PhD THESIS

STUDY OF QUALITATIVE AND QUANTITATIVE TRAITS VARIABILITY FROM A RED CLOVER CULTIVARS COLLECTION (*TRIFOLIUM PRATENSE* L.)

(SUMMARY OF THE PhD THESIS)

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INTRODUCTION

Red clover (*Trifolium pratense* L.) is considered one of the main domesticated species within the *Fabaceae* family, having been cultivated since the 3rd century BC as a primary source for the production of high-quality forage and later for use in traditional medicine due to its medicinal properties.

Given its essential role in both animal nutrition and human medicine, red clover breeding programs primarily aim to increase vegetative biomass (Muntean, 2002) and to improve inflorescence production, due to its melliferous and therapeutic value. In addition to its fundamental importance in animal feeding and medicine, red clover also contributes significantly to soil nitrogen enrichment (Watson & Stoddard, 2017; Petrauskas *et al*., 2023), owing to the formation of symbiotic relationships with soil microorganisms (e.g., bacteria of the genus *Rhizobium*) capable of fixing atmospheric nitrogen (Vasiljević *et al*., 2022). Consequently, red clover is distinguished by its considerable importance from agronomic, ecological, and medical perspectives.

THESIS STRUCTURE

The doctoral thesis entitled **"Study on the Variability of Qualitative and Quantitative Traits within a Cultivated Collection of Red Clover (*Trifolium pratense* L.)"** comprises 143 pages and is structured into two main parts, encompassing 14 chapters, 62 figures, 30 tables, and 242 bibliographic references. **The first part** of the thesis provides a synthesis of the current state of knowledge on red clover and is organized into three chapters, covering 28 pages (25.0%). **The second part** of the thesis, dedicated to the author's original contribution, presents the results of experimental research and extends over 84 pages (75.0%), organized into 11 chapters.

1. STATE OF ART:

It includes the first three chapters of the thesis, which present general aspects and origin, biological and genetic characteristics, together with the chemical composition and uses of red clover.

2. PERSONAL CONTRIBUTION:

It comprises the remaining eleven chapters, which present the aim and objectives of the study, the natural research framework, the variability of morphological traits in red clover, its productivity, forage and medicinal quality, the interrelationships between production and quality parameters and PCA analysis, genotypic diversity, and the mycorrhizal activity of this species. This part of the thesis also includes the chapters on conclusions and recommendations, as well as the chapter addressing the originality and innovative contributions of the thesis.

RESEARCH AIM AND OBJECTIVES

The aim of this doctoral thesis was to evaluate the phenotypic and genotypic variability of a collection of red clover (*Trifolium pratense* L.) genotypes, with the objective of identifying potentially valuable parents for breeding programs aimed at developing new cultivars with high productivity, improved forage quality, and enhanced therapeutic potential.

To achieve this aim, the following objectives were established:

1. Phenotypic characterization of the red clover collection at USAMV Cluj-Napoca, including morphological traits, productivity, forage and medicinal quality parameters;
2. Analysis of the interrelationships between production components and quality traits using correlation analysis;
3. The stability of quality traits and production elements study, as well as the identification of cultivars with the best perenniality;
4. Identification of the main directions of variation in production and quality traits through PCA (Principal Component Analysis);
5. Genotypic characterization of the red clover collection at USAMV Cluj-Napoca using SSR molecular markers;
6. Determination of the mycorrhizal activity of red clover.

STUDY 1 – ANALYSIS OF THE MORPHOLOGICAL TRAIT VARIABILITY IN RED CLOVER

Introduction: The morphological characters of red clover are essential for species identification and evaluation, and these traits vary depending on environmental conditions, cultivar, and type of cultivation. Among these characters are: plant height, leaflet dimensions, number of shoots per plant, and number of branches per shoot.

The aim of the study was to evaluate and identify superior red clover cultivars from the perspective of morphological characters, with a view to selecting them for the development of new cultivars with improved traits suitable for cultivation.

Materials and Methods For the analysis of morphological characteristics, 90 red clover cultivars (70 diploid and 20 tetraploid) originating from Romania as well as from other European and American countries were tested over two experimental years (2022 and 2023). Measurements were performed in the field using a measuring tape for: plant height (cm), leaflet length and width (cm), number of shoots per plant, and number of branches per shoot.

Results and Conclusions The analyses revealed that in the second year of vegetation, tetraploid cultivars exhibited greater plant height compared to diploids (e.g., Corvus). However, in the third year, diploids surpassed tetraploids, indicating a progressive decline in the vigor of tetraploids after the second year. Although climatic conditions were favorable in the third year (wetter year), plant height was lower than in the second

year, a phenomenon attributed to plant aging. Leaflet length showed similar values between the two categories, with tetraploids recording higher values in cultivars such as Ilte and Magura, which remained relatively stable across both years. Leaflet width was greater in tetraploids during the second year, with an extreme value observed in the tetraploid cultivar Ilte, but it decreased in the third year compared to diploids. The number of shoots per plant and the number of branches per shoot showed similar values between diploids and tetraploids in the second year; tetraploids presented higher values (e.g., Sadunai). However, in the third year, diploids outperformed tetraploids, again due to the progressive decline in tetraploid vigor. Similar results regarding morphological characters have been reported in other studies, where tetraploids generally exhibited higher values compared to diploids (Muntean, 2008; Vasiljević et al., 2022), confirming the initially superior development of tetraploid cultivars.

Regarding the variability of morphological traits, both diploid and tetraploid cultivars displayed moderate variability for plant height and leaflet width, which allows for the identification of potential parental lines for plant breeding programs.

In conclusion, the morphological analysis demonstrated that, during the second year of vegetation, tetraploid cultivars recorded higher values for plant height, leaflet width, number of shoots per plant, and number of branches per shoot compared to diploid cultivars, due to their initially greater vigor. In contrast, during the third year, diploid cultivars exhibited superior performance for most characters, owing to their better persistence and longevity compared to tetraploids.

STUDY 2 – PRODUCTIVITY OF RED CLOVER

Introduction: Red clover production represents an essential factor in ensuring a sustainable source of animal feed and constitutes a key element in livestock nutrition. Among the most important aspects of production are the quantity of green biomass and hay obtained, as well as the weight of flower heads per plant.

The aim of the study was to evaluate and identify red clover cultivars with high production potential, with the purpose of selecting them as genetic material for the development of new cultivars with superior yields.

Materials and Methods For the assessment of productivity, green vegetative biomass (kg/m^2) was measured in the field using a handheld scale. The values of green vegetative mass were subsequently converted from kg/m^2 to t/ha (1 kg/m^2=10 t/ha). Additionally, red clover flower heads were weighed, with the weight reported in grams per plant. To determine the quantity of dry vegetative mass (hay), a 4:1 ratio between green vegetative mass and dry mass was applied, according to specialized literature (Resmeriță *et al*., 1973), for plants at approximately 50% flowering stage.

Results and Conclusions The analyses revealed that, in the second year of vegetation, tetraploid cultivars recorded higher productivity, expressed through superior values of both green and dry vegetative mass. This was attributed to their genetic structure, which

favored enhanced growth and development processes. In the third year, diploid cultivars outperformed tetraploids, due to the progressive decline in tetraploid vigor. In both years, certain diploid cultivars exhibited extremely low production values: Gandalf in the second year and Marieta in the third year, for both green and dry vegetative mass. Regarding the weight of flower heads per plant, tetraploids showed higher values across both experimental years, while diploids produced inflorescences of smaller size. The higher productivity of tetraploids is also supported by findings reported in the scientific literature (Muntean, 2002; Muntean, 2008; Liatukas & Bukauskaitė, 2012).

Regarding the variability of productivity traits, both diploid and tetraploid cultivars displayed medium to high coefficients of variation. This level of variability is valuable for the selection of parental lines in breeding programs.

In conclusion, during the second year of vegetation characterized by low precipitation and high temperature, tetraploid cultivars achieved superior performance in green and dry vegetative mass as well as in the weight of flower heads per plant. In contrast, during the third year characterized by high precipitation and high temperatures diploid cultivars exhibited higher production of vegetative mass, owing to their better persistence and longevity.

STUDY 3 – FORAGE QUALITY OF RED CLOVER

Introduction: The chemical characteristics of red clover, such as protein content, ADF and NDF, are essential for evaluating its nutritional value and significantly influence the forage quality used in animal feeding.

The aim of the study was to evaluate and identify red clover cultivars exhibiting high protein content and low ADF and NDF values, with the purpose of selecting the most performant parental genotypes for the development of new, improved cultivars.

Materials and Methods: For the assessment of forage quality, plant material was harvested in the second year of vegetation from all 90 cultivars included in the study. Crude protein content was determined using the Kjeldahl method, adapted according to Dhont & Vanden Berghe (2003a), for all cultivars. For a more detailed evaluation of the chemical profile, a second analysis was performed, focusing on two additional parameters: ADF and NDF, determined using the Van Soest method (Dhont & Vanden Berghe, 2003b). Based on the highest protein values, five diploid and five tetraploid cultivars were selected for this supplementary analysis. The crude protein content (% dry matter) was converted into t/ha by multiplying the percentage obtained via the Kjeldahl method by the dry matter yield (t/ha).

Results and Conclusions: Tetraploid red clover cultivars exhibited higher crude protein content compared to diploid cultivars. This difference is attributed to their better-developed photosynthetic apparatus and a more favorable leaf-to-stem ratio, as leaves generally contain substantially higher protein levels than stems. The lowest protein content values were recorded in the cultivars Gandalf (diploid) and Lars (tetraploid), both of Norwegian origin. The trend toward higher protein content in

tetraploids is also supported by findings reported in the scientific literature (Drobná & Jančovič, 2006; Marković et al., 2022). Regarding fiber fractions, diploid cultivars showed higher values of both NDF and ADF compared to tetraploids.

The three key forage quality parameters, protein, ADF, and NDF in tetraploid cultivars displayed high coefficients of variation, indicating a stronger genotype-environment interaction. This aspect is particularly relevant for the identification of parental material in breeding programs. In diploid cultivars, high variability was observed for protein content, and moderate for ADF and NDF.

In conclusion, the analysis of forage quality parameters demonstrated that tetraploids achieved higher protein content and lower ADF and NDF values compared to diploid cultivars. As a result, tetraploids produced forage of superior quality.

STUDY 4 – MEDICINAL QUALITY OF RED CLOVER

Introduction: The chemical characteristics of red clover, particularly the content of flavonoids and total polyphenols, are essential for assessing the medicinal quality of the plant. Due to their antioxidant properties, these compounds contribute to protecting cells against oxidative stress.

The aim of the study was to evaluate and identify red clover cultivars exhibiting superior contents of flavonoids and polyphenols, as well as high antioxidant activity, with the purpose of selecting cultivars with significant therapeutic potential to serve as parental material for the development of new cultivars with improved properties.

Materials and Methods: To determine total flavonoid content, total polyphenol content, and antioxidant activity, all cultivars available in the collection were analyzed using the methods described by Bunea *et al*. (2011), specifically, the colorimetric method for total flavonoid content, ABTS method for antioxidant activity, and Folin-Ciocalteu method for total polyphenol content. For a more detailed evaluation of the chemical profile, HPLC analysis was also performed using the HPLC-DAD-ESI+ method for phenolic compounds. For this detailed analysis, the 20 most performant cultivars (diploid and tetraploid), characterized by high total polyphenol content, were selected.

Results and Conclusions: For total flavonoids and antioxidant activity, diploid cultivars recorded higher values than tetraploid cultivars. Extreme values for flavonoids were observed in the cultivars AberClaret, Callisto (C2), and Radviliai. Among the tetraploids, only the cultivar Bivoj stood out for high antioxidant activity. The higher levels of flavonoids and antioxidant activity in diploids may be associated with greater sensitivity to abiotic stress, leading to the intensification of defense mechanisms against stress factors. In contrast, regarding total polyphenol content, tetraploid cultivars exhibited significantly higher values compared to diploids. This effect is attributed to polyploidy, which can enhance the expression of genes involved in the biosynthesis of phenolic compounds. Among the diploids, the cultivars AberClaret, Diplo, and Callisto (C1) were notable for their high polyphenol content. A study conducted by Mikulić et al. (2023) on

red clover cultivars also revealed significant differences in the content of medicinally relevant substances between the two ploidy levels: tetraploids showed higher polyphenol content, while diploids exhibited elevated antioxidant activity. Regarding the individual phenolic compounds identified in red clover extracts, tetraploids showed higher contents of hydroxybenzoic acids, genistein, kaempferol, and biochanin A. Diploids, on the other hand, showed higher values for quercetin and other flavonols.

Regarding the variability of medicinal quality traits in the studied cultivars, both diploid and tetraploid groups displayed high variability for total polyphenol content, total flavonoid content, and antioxidant activity. This substantial variability is highly valuable for the identification of suitable parental genotypes in plant breeding.

In conclusion, the analysis of medicinal quality parameters revealed that the highest values for antioxidant activity and total flavonoid content were recorded in diploid cultivars. Tetraploid cultivars outperformed diploids only in terms of total polyphenol content.

STUDY 5 – INTERRELATIONSHIPS BETWEEN PRODUCTION AND QUALITY PARAMETERS IN RED CLOVER AND PCA ANALYSIS

Introduction: The production and quality parameters of red clover are key determinants in evaluating the agronomic potential of the species. Their expression is closely linked to environmental conditions, cultivar, and the cultivation technologies applied. The use of multivariate statistical methods, such as correlation analysis and principal component analysis (PCA), enables an integrated description of the interrelationships among morphological, productivity, and quality traits by identifying relevant character combinations. These approaches are valuable for optimizing cultivation strategies and supporting breeding programs in red clover.

The aim of the study was to perform an integrated analysis of the relationships between morphological traits, forage value, and medicinal value in a collection of 90 diploid and tetraploid red clover cultivars. To achieve this goal, the following specific objectives were pursued:

1. highlighting the relationships between plant morphology, productivity traits, and forage and medicinal quality indices;
2. comparing how these relationships manifest depending on ploidy level;
3. identifying cultivars with a favorable combined profile (high production associated with superior quality indicators) that can be used as genitors.

Materials and Methods To determine significant relationships among the studied characteristics at the two ploidy levels, Spearman correlation coefficients were calculated and heatmap visualizations were generated separately for diploids and tetraploids. Principal Component Analysis (PCA) was performed on the following variables: green biomass, dry biomass, flower head weight per plant, crude protein content, total polyphenols, total flavonoids, and antioxidant activity. The analysis was

conducted separately for diploid and tetraploid groups, with results presented using biplot graphics.

Results and Conclusions: Both in diploid and tetraploid cultivars, significant positive correlations were identified among morphological traits, productivity parameters, and forage and medicinal quality traits, with no negative correlations observed. The strongest associations involved green and dry vegetative mass, flower head weight per plant, crude protein content, total polyphenols, leaflet length and width, as well as flavonoids and total polyphenols. The PCA proved to be appropriate (Bartlett's test $p < 0.001$; KMO = 0.648) and showed that the first two principal components were mainly represented by productivity traits (vegetative mass, flower head weight) and polyphenol content. In the PCA performed for forage profile, PC1 (66.69%) separated cultivars mainly according to vegetative biomass, while PC2 (33.31%) was associated with protein content. The lower-left quadrant grouped cultivars with superior forage profiles, among which the diploid cultivar Diadem stood out due to high values for both yield and protein content. In the PCA conducted for medicinal quality, PC1 (57.26%) accounted for variation in polyphenols, flavonoids, and flower head weight, while PC2 (22.93%) was primarily related to antioxidant activity. The lower-left quadrant included cultivars with favorable medicinal profiles, among which the diploid cultivars Diplo and Callisto (C1) were particularly notable.

In conclusion, the positive correlations observed between productivity and forage quality, together with a significant association between vegetative biomass and polyphenol content, indicate that, in general, more productive red clover cultivars also tend to exhibit higher polyphenol levels.

STUDY 6 - GENOTYPIC DIVERSITY OF RED CLOVER

Introduction: Genetic analysis of red clover is essential for evaluating diversity and improving varieties. Due to outcrossing reproduction, populations exhibit high genetic variability. SSR molecular markers are used to characterize diversity, establish kinship, and identify valuable germplasm sources.

The study aimed to analyze the genetic diversity among red clover cultivars using the SSR marker, to identify genetically distinct genotypes useful as parental material in breeding.

Materials and methods: The study included 36 red clover cultivars (18 diploid and 18 tetraploid) originating from various regions of Europe and America, selected for varied values of productivity and forage and medicinal quality. Young leaves were collected from several plants in the field and stored in a freezer until DNA extraction. SSR analysis used eight primer pairs and consisted of DNA isolation, quality evaluation, and genetic analysis of the cultivars. Data were processed to calculate genetic characteristics (number/effective number of alleles; observed/expected heterozygosity; Nei's index; Shannon's index; polymorphic information content and genetic distance). Based on the

matrix of genetic distances (using the UPGMA method) and separately based on the combination of values of three characteristics (vegetal mass, protein content, and total flavonoids), circular dendrograms were generated for both ploidy levels. Results and conclusions: SSR molecular markers showed high genetic diversity in red clover, influenced by the ploidy level. In diploids, most markers were polymorphic, with high Na, Ne, and PIC values; only gtrs171 and TRSSRA02B08 were monomorphic. In tetraploids, Na and Ne were higher, all markers were polymorphic, and Ha, PIC, and Shannon confirmed greater diversity compared to diploids. Cluster analysis grouped diploids into five clusters where Callisto (C2), Sepia, and Diplo had a common genetic background, while Kindia was more isolated. Grouping based on vegetal mass, proteins, and flavonoids also created five phenotypic clusters. In tetraploids, genetic clustering created five groups where Sadunai, Magura, and Lars had a common genetic background, while Ilte was more isolated.

In conclusion, the identified SSR molecular markers were considered adequate for evaluating genetic diversity in both ploidy groups. Cluster analysis highlighted significant genetic variability among the analyzed diploid and tetraploid cultivars, which were grouped into five distinct clusters. Comparing the two types of analyses showed that genetic proximity was not always reflected in the similarity of biochemical characteristics, suggesting the influence of environmental factors and genetic regulatory mechanisms on phenotypic expression.

STUDY 7 – MYCORRHIZAL ACTIVITY OF RED CLOVER

Introduction: The mycorrhizal activity of red clover represents an important aspect of the interaction between the host plant and the mycorrhizal fungi in the soil, significantly influencing its development.

The study aimed to analyze the interactions between red clover cultivars and the types of mycorrhizae present at the root level and to select cultivars with high resistance and efficiency in utilizing soil resources.

Materials and methods: It consists of the roots of 10 diploid and 10 tetraploid cultivars. As selection, the first 5 diploid and tetraploid cultivars with high protein and flavonoid content were chosen, followed by 5 diploid and tetraploid cultivars characterized by low protein and flavonoid content. To determine the presence of mycorrhizae at the root level and mycorrhizal activity, samples were collected from the experimental field in three replications and analyzed using the method described by Stoian *et al*. (2022).

Results and conclusions: The highest values of mycorrhizal intensity, frequency, and degree of colonization were recorded in the tetraploid cultivars. Among these, Lasang, Linus, and Rezista showed the highest values of mycorrhizal parameters, but a lower content of biochemical compounds. In contrast, the Bivoj cultivar stood out for a more efficient use of the mycorrhizal symbiosis, associated with a better nutrient uptake. No significant correlations were found between mycorrhizal activity and productivity and quality traits (vegetative mass, protein content, and flavonoids), regardless of the ploidy.

In conclusion, the results showed that high mycorrhizal activity cannot guarantee superior biochemical performance; the efficiency of the symbiosis also depends on the specific genotype–fungus compatibility, not just on the level of colonization.

RECOMMENDATIONS

For the improvement of forage quality, the diploid cultivars **Diadem, AberChianti,** and **Tășnad**, as well as the tetraploid cultivars **Bivoj, Vesna, and Sigord**, are recommended as parental forms. These cultivars are characterized by high forage biomass yields and superior protein content (t/ha) throughout the experimental years. They were selected due to their high production potential and enhanced nutritional value. Possible crosses among the studied cultivars to obtain high-performing progeny include Diadem × AberChianti (2n) and Bivoj × Sigord (4n).

Regarding the improvement of medicinal quality, the diploid cultivars **AberClaret, Callisto (C2),** and **Diplo**, together with the tetraploid cultivars **Bivoj, Tornado,** and **Vesna**, were identified as valuable parental genotypes. These cultivars are characterized by higher capitulum weight/plant and higher levels of total flavonoids per plant. They were selected as parents due to their high capitulum yield and increased content of bioactive compounds. Possible crosses aimed at obtaining progeny with superior medicinal potential include Callisto (C2) × Diplo (2n) and Bivoj × Vesna (4n).

In conclusion, the obtained results highlight the existence of valuable genetic resources for both forage quality improvement and the enhancement of medicinal potential, providing clear directions for future breeding programs depending on the targeted objective.

INNOVATIVE CONTRIBUTIONS OF THE THESIS

The present research brings original contributions to the field of red clover breeding and valorization through an integrated approach that analyzed the morphological, productive, biochemical, genetic, and mycorrhizal traits of diploid and tetraploid cultivars under specific pedoclimatic conditions.

The originality of the study lies in the comparative evaluation of the two ploidy levels over two years of vegetation, highlighting the superior vigor of tetraploids and the better persistence of diploids.

The results revealed high variability in morphological and productivity traits, relevant for the selection of cultivars adapted to climatic stress conditions. The analysis of forage and medicinal quality demonstrated the superiority of tetraploids for forage use (through protein content and digestibility) and of diploids for medicinal use (through their capacity to accumulate bioactive compounds).

The integration of genetic and mycorrhizal analyses showed that the intensity of mycorrhizal colonization is not directly correlated with biochemical performance,

and genetic proximity does not always reflect functional similarities. Through its multidisciplinary approach, the study provides a solid scientific foundation for modern breeding strategies and differentiated utilization of red clover in sustainable agriculture.

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