_s	seasonal variances
AMV	alfalfa mosaic virus
ANOVA	analysis of variance
APLS	area of plant spread
AUDPC	area under the disease progress curve
BLUP	best linear unbiased predictor
BYMV	bean yellow mosaic virus
CEC	cation exchange capacity
cmol+/kg	centimoles of positive charge per kilogram
CO^2	carbon dioxide
CRD	completely randomized design
cv	cultivar
CYVV	clover yellow vein potyvirus
DAP	days after planting
DI	Disease incidence
DNA	deoxyribonucleic acid
DSI	disease severity index
ECV%	error coefficient of variation percentage
EURISCO	European Internet Search Catalogue
F pr	probability value associated with F statistics
GEI	genotype-by-environment interaction

GRIN	germplasm resources information network
IAN	intensity of anthocyanin pigmentation on stem and leaf
IWC	intensity of white color on leaf
LC	leaf color
LS	leaf size
LSD	least significant difference
MAF QT	Ministry of Agriculture and Fisheries Quicktest
Mb	megabases
mg/kg	milligram per kilogram
mg/l	milligram per litre
MLL	medial leaf length
MLW	medial leaf width
N	nitrogen
NIWA	National Institute of Water and Atmosphere
NPGS	National Plant Germplasm System
n_b	number of replications
n_s	number of seasons
NZ	New Zealand
PC	principal component
PCA	principal components analysis
PDA	potato dextrose agar
PGH	plant growth habit
PLG	plant growth for visually assessed forage yield
PLH	plant height
PLS	plant survival
PStrV	pea streak carlovirus
PSV	peanut stunt cucumovirus
QDR	quantitative disease resistance
R^2	repeatability
R_1^2	repeatability for each season
R_2^2	repeatability across season

r	Pearson’s correlation coefficient
RCVMV	red clover vein mosaic carlovirus
REML	residual maximum likelihood
r_p	Phenotypic correlation coefficients
SE	standard errors
SINGER	system-wide information network for genetic resources
SML	shape of medial leaflet
STN	stem number
St. Dev	standard deviation
TFF	time to first flowering
TFL	tendency to flower
TL	tube luminescent or luorescent light
USDA	United States Department of Agriculture
WCMV	white clover mosaic potyvirus

APPENDICES

Appendix 1. Geographical information of 40 red clover germplasm accessions in the Margot Forde Forage Germplasm Centre used in the field trial to assess their genetic diversity and adaptation to New Zealand conditions

Code	Accesion No.	Country of origin	Collection Latitude	Collection Longitude	Altitude (m)
1	1_3857	Armenia	39.4058	46.3889	1389
12	2_3860	Armenia	40.1508	44.7933	1833
23	3_3862	Armenia	39.7278	45.3961	1153
34	4_3885	Armenia	40.4533	44.7219	1798
36	5_3895	Armenia	39.6342	45.9025	2090
37	6_3898	Armenia	39.2481	46.4775	1066
38	7_3470	Azberbaijan	40.7833	46.1	
39	8_3473	Azberbaijan	41.2667	48.8667	146
40	9_3474	Azberbaijan	41.1667	48.6	802
2	10_3476	Azberbaijan	40.6833	48.6333	1089
3	11_2465	Caucasus	42.475826	44.478515	
4	12_3269	Caucasus	41.372423	48.53876	
5	13_3270	Caucasus	40.636144	48.60992	
6	14_4088	Caucasus	44.8886	41.999	310
7	15_4095	Caucasus	43.6694	43.4892	498
8	16_4097	Caucasus	43.3731	43.1683	1084
9	17_4077	Greece	40.8966	21.8168	1814
10	18_4079	Greece	41.4111	24.67	1475
11	19_4080	Greece	41.315	22.9905	925
13	20_4084	Greece			
14	21_4085	Greece	40.7447	21.1985	1020
15	22_4087	Greece	41.3105	23.0472	175
16	23_2496	Portugal	41.822953	-6.75451	
17	24_2498	Portugal	41.641566	-8.439756	
18	25_3511	Portugal	37.1467	-7.89417	
19	26_3513	Portugal	37.3433	-8.55667	
20	27_3514	Portugal	37.5733	-8.48639	
21	28_3592	Portugal	41.7728	-7.76167	
22	29_2507	Spain	43.666886	-7.604921	
24	30_2538	Spain	43.436632	-7.382728	
25	31_2647	Spain	43.397061	-8.158808	
26	32_3590	Spain	42.6236	-3.72745	998
27	33_3591	Spain	43.0838	-1.6075	467
28	34_3594	Spain	43.2328	-7.7297	424
29	35_3935	Tajikistan	37.3856	71.6603	2949
30	36_3941	Tajikistan	37.9906	71.7036	2299
31	37_3942	Tajikistan	38.7881	70.2952	1484
32	38_3945	Tajikistan	38.7692	69.8492	1218
33	39_3947	Tajikistan	38.5822	69.395	1378
35	40_3949	Tajikistan	37.4101	71.4918	2844

Appendix 2. Red clover germplasm grouped into different clusters with respective means for various traits and country of origin under winter assessment.

Cluster	Accession No.	Country of origin	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
1	11_2465	Caucasus	1.74	8.00	2.44	7.78	0.87	7.48	7.76	0.90	0.01	2.98
1	16_4097	Caucasus	1.01	8.99	1.08	5.94	0.50	6.84	5.95	1.03	0.00	2.95
1	17_4077	Greece	2.84	6.99	2.49	11.72	1.19	8.64	7.68	0.90	0.02	2.97
1	18_4079	Greece	1.73	8.03	1.97	10.53	0.87	6.81	6.90	0.99	0.01	2.99
1	35_3935	Tajikistan	2.02	7.98	2.06	6.40	1.19	8.15	7.44	1.60	0.01	2.92
1	5_3895	Armenia	2.05	8.01	2.01	5.46	0.82	7.94	7.39	1.05	0.01	3.07
2	1_3857	Armenia	3.80	8.02	5.34	8.65	2.93	12.59	11.09	1.15	0.02	3.04
2	19_4080	Greece	2.85	9.01	3.03	9.03	1.32	9.50	10.25	1.48	0.02	2.97
2	20_4084	Greece	3.76	8.03	4.03	11.83	1.94	11.45	10.25	1.98	0.03	2.98
2	21_4085	Greece	2.80	8.03	2.57	10.41	1.19	9.06	8.60	2.00	0.02	3.11
2	32_3590	Spain	3.83	8.99	4.44	11.45	1.51	11.70	10.73	2.04	0.03	3.00
2	36_3941	Tajikistan	2.52	8.99	2.88	6.50	1.25	9.39	8.92	1.14	0.01	2.98
2	37_3942	Tajikistan	3.27	8.01	4.55	8.65	2.13	13.18	14.97	1.06	0.04	2.53
2	38_3945	Tajikistan	3.72	7.99	4.54	9.89	2.38	11.48	12.85	1.40	0.02	3.06
2	39_3947	Tajikistan	2.89	7.98	3.96	8.50	2.13	11.48	11.30	0.89	0.02	2.97
2	40_3949	Tajikistan	3.00	9.00	3.39	9.20	1.39	10.96	10.33	1.03	0.02	2.49
3	15_4095	Caucasus	2.65	9.03	3.81	11.53	2.12	9.29	9.77	0.96	0.03	1.93
3	22_4087	Greece	2.77	8.99	3.25	10.73	1.87	9.59	11.54	2.12	0.02	1.90
4	14_4088	Caucasus	4.03	6.99	4.54	15.13	3.19	10.78	11.75	2.57	0.04	2.50
4	28_3592	Portugal	5.05	7.99	5.59	11.34	2.76	13.93	14.85	1.04	0.05	1.99
4	3_3862	Armenia	5.23	7.99	5.84	11.52	3.19	16.53	17.57	1.53	0.06	3.07
4	4_3885	Armenia	4.81	7.00	5.80	12.26	3.19	16.50	15.67	1.89	0.06	2.63
4	6_3898	Armenia	4.99	7.02	5.58	12.81	2.81	15.65	15.34	1.65	0.04	2.94
4	8_3473	Azberbaijan	4.39	7.02	6.25	11.29	3.88	12.50	14.00	1.69	0.04	1.89
4	9_3474	Azberbaijan	4.28	7.99	5.06	14.87	3.37	13.34	14.38	1.45	0.04	3.01
5	29_2507	Spain	6.98	8.99	4.76	14.95	4.63	11.93	12.42	2.90	0.10	3.04
5	30_2538	Spain	6.68	8.99	4.88	15.44	3.43	12.85	13.17	2.43	0.08	3.03
6	10_3476	Azberbaijan	6.09	7.98	6.13	15.74	3.69	15.83	16.36	2.06	0.06	2.58
6	12_3269	Caucasus	4.94	8.01	5.88	16.03	3.00	15.03	14.72	2.59	0.06	1.99
6	2_3860	Armenia	4.12	9.00	5.10	11.70	2.75	13.44	16.64	2.12	0.04	2.46
6	23_2496	Portugal	5.88	8.98	5.48	11.99	2.81	14.99	17.34	2.81	0.09	1.54
6	26_3513	Portugal	7.53	8.98	7.04	14.16	3.88	18.84	20.40	2.58	0.07	2.49
6	27_3514	Portugal	5.24	8.98	5.77	14.58	2.25	13.72	15.83	1.91	0.05	2.54
6	33_3591	Spain	5.57	8.00	5.72	16.62	3.50	13.43	15.68	1.49	0.06	1.93
6	7_3470	Azberbaijan	5.57	8.00	6.58	15.90	3.44	16.38	18.53	1.85	0.07	1.97
6	Broadway	NZ	7.03	8.33	6.37	15.09	4.21	17.51	18.92	1.35	0.08	2.48
7	13_3270	Caucasus	7.00	8.99	6.68	13.23	4.56	18.65	20.15	1.86	0.16	3.03
7	24_2498	Portugal	7.36	9.00	5.58	13.93	4.81	16.08	18.22	2.02	0.15	2.05
7	31_2647	Spain	8.50	7.99	7.06	15.40	5.50	17.12	21.74	1.89	0.11	2.53
8	25_3511	Portugal	8.32	5.99	6.73	16.62	6.81	20.45	24.17	1.50	0.10	2.43
8	34_3594	Spain	6.21	7.02	6.93	12.14	5.32	18.93	24.07	2.03	0.07	2.52
8	Relish	NZ	6.63	7.00	6.98	15.67	4.63	16.89	19.48	1.85	0.07	2.25
8	Sensation	NZ	6.14	7.33	6.56	12.70	5.50	18.25	21.24	2.00	0.07	2.20

Appendix 3. Red clover germplasm grouped into different clusters with respective means for various traits and country of origin under spring assessment.

Cluster	Accession No.	Country of origin	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
1	10_3476	Azberbaijan	8.76	6.50	5.82	24.90	12.54	23.03	31.30	2.00	0.24	2.01
1	12_3269	Caucasus	8.38	8.78	6.23	20.79	9.29	23.29	33.55	3.14	0.17	2.01
1	13_3270	Caucasus	8.80	7.23	5.98	33.00	15.47	27.17	34.81	1.94	0.37	2.01
1	2_3860	Armenia	8.11	7.18	6.32	24.75	10.15	21.80	29.13	2.02	0.16	2.01
1	23_2496	Portugal	8.71	9.21	5.97	19.93	9.84	21.57	31.41	2.49	0.31	2.01
1	24_2498	Portugal	8.97	7.13	6.22	22.81	12.82	24.51	33.87	2.07	0.11	2.01
1	25_3511	Portugal	8.99	5.19	6.95	25.62	15.49	31.81	43.06	1.63	0.21	2.01
1	26_3513	Portugal	8.85	7.68	6.48	33.69	12.29	25.66	33.30	1.84	0.36	2.01
1	27_3514	Portugal	8.47	7.87	5.00	44.95	10.50	20.66	28.57	1.27	0.35	1.16
1	28_3592	Portugal	8.51	7.28	5.75	26.32	9.25	22.44	29.57	1.47	0.12	2.01
1	3_3862	Armenia	8.43	6.93	5.98	26.42	10.88	22.79	34.37	1.44	0.20	2.01
1	31_2647	Spain	8.67	7.20	6.24	22.48	11.84	25.37	34.69	1.88	0.18	2.01
1	33_3591	Spain	8.87	6.83	6.00	43.34	10.93	20.53	33.76	1.38	0.27	1.16
1	34_3594	Spain	8.62	5.13	6.73	32.06	16.62	25.21	36.22	2.10	0.12	2.01
1	38_3945	Tajikistan	7.76	6.92	5.30	20.15	10.43	21.69	29.06	2.02	0.11	2.01
1	4_3885	Armenia	8.45	7.25	6.19	21.27	9.79	24.81	29.14	2.18	0.21	2.01
1	7_3470	Azberbaijan	8.25	9.11	6.31	38.02	11.21	26.10	37.17	1.86	0.24	2.01
1	8_3473	Azberbaijan	8.81	6.80	6.31	23.62	11.12	25.46	32.87	1.45	0.16	2.01
1	9_3474	Azberbaijan	8.08	8.08	5.26	28.48	9.90	22.05	26.60	2.00	0.17	2.01
1	Broadway	NZ	8.60	6.81	6.18	31.98	12.09	23.87	30.12	0.93	0.19	2.03
1	Relish	NZ	8.56	5.68	6.18	33.20	12.99	24.71	33.07	1.81	0.24	2.03
1	Sensation	NZ	8.26	5.61	6.36	28.14	13.68	25.03	34.32	1.71	0.18	2.03
2	1_3857	Armenia	7.52	8.29	5.73	18.97	11.35	25.40	28.72	1.99	0.13	2.87
2	14_4088	Caucasus	7.26	7.93	5.49	20.26	8.09	20.27	27.42	1.94	0.11	1.59
2	15_4095	Caucasus	7.03	8.58	4.98	23.54	7.17	19.66	25.09	0.91	0.20	2.01
2	20_4084	Greece	6.10	7.88	4.72	21.70	7.48	19.77	20.77	2.54	0.09	2.87
2	29_2507	Spain	7.87	8.85	4.48	47.85	7.65	17.19	18.56	3.10	0.11	2.87
2	30_2538	Spain	8.23	8.83	4.96	40.12	7.77	17.15	21.68	2.66	0.19	2.87
2	32_3590	Spain	6.84	8.31	5.47	19.66	7.67	20.88	21.80	1.94	0.15	2.87
2	35_3935	Tajikistan	5.86	7.74	4.97	13.20	6.04	18.49	20.61	0.89	0.07	2.87
2	36_3941	Tajikistan	6.41	7.49	5.29	13.51	6.57	20.02	24.14	0.92	0.11	2.44
2	37_3942	Tajikistan	6.46	9.06	5.49	13.00	6.49	20.83	26.45	1.23	0.07	2.87
2	39_3947	Tajikistan	6.16	7.19	5.47	12.47	6.99	21.28	24.11	0.98	0.18	2.87
2	40_3949	Tajikistan	6.28	8.29	5.50	16.73	6.86	21.64	24.95	1.97	0.10	2.01
2	6_3898	Armenia	7.88	8.34	5.99	38.15	8.19	22.56	25.55	1.20	0.28	2.44
3	11_2465	Caucasus	3.99	8.67	2.99	11.21	4.53	15.57	17.68	0.88	0.05	2.87
3	16_4097	Caucasus	3.41	6.48	3.60	11.89	3.99	14.92	16.18	1.06	0.04	2.87
3	17_4077	Greece	4.32	7.90	3.51	18.56	5.61	13.55	13.46	1.70	0.06	2.87
3	18_4079	Greece	2.97	9.86	1.99	20.12	3.89	11.08	12.57	1.67	0.09	2.44
3	19_4080	Greece	5.90	9.23	4.24	17.44	4.39	17.24	19.57	1.60	0.06	2.87
3	21_4085	Greece	5.06	7.44	3.52	18.28	4.16	15.75	15.09	2.08	0.07	2.87
3	22_4087	Greece	5.83	9.17	3.55	23.23	4.94	15.48	20.08	1.46	0.18	2.87
3	5_3895	Armenia	4.34	9.16	3.98	12.25	4.98	17.55	19.04	1.92	0.04	2.87

Appendix 4. Red clover germplasm grouped into different clusters with respective means for various traits and country of origin under summer assessment.

Cluster	Line	Country of origin	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML	TFL	FLC
1	10_3476	Azerbaijan	8.3	6.9	4.6	37.0	32.9	18.8	29.4	1.5	0.3	1.5	6.5	3.5
1	16_4097	Caucasus	4.5	6.8	3.8	15.7	25.9	20.4	30.9	2.4	0.2	1.5	3.5	2.5
1	17_4077	Greece	6.0	7.0	4.3	61.0	40.6	19.7	32.9	3.2	0.3	1.1	5.1	2.0
1	18_4079	Greece	4.5	7.1	4.0	58.1	23.6	15.6	24.1	2.1	0.3	1.1	4.5	3.0
1	19_4080	Greece	5.0	7.9	4.6	35.2	31.9	19.5	36.1	2.1	0.2	1.1	3.1	2.0
1	20_4084	Greece	5.5	5.1	4.3	29.0	39.9	17.3	31.6	3.3	0.4	1.4	3.8	2.5
1	21_4085	Greece	5.3	6.0	4.0	23.1	35.3	15.1	31.2	2.3	0.5	1.5	3.4	3.0
1	22_4087	Greece	5.0	7.9	4.5	40.3	20.1	15.7	34.8	1.9	0.4	0.9	4.0	2.5
1	29_2507	Spain	6.8	8.0	4.0	52.1	26.8	16.3	26.4	2.6	0.4	2.6	3.4	3.0
1	30_2538	Spain	7.0	6.9	4.6	51.6	30.4	15.5	23.2	2.2	0.3	1.6	4.9	4.5
2	1_3857	Armenia	8.3	5.0	5.5	28.9	36.4	21.9	34.8	2.1	0.5	1.1	6.0	3.0
2	11_2465	Caucasus	6.5	6.0	5.1	39.4	38.4	17.8	29.7	2.5	0.5	1.0	5.3	3.2
2	13_3270	Caucasus	8.3	5.9	4.9	41.1	33.3	19.6	34.0	2.5	0.5	1.1	7.0	2.5
2	2_3860	Armenia	9.0	4.9	4.7	41.5	34.5	22.3	44.6	2.0	0.6	0.9	6.6	3.0
2	27_3514	Portugal	8.8	6.1	3.3	40.2	37.0	14.9	30.6	2.3	0.8	1.1	6.3	2.5
2	3_3862	Armenia	9.0	5.1	5.7	32.2	46.4	22.7	45.8	2.2	0.7	1.0	4.6	3.0
2	33_3591	Spain	8.5	6.1	3.6	46.5	36.6	20.9	38.1	2.7	0.4	0.9	7.1	3.0
2	36_3941	Tajikistan	8.3	3.9	5.0	34.5	47.0	21.9	41.3	2.2	0.8	1.1	6.5	2.3
2	38_3945	Tajikistan	8.5	4.0	5.1	33.8	40.4	19.9	35.0	1.9	0.5	0.9	7.0	3.0
2	4_3885	Armenia	9.0	5.0	6.0	31.8	42.3	21.4	40.3	2.1	0.5	0.9	6.4	3.0
2	7_3470	Azerbaijan	8.0	6.0	4.7	49.0	41.6	17.1	38.6	2.5	0.7	0.8	6.1	3.0
2	9_3474	Azerbaijan	8.3	6.1	4.4	51.8	32.1	19.7	31.0	2.3	0.4	1.1	6.8	2.5
3	12_3269	Caucasus	8.0	6.0	4.9	33.0	37.0	20.9	39.1	1.9	0.5	2.0	6.2	3.0
3	14_4088	Caucasus	8.3	5.0	6.0	31.3	48.3	23.4	40.3	2.2	0.6	2.0	3.7	2.0
3	15_4095	Caucasus	8.3	6.1	5.9	32.6	38.0	23.0	40.0	1.9	0.4	1.5	3.1	2.0
3	23_2496	Portugal	8.3	5.9	4.9	43.6	44.9	21.0	39.1	2.0	0.6	1.4	5.7	2.5
3	24_2498	Portugal	8.3	5.0	5.0	29.0	41.8	22.6	36.1	2.4	0.4	2.0	6.2	3.5
3	25_3511	Portugal	9.0	3.0	6.8	34.1	50.0	26.0	47.6	1.6	0.4	1.0	4.9	2.0
3	26_3513	Portugal	8.5	7.0	4.6	39.1	43.1	17.6	33.4	1.0	0.9	1.0	5.2	3.0
3	28_3592	Portugal	8.0	6.0	6.3	57.0	32.4	21.3	43.1	1.8	0.5	2.1	4.1	3.0
3	31_2647	Spain	8.8	5.1	5.9	41.8	45.1	19.8	37.0	0.7	0.6	1.4	4.9	2.5
3	32_3590	Spain	7.0	7.0	4.4	26.0	40.8	17.9	35.4	0.7	0.6	1.6	3.6	2.0
3	34_3594	Spain	9.0	3.0	6.8	33.4	47.1	24.2	47.5	2.0	0.4	1.0	4.8	2.5
3	35_3935	Tajikistan	7.0	6.1	5.1	19.5	48.5	20.4	37.6	1.2	0.4	1.5	4.7	2.5
3	37_3942	Tajikistan	8.0	5.0	5.2	27.7	41.0	22.6	40.3	1.6	0.5	1.0	6.4	2.0
3	39_3947	Tajikistan	7.5	6.0	5.3	28.8	35.9	23.1	39.1	2.1	0.5	1.6	5.5	2.0
3	40_3949	Tajikistan	7.3	4.9	5.7	23.9	38.3	18.7	38.1	1.6	0.4	1.5	6.5	3.0
3	5_3895	Armenia	6.8	6.1	4.5	28.4	36.5	19.3	34.1	2.1	0.5	1.9	4.8	2.0
3	6_3898	Armenia	8.0	7.1	5.4	39.2	31.5	21.2	37.7	2.1	0.9	1.4	3.5	2.5
3	8_3473	Azerbaijan	9.0	3.0	6.4	22.7	57.6	19.5	37.4	1.2	0.5	1.3	5.1	3.0
3	Broadway	NZ	8.8	5.3	5.9	52.8	45.2	22.5	37.9	1.5	0.5	1.4	4.7	2.7
3	Relish	NZ	8.9	4.0	6.1	53.4	48.0	22.4	41.7	1.1	0.5	1.8	4.7	3.0
3	Sensation	NZ	8.9	3.3	6.7	35.1	49.5	24.5	44.1	1.8	0.5	1.6	4.4	2.8

Appendix 5. Red clover germplasm grouped into different clusters with respective means for various traits and country of origin assessed across seasons.

Cluster	Accession	Country of origin	PLG	PGH	LS	STN	PLH	MLW	MLL	IAN	APLS	SML
1	29_2507	Spain	7.06	8.28	4.62	32.79	15.09	15.54	20.38	2.26	0.22	2.43
1	30_2538	Spain	7.17	7.99	4.89	31.38	15.62	15.6	20.74	2.11	0.21	2.28
2	11_2465	Caucasus	4.37	7.36	3.86	21.78	15.92	14.5	19.94	1.66	0.21	2.19
2	16_4097	Caucasus	3.47	7.43	3.3	16.73	13.83	14.71	19.37	1.68	0.15	2.28
2	17_4077	Greece	4.68	7.25	3.83	27.79	16.38	14.65	19.78	1.87	0.18	2.2
2	18_4079	Greece	3.50	7.94	3.2	27.57	13.43	12.31	17.22	1.7	0.19	2.12
2	19_4080	Greece	4.85	8.32	4.17	22.25	15	15.8	22.74	1.77	0.16	2.2
2	20_4084	Greece	5.31	7.14	4.54	21.9	16.68	16.39	21.87	2.13	0.21	2.27
2	21_4085	Greece	4.69	7.28	3.72	20.28	15.45	14.09	19.96	1.93	0.21	2.28
2	22_4087	Greece	4.77	8.26	3.99	24.83	13.26	14.55	22.34	1.82	0.22	2.05
2	5_3895	Armenia	4.70	7.58	3.84	19.5	15.66	15.43	21.52	1.75	0.2	2.35
3	15_4095	Caucasus	6.02	7.67	4.95	23.34	16.51	17.43	25.15	1.59	0.23	1.97
3	32_3590	Spain	5.95	7.96	4.86	21.16	16.86	17.06	23.38	1.72	0.24	2.28
3	35_3935	Tajikistan	5.16	7.26	4.27	17.76	17.83	16.19	22.48	1.58	0.2	2.27
3	36_3941	Tajikistan	5.84	6.96	4.55	21.18	17.61	17.35	24.93	1.62	0.27	2.14
3	37_3942	Tajikistan	5.99	7.31	5.07	19.47	16.81	18.67	27.22	1.55	0.21	2.13
3	39_3947	Tajikistan	5.64	7.02	4.96	19.92	16.11	18.51	25	1.58	0.24	2.28
3	40_3949	Tajikistan	5.65	7.46	4.9	19.96	16.38	17.18	24.53	1.69	0.19	2.06
4	13_3270	Caucasus	7.86	7.25	5.68	27.07	17.34	21.25	29.19	1.94	0.3	2.06
4	23_2496	Portugal	7.46	7.77	5.4	24.79	18	19.01	28.48	2.09	0.28	1.9
4	26_3513	Portugal	8.05	7.74	5.82	27.67	18.35	20.33	28.26	1.82	0.34	1.97
4	27_3514	Portugal	7.34	7.53	4.78	29.95	16.84	16.7	25.26	1.81	0.32	1.83
4	33_3591	Spain	7.52	7.06	5.05	31.1	17.06	18.12	28.5	1.82	0.24	1.75
4	6_3898	Armenia	6.94	7.39	5.48	27.81	15.73	19.37	26.39	1.72	0.34	2.2
4	7_3470	Azberbaijan	7.21	7.57	5.67	30.07	17.94	19.61	29.98	1.94	0.29	1.89
5	1_3857	Armenia	6.49	7.07	5.46	21.67	16.97	19.81	25.38	1.82	0.22	2.2
5	10_3476	Azberbaijan	7.55	7.17	5.39	25.81	16.76	19.01	25.99	1.87	0.21	2.05
5	12_3269	Caucasus	6.98	7.55	5.57	23.74	16.82	19.48	28.32	2.12	0.24	2.06
5	14_4088	Caucasus	6.52	6.75	5.31	23.32	18.38	18.26	26.24	2.03	0.24	2.05
5	2_3860	Armenia	7.00	7.03	5.32	25.57	16.49	19.21	28.79	1.91	0.25	1.97
5	24_2498	Portugal	7.97	7.05	5.48	23.54	18.37	20.8	28.84	1.96	0.24	2.05
5	28_3592	Portugal	7.11	7.06	5.64	28.86	16	19.09	28.11	1.67	0.23	2.05
5	38_3945	Tajikistan	6.64	6.53	4.98	22.66	17.25	17.67	25.51	1.79	0.24	2.05
5	4_3885	Armenia	7.28	6.58	5.84	22.98	17.73	20.49	27.83	1.89	0.26	1.98
5	9_3474	Azberbaijan	6.85	7.32	4.96	28.7	16.25	18.29	24.67	1.84	0.23	2.06
6	25_3511	Portugal	8.45	5.25	6.51	25.19	20.37	24.74	36.18	1.68	0.23	1.98
6	3_3862	Armenia	7.41	6.82	5.73	24.43	18.54	20.43	31.4	1.75	0.28	2.05
6	31_2647	Spain	8.42	6.83	6.09	25.9	18.83	20.4	30.14	1.71	0.26	2.04
6	34_3594	Spain	7.72	5.57	6.45	25.33	19.73	21.98	33.58	1.9	0.22	1.98
6	8_3473	Azberbaijan	7.28	6.04	5.96	21.3	20.43	19.01	27.44	1.62	0.23	1.97
6	Broadway	NZ	7.96	6.87	5.93	31.69	19.06	20.89	28.74	1.54	0.26	2.01
6	Relish	NZ	7.88	5.81	6.14	32.23	19.89	20.94	30.39	1.69	0.26	2.05
6	Sensation	NZ	7.61	5.71	6.24	25.33	20.36	22.07	32.06	1.82	0.23	2.01