

BREEDING PROGRESS REVIEW ON THE TRIFOLIUM REPENS

Ramona Loredana TOPORAN¹; M. HORABLAGA^{1,2}; I. SAMFIRA¹;

¹University of Life Science „King Mihai I” from Timisoara, Faculty of Agriculture, Calea Aradului, no. 119, Timisoara, Romania

²Lovrin Agricultural Research and Development Station, Romania

Corresponding author: ramo27lore@yahoo.com; ionelsamfira@usvt.ro

Abstract. White clover (*Trifolium repens* L.) is a perennial species, it is part of the Fabaceae family, it grows as a creeping, branched perennial plant. It is widespread in a wide range of climates in the temperate and subtropical regions of the northern and southern hemispheres. Sustainable agricultural practices include the use of white clover or legumes to supply nitrogen to the soil in a crop rotation strategy to limit the use of agrochemicals. The species is frequently used as a cover crop, which serves as manure and is also included in mixtures with other forage plants in cattle feed. Several studies have reported the suppressive effects of white clover on weed and disease pressure or unexplained failure of overseeded grasses in clover-dominated pastures. The polyploidy and genetic diversity of white clover have made it difficult to make rapid progress in genetic and genomic studies. Molecular genetic studies have been carried out for the last quarter of a century, and at the initial stage, segregation, and diversity analyses were performed with restriction fragment length polymorphism (RFLP) markers, random amplified polymorphic DNA (RAPD), and markers by amplified fragment length polymorphism (AFLP). The first linkage map was constructed with 78 SSR and 57 AFLP markers using an F2 mapping population cross between parental inbred lines. A linkage map suggesting homeologous pairing of linkage groups was subsequently reported, which consisted of 493 SSR loci on 16 homeologous linkage groups of 1144 cM.

Keywords: *Trifolium repens*, breeding of the productive traits, genetics features.

INTRODUCTION

Trifolium repens is also known as white clover, creeping clover, honeysuckle clover, or ladino clover. It is native to the Mediterranean Sea and during the early Miocene, 16-23 million years ago, it spread to Western Asia and Europe through animal and human migration. The cultivation of white clover started about 400 years ago, it is currently considered a naturalized plant, it tends to acclimatize in the temperate region of the globe where the annual precipitation is over 750 mm.

The distribution of the species is wide due to its nature of edaphic tolerance, it grows on soils that vary from acidic to calcareous (AHMAD and ZEB, 2020). It grows well where the annual precipitation is in the range of 31–191 cm, the average temperature is between 4.3°–21.8 °C, and the soil pH is between 4.5–8.2 (SPRINGER and AIKEN, 2015). White clover is mainly grown in perennial pastures together with other forage plants, using direct grazing or mechanically harvested in hay and silage. However, flowering always reduces the number of axillary buds growing into branch stolons and leaf production, producing a sharp drop in nutritional value and digestibility.

Therefore, delaying the flowering period and extending the vegetative growth period of *T. repens* will greatly improve yield and forage quality. Moreover, flowering at the right time and well-developed inflorescence are not only an important guarantee for seed production but also increase the opportunity for reseeding in the pasture under unfavourable conditions and increase persistence. Therefore, it is important to study the regulation of flowering time and inflorescence development for future genetic improvement and breeding programs, however, the molecular mechanism remains unknown due to the limited availability of genome resources (MA et al., 2022). Seed yield is an important criterion for determining the

commercial acceptability of new varieties. Often, the seed production capacity of a forage variety is tested only at the final stages of its development, but the most effective strategy would be to select and select plants for seed yield at an early stage of reproduction (JAHUFER and GAWLER, 2000).

Through the use of improved cultivars, *Trifolium repens* has become the most important pasture legume worldwide. The ancestors of the species were diploids that were identified by DNA sequence phylogeny as *T. pallescens* and *T. occidentale*. This is how the hypothesis appears that the white clover appeared as a hybrid between *T. pallescens* and *T. occidentale* (WILLIAMS et. al., 2012). The species has an outcross breeding system and strong gametophytic self-incompatibility, causing high levels of genetic heterozygosity in natural and synthetic populations. However, the *Trifolium repens* species has excellent resistance to cold, drought, trampling, and weed infestation, therefore it is important for the improvement and reproduction of new varieties. It is necessary to construct a high-quality white clover genome to accelerate genetic research and fully utilize the genetic potential in breeding excellent varieties (WANG et. al., 2023). Also, the persistence and stress tolerance of white clover is affected by several viruses, including the alfalfa mosaic virus (LANE et. al., 2000; de LUCAS et. al., 2015).

RESULTS AND DISCUSSION

Genetics and reproduction

The species reproduces vegetatively using stolons that can root at each node and produce a new shoot that remains dormant or can develop into a new stolon or terminal inflorescence. Clones have a high degree of physiological integration, including both acropetal and basipetal translocation of resources between shoots formed along the stolon (Du et. al., 2009). This growing system ensures a high biomass production which together with a strong adaptability makes the average production 4500–6000 kg of green mass per hectare, with a tolerance to mowing and good regeneration (SHANG et al., 2019).

Trifolium repens is a heterotetraploid rich in various nutrients and mineral elements, it has high nutritional, ecological, genetic, and medicinal values. The forage has good palatability for herbivorous animals due to its high carbohydrate and protein content, being used as feed for ruminants in many parts of the world. It has excellent growth when mixed with forages from the *Gramineae* family, and plays an integral role in intensive grazing in terms of animal performance and forage production, thus suggesting its important role in the stable development of the grassland ecosystem.

White clover is a plant capable of both asexual (seed production and dispersal) and vegetative (stolon generation) reproduction. Each plant produces about 10 inflorescences grouped in globular racemes, and each inflorescence has 30-70 florets, which are white with shades of pink and begin to bend slowly after flowering, and four seeds per pod can be produced after pollination. Because *T. repens* is predominantly self-incompatible, cross-pollination is essential for a significant seed set. Pollen is not easily dispersed by wind and any airborne pollen does not result in efficient pollination, pollinating insects are required to transfer pollen between individual clover plants, and bees (*Apis mellifera*) are the most important pollinator of *T. repens* (HOU et al., 2002). The species is considered to be of the perennial cyanogenic type, the polymorphism of cyanogenesis is controlled by the alleles of two independent loci (the Ac locus controls the synthesis of the cyanogenic precursors, linamarin and lotaustralin, from hydrophobic proteinogenic amino acids and the Li locus which controls the production of the enzyme linamarase, responsible for splitting glycosides cyanogenic following mechanical damage to plant tissues).

Variable expression of Ac and Li alleles produces four cyanotypes (AcLi, Acli, acLi, and acli) leading to a substantial range of efficient cyanogenesis among white clover genotypes (BALLHORN and ELIAS, 2014). A large-scale study found that Li exists as a single-copy gene in plants that have linamarase activity, and the absence of enzyme activity in li/li plants correlates with the absence of a large part or all of the gene in the white clover genome (OLSEN et al., 2007).

From a cytogenetic point of view, most species of white clover are diploid, and the number of chromosomes is 16, polyploidy is less frequent. Only 16% of the 248 *Trifolium* species worldwide are polyploid. However, 70% of known polyploids occur in the subgenus *Amoria*, which is considered to be one of the most primitive and unspecialized subgenera.

In 1987, Williams considered *Trifolium* to be an allotetraploid, and in 1999 the allopolyploid origin of white clover was also supported. In 2003, a framework genetic map of white clover was constructed using SSR and AFLP molecular markers (MAJUMDAR et al., 2004). A set of F₂ progeny derived from the crossing of fourth and fifth-generation inbred genotypes carrying a self-fertile (Sf) mutation was used to construct the genetic map. White clover SSR (TRSSR) and AFLP markers were used to obtain a map with 135 markers (78 TRSSR loci and 57 AFLP loci) assigned to 18 linkage groups (LGs) with a total map length of 825 cM.

Substantial segregation bias was observed for both mapped and unmapped loci, with a bias toward heterozygous types and a preponderance of skewed markers on certain LGs. This observation, and the high degree of residual heterozygosity within the inbred parental genotypes, suggests reduced individual fitness due to the loss of heterosis which is a major effect for white clover and which will limit the applicability of F₂ or backcross mapping strategies for this species (JONES et al., 2003). The genetic map of white clover was used to assess the genetic control of agronomically important traits that vary in the F₂(I.4R×I.5J) mapping family. Molecular genetic dissection of agronomic traits in white clover provides the basis for equivalent studies in more complex populations, the design of marker-assisted selection strategies, and comparative genetics with legume model species (COGAN et al., 2006; OLSEN and UNGERER, 2008).

T. nigrescens was shown to be the donor of the Li gene to *T. repens*, as there are at most two active Li alleles in the amphidiploid genome of *T. repens*, making it unlikely that the other parent species contributed an active Li allele (KAKES and CHARDONNENS, 2000). Polymorphism has long been a model system for studying the maintenance of genetic variability. The prevailing model holds that opposing selective forces maintain polymorphism, which involves a cost of defence against cold or drought conditions (HAYDEN and PARKER, 2002).

White clover (*Trifolium repens* L.) has an allotetraploid genome ($2n=4\times=32$) estimated at 1093 Mb. Several linkage maps of various sizes are available; however, no integrated map and marker set have explored the consistency of linkage analysis between unrelated mapping populations. This integrative analysis requires tools for homologous matching between populations, and the development of these tools provides a consistent framework map of the white clover genome and facilitates in silico alignment with the model forage legume, *Medicago truncatula* (ISOBE et al., 2012; GRIFFITHS et al., 2013).

Breeding objectives past and future

Increase in fodder production and adaptability to climate change Genetic control of the growth performance of white clover is of great importance to dairy industries due to increasing soil salinity problems (WANG et al., 2010). The application of molecular markers to

identify quantitative trait loci (QTL) associated with key root traits is expected to improve white clover breeding programs, improve traits such as increased efficiency in phosphorus (P) uptake and tolerance to summer moisture stress (JAHUFER et al., 2006). Under suitable climatic conditions, white clover+grasses mixtures can produce high yields and good forage quality. However, it has been shown that a good water supply is needed for growth. This may become a challenge following climate change, as summer rains are expected to become rare (KÜCHENMEISTER et. al., 2012; BUTNARIU et.al., 2015; CAUNII et.al., 2015).

To increase the genetic variation of white clover, attempts have been made to create interspecific hybrids with 11 related clover species, notably *T. nigrescens*, *T. uniflorum*, *T. occidentale*, *T. pallescens* and *T. ambiguum*. These species range from annuals to hardy perennials, some having adaptations to stressful environments and providing new traits for breeding more hardy white clover cultivars on seasonally dry infertile pastures.

However, at present, only the variety called Aberlasting, derived from interspecific hybridization, has been commercially released, and adequate seed production remains a major obstacle to the successful commercialization of *Trifolium* hybrids (CARADUS et. al., 2023). Interspecific breeding programs in white clover have been carried out for more than 50 years, in many cases involving embryo rescue or egg culture techniques. Their goals were to help understand the evolutionary relationships within the genus and to introduce useful traits, predominantly in white or red clover. The greatest efforts have been made in crossing *Trifolium nigrescens* and *Trifolium ambiguum*, developing new germplasm, and transferring useful traits, although no material has yet been commercialized, and this work has not yet led to varieties. Larger crossbreeding programs in recent years with DNA level comparisons have shed considerable light on the systematics and evolution of the genus, discussing the future of interspecific hybridization for agricultural clover improvement and the role of molecular approaches in this regard (ABBERTON, 2007).

Table 1

Chromosome number, ploidy, reproductive system, and perenniality of *Trifolium repens* (ABBERTON, 2007).

Species	The number of chromosomes	Ploidy	Perennial/annual
<i>Trifolium repens</i>	32	4x	Perennial plant

Cold tolerance is an important trait of white clover, which has been characterized in some morphological, physiological, and genetic breeding research.

However, as a non-model plant, there are few published reports on cold stress or cold tolerance. With the limitation of the allotetraploid genome and high genetic heterozygosity, the molecular mechanisms involved in the response to cold stress are still poorly understood. With the development of high-throughput sequencing technologies, RNA-seq is a cost-effective technology for measuring gene expression profiles in plants, especially non-model plants (ZHANG et. al., 2022).

Genetic relationships between individuals were compared using phenotypic clustering, and those between cultivars were compared using nonmetric multidimensional scaling. Intrapopulation variability exceeded interpopulation variability, with substantial overlap between populations and poor differentiation between populations. The number of parental genotypes used to obtain each cultivar was not a major determinant of genome-wide genetic diversity. The results of the assessment of genetic variation in elite white clover germplasm pools are important in the feasibility of molecular marker-based cultivar discrimination and

will be used to design linkage disequilibrium mapping strategies for marker-trait association (GEORGE et. al., 2006).

Studies on increasing crude protein content

The nutritional value of forage plants depends on their chemical composition, and the main components are protein and crude fibre. Neutral detergent fibre (NDF) and acid detergent fibre (ADF) are the most important indicators to determine the nutritional value of forage plants, as they have a significantly negative relationship with digestibility.

The content of NDF and ADF increases as the plants mature. The stems and shoots of plants contain a higher level of crude fibre than the leaves. The increase in crude fiber content of plants as they grow is due to a decrease in the leaf/stem ratio. The NDF and ADF content is generally lower in legumes than in other types of forage plants, but legumes generally have a higher macronutrient content than other forage plants.

The mineral content of plants varies according to their growth stage, and at maturity, it decreases (KOUTSOUKIS et. al., 2019). A research study of the dry matter of *Trifolium repens* found it to be high in total protein and optimal in crude fibre, and an analysis of the mineral composition revealed optimal levels of phosphorus, magnesium, and sodium, excessive of potassium, calcium, and manganese and a deficiency of copper, zinc, and iron.

It was also confirmed that there is a significant relationship between soil chemical properties and the content of total protein, crude fibre, and mineral components in white clover dry matter (ALBERSKI and Olszewska, 2015). White clover can contain between 168 to 270 g of crude protein kg⁻¹ DM and can fix 57 to 232 kg of nitrogen ha⁻¹, and the best association can be made with forage plants such as perennial ryegrass and sedge (SAMFIRA et.al., 2013; BARBAT, et.al., 2013; PERALTA et.al., 2017).

Some nutritive value data showed that white clover has high nutritive value in the cold season, a progressive decline through spring, and uniformly lower nutritive value in summer. The results of laboratory determinations of the degradability studies and following the digestibility study on white clover harvested at the early flower, full flower, and ripe seed stages were consistent with the results of the nutritive value data set. The onset of maturity during the primary growth phase in spring brought large changes to nutrient values: neutral detergent fibre (NDF) had values ranging from 184 to 301g kg⁻¹ dry matter (DM), nitrogen (N) decreased from 36 to 20 g kg⁻¹ DM, in vitro digestibility decreased from 0.74 to 0.65 and metabolizable protein content decreased from 144 to 67g kg⁻¹ DM from the early flowering stage to the seed stage ripe. These changes in nutritive value were accompanied by a decrease in in vivo digestibility at a rate of 0.0032 d⁻¹ and a 0.2 reduction in voluntary intake (TURNER, 1998).

It has high crude protein content, is widely grown as a legume forage crop, and improves soil fertility by fixing atmospheric nitrogen with the help of symbiotic bacteria (CHU et. al., 2022), however, white clover growth is often severely reduced during summer drought (BALLIZANY et. al., 2012), and productivity and survival are limited in drought and dry conditions (NICHOLS S. N. et. al., 2014). In addition to nitrogen-fixing capacity, white clover is also one of the most palatable legumes used in pastures to improve forage quality (ZHANG et. al., 2007). Secondary metabolites have been suspected to cause these effects, and therefore basic knowledge of the active or passive release of biologically active secondary metabolites from white clover is needed (CARLSEN et. al., 2012).

CONCLUSIONS

The importance of white clover as a pasture legume has led to major genetic advances in ecophysiology research, germplasm conservation, and white clover improvement.

In the current varieties of white clover, the fact that biomass production fluctuates a lot from year to year, especially in dry environments, is a problem and a target for improvement, mostly due to poor survival during summer moisture stress. Factors such as edaphic constraints, intolerance to the competition of forage plants, intensive grazing, and lack of growth during the winter, limit the contribution of white clover to the productive performance of pastures.

White clover breeding programs have sought to increase dry matter yield, improve forage quality and persistence, or a combination of these. Selections for improved drought tolerance have been marginally successful.

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