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INDICADORES FISIOLÓGICOS DE ADAPTABILIDAD AL DÉFICIT HÍDRICO DE *Lotus corniculatus* L. EN ZONAS ÁRIDAS DEL NORTE DE MÉXICO

PHYSIOLOGICAL INDICATORS OF ADAPTABILITY TO WATER DEFICIT OF *Lotus corniculatus* L. IN ARID AREAS OF NORTHERN MEXICO

THESIS

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Physiological indicators of adaptability to water deficit of *Lotus corniculatus* L. in arid areas of northern Mexico

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ABBREVIATIONS LIST

A	Absorbance
ATPase	F0F1-ATP Synthase
CAM	Crassulaceae acid metabolism
Car	Carotenoids
Chl	Chlorophyll
Chl a	Chlorophyll a
Chl b	Chlorophyll b
Chl t	Chlorophyll total
CT	Condensed Tannin
DAT	Days after transplanting
DPPH	2,2-Diphenyl-1-picrylhudrazyl
EC	Electric Conductivity
EWU	Efficient Water Use
FC	Field Capacity
GAE	Gallic Acid Equivalent
gFW	Gram of fresh weigth
MPa	Megapascal
NWD	Non-Water Deficit
PAR	Photosynthetically active radiation
PASW	Statistical program for Windows
PC1	Principal component 1
PC2	Principal component 2
PCA	Principal Component Analysis
PMP	Permanent Wilting Point
PSI	Photosystem I
PSII	Photosystem II
QE	Quercetin Equivalent
RCI	Relative Chlorophyll index
ROS	Reactive Oxygen species
RSA	Radical Scavenging Activity
RWC	Relative Water Content

SWC	Soil Water Content
T	Temperature
TAA	Total Antioxidant Activity
TEAC	Trolox Equivalent Antioxidant Capacity
TFC	Total Flavonoid Concentration
TH	Hydrolysable Tannin
TPC	Total Phenol Concentration
TSC	Total Saponin Concentration
TTC	Total Tannin Concentration
UV	Ultraviolete
UV-B	Ultraviolete B
WD	Water deficit

DEDICATED TO

To my family for their support and support during these four years of work.

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PHYSIOLOGICAL INDICATORS OF ADAPTABILITY TO WATER DEFICIT OF *Lotus corniculatus* L. IN ARID AREAS OF NORTHERN MEXICO

GENERAL ABSTRACT

This study aims to investigate the impact of water limitation on *Lotus corniculatus* L., focusing on secondary metabolites production and antioxidant activity in response to water deficit (WD) across different seasonal periods. Moreover, this research assessed physiological responses, particularly changes in relative water content and photosynthetic pigments as indicators of plant resilience. Employing a randomized block experimental design with three replications, the study examined two levels of soil water content and five ecotypes arranged in a factorially (2 × 5) to generate ten treatments. Results reveal significant variations among ecotypes under WD, particularly the ecotype 255301, which exhibited elevated concentrations of total phenols, flavonoids, tannins, and radical scavenging activity during winter. Ecotype 255305 demonstrated increased radical scavenging activity and total tannin concentration in both winter and spring under WD. Additionally, ecotype 255301 showed higher total saponin concentration during summer under WD, collectively indicating adaptive responses to mitigate water deficit and extreme temperatures.

Photosynthetic pigments displayed significant effects, particularly total chlorophyll, with notable increases in chlorophyll *a* concentration in ecotype 255301 under WD compared to other ecotypes. Carotenoid concentrations were also influenced by water deficit, showing higher levels in specific ecotypes and the Estanzuela Ganador variety during seasonal periods. Principal component analysis reveals that soil moisture content significantly affects chlorophyll and carotenoid concentrations, with optimal moisture conditions correlating with higher pigment levels.

In conclusion, understanding the physiological response of *L. corniculatus* to water deficit stress in this study underscores insights into its potential as a water-saving forage crop for sustainable agriculture and enhancing food security.

Keywords: drought, soil moisture, plant physiology, forage, biochemical response

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INDICADORES FISIOLÓGICOS DE ADAPTABILIDAD AL DÉFICIT HÍDRICO DE *Lotus corniculatus* L. EN ZONAS ÁRIDAS DEL NORTE DE MÉXICO

RESUMEN GENERAL

El impacto de la limitación de agua en materiales de *Lotus corniculatus* L., fue el objetivo de este estudio, centrándose en la producción de metabolitos secundarios y la actividad antioxidante en respuesta al déficit hídrico (DH) en diferentes períodos estacionales. Además, esta investigación evaluó las respuestas fisiológicas, enfocada en los cambios en el contenido relativo de agua y los pigmentos fotosintéticos como indicadores de la resiliencia de las plantas. El diseño experimental empleado fue en bloques aleatorios con tres repeticiones, el estudio examino dos niveles de contenido de agua en el suelo y cinco ecotipos ordenados de manera factorial (2 × 5) para generar diez tratamientos. Los resultados revelaron variaciones significativas entre los ecotipos bajo DH, particularmente el ecotipo 255301 que exhibió concentraciones elevadas de fenoles totales, flavonoides, taninos y actividad eliminadora de radicales durante el invierno. El ecotipo 255305 demostró una mayor actividad eliminadora de radicales y una concentración total de taninos en invierno y primavera bajo DH. Además, el ecotipo 255301 exhibe una mayor concentración de saponina total durante el verano bajo DH, lo que indica colectivamente respuestas adaptativas para mitigar el déficit de agua y las temperaturas extremas.

Los pigmentos fotosintéticos mostraron efectos significativos, particularmente en la clorofila total, con notables incrementos en la concentración de clorofila *a* en el ecotipo 255301 bajo DH en comparación con otros ecotipos. Las concentraciones de carotenoides también están influenciadas por el déficit hídrico, observándose niveles más altos en ecotipos específicos y en la variedad Estanzuela Ganador durante los períodos estacionales. El análisis de componentes principales revela que el contenido de humedad del suelo afecta significativamente las concentraciones de clorofila y carotenoides, y las condiciones óptimas de humedad se correlacionan con niveles más altos de pigmento.

En conclusión, la importancia de comprender las respuestas fisiológicas de *L. corniculatus* al estrés por déficit hídrico en esta investigación subraya información sobre su potencial como cultivo forrajero que ahorra agua para la agricultura sostenible y la mejora de la seguridad alimentaria.

Palabras clave: sequía, humedad del suelo, fisiología vegetal, forraje, respuesta bioquímica

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CHAPTER I: GENERAL INTRODUCTION

Water, a fundamental element of subsistence, stands at the core of global agricultural systems, profoundly influencing food production and sustainability. Agricultural practices worldwide consume more than 70% of the available freshwater resources, far surpassing the mere 20 liters typically allotted for human consumption (Saha et al., 2022). However, this vital resource is under increasing pressure due to a tripled world population during the 20th century, exacerbating water scarcity issues, particularly in developing regions where approximately 35% of the global population resides (Kramina et al., 2018; Wagoner, 1990; G. Wang et al., 2016). Prolonged droughts, symptomatic of climate change, have inflicted severe damage on vital crops in major agricultural regions, further exacerbating the strain on water resources (Sinclair et al., 2019; Yung et al., 2022). In arid regions, where up to 90% of available water is utilized, it becomes imperative to explore innovative strategies to enhance water use efficiency in agriculture (Flörke et al., 2018).

One such strategy involves the exploration and promotion of alternative crops suitable for arid and semi-arid regions. In Mexico, for instance, approximately 10 million hectares of agricultural land are considered unproductive due to extreme aridity, primarily affecting the northern and northwest regions (Pedroza Sandoval et al., 2014). Extensive livestock farming, reliant on native vegetation and unrestricted grazing, exacerbates environmental degradation and loss of biodiversity in these areas (Hasanuzzaman, 2020; Pérez-Espejo et al., 2016; Sinclair et al., 2019). Introducing alternative forages, such as *Lotus corniculatus* L. (Bird's-foot trefoil), holds promise in mitigating these challenges due to its drought tolerance and adaptability to various soil conditions (Blumenthal & McGraw, 1999; Grant & Small, 1996).

Lotus corniculatus L., a member of the Fabaceae family, boasts extensive biological diversity with over 18,000 species globally (Álvarez-Vázquez et al., 2018). Recognized for its resilience in poorly drained and sandy soils, Bird's-foot trefoil offers high-quality forage suitable for silage production, straw production,

and direct consumption (Carter et al., 1999; Morris et al., 2021; Santacoloma-Varón et al., 2017). However, despite its global significance, there remains a need to enhance its drought and salt tolerance to ensure its productivity and sustainability, particularly in water-limited regions.

Understanding the physiological and genetic mechanisms enabling plants to tolerate water deficit is crucial for designing efficient cropping systems. Plant responses to water stress involve intricate mechanisms, including stomatal regulation, photosynthetic adaptation, and antioxidant defense systems (Farooq et al., 2009; Flexas et al., 2004; Kostopoulou & Karatassiou, 2017). Efficient water use, characterized by the relationship between accumulated plant dry matter and transpired water, serves as a vital parameter for identifying drought-tolerant genotypes and developing plant improvement programs (Balazadeh et al., 2021; Inostroza et al., 2015; Islam et al., 2021).

Moreover, the antioxidant system plays a pivotal role in mitigating oxidative stress induced by water deficit, safeguarding cellular components from damage caused by reactive oxygen species (Talaat, 2019). Exploring non-enzymatic antioxidant systems, such as phenolic compounds, flavonoids, tannins, and saponins, sheds light on their diverse roles in plant defense mechanisms and environmental adaptations (Abderrahmane et al., 2014; Banu & Cathrine, 2015).

Additionally, understanding the impact of water stress on photosynthetic pigments provides valuable insights into plant health and productivity. Chlorophyll and carotenoid concentrations serve as essential indicators of stress events and adaptation strategies in response to biotic and abiotic stressors (Barickman et al., 2019; Croft et al., 2020; Kume et al., 2018; Sheikh et al., 2017). Considering these, this thesis aims to investigate strategies for enhancing water use efficiency and adaptation mechanisms in agriculture, with a particular focus on *Lotus corniculatus*. Through a comprehensive exploration of physiological, genetic, and biochemical mechanisms, this research endeavors to contribute to sustainable agricultural practices in water-limited regions, ensuring food security and environmental resilience in the face of climate change challenges.

1.2 JUSTIFICATION

Water scarcity is a pressing issue globally, particularly in arid and semi-arid regions where agricultural practices are heavily dependent on limited water resources. With over 70% of freshwater being utilized for agricultural purposes, innovative strategies are imperative to enhance water use efficiency and ensure sustainable food production. Introducing alternative crops resilient to drought conditions presents a viable solution to mitigate water scarcity challenges.

Lotus corniculatus, commonly known as Bird' s-Foot trefoil, emerges as a promising alternative forage crop due to its drought tolerance and adaptability to various soil conditions. Exploring and promoting such alternative crops offers a pathway to alleviating the pressure on water resources, especially in regions like Mexico, where extensive agricultural lands remain unproductive due to extreme aridity.

This study underscores the importance of understanding the physiological and genetic mechanisms underlying plant responses to water deficit. By evaluating different ecotypes and varieties of *L. corniculatus* under varying soil water content levels, the research sheds light on the production of secondary metabolites and antioxidant activity in response to water limitation.

The findings reveal significant increases in concentrations of total phenols, flavonoids, tannins, and radical scavenging activity in specific ecotypes of *L. corniculatus* under water deficit conditions during different seasons. These responses demonstrate the plant's adaptive strategies to mitigate the effects of water scarcity and extreme temperatures, thereby enhancing its resilience and productivity in arid environments.

The study's results provide valuable insights into the biochemical mechanisms employed by *L. corniculatus* to cope with water stress, including the role of non-enzymatic antioxidant systems and the impact on photosynthetic pigments. Such knowledge is crucial for developing strategies to improve water use efficiency and enhance crop resilience in water-limited regions, contributing to sustainable

agricultural practices and ensuring food security amidst climate change challenges.

1.3 GENERAL OBJECTIVE

To identify physiological responses of *L. corniculatus* ecotypes and a variety in two soil moisture contents under semi-controlled conditions.

1.3.1 Specific objectives

- To evaluate different ecotypes and one variety of *L. corniculatus* in terms of their ability to produce secondary metabolites with antioxidant activity under water deficit through seasonal times.

- To evaluate the performance of different ecotypes and a variety of *L. corniculatus* by the relative water content, the concentration of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and total chlorophyll), as well as the relative chlorophyll index in response to water deficit during the different seasonal times.

1.4 HYPOTHESIS

Some ecotypes or varieties of *L. corniculatus* present favorable physiological and biochemical changes under water-limiting conditions.

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CHAPTER II: THEORETICAL-CONCEPTUAL FRAMEWORK

2.1 Water

Water is a fundamental element of subsistence. The average amount of water consumed worldwide for agricultural use is more than 70% (Flörke et al., 2018). Water required in agricultural production systems (crop, livestock, and aquaculture) is more significant than the 20 L usually needed for human consumption (Salter, 2017). Freshwater supplies in developing countries that are used to produce food are estimated at between 70 and 90%. However, the volume of water available is directly affected by population increase (Clemensen et al., 2020). The world population tripled during the 20th century, increasing the pressure on water resources, so water withdrawals increased sixfold, generating serious situations in regions where water scarcity was already a problem, where about 35% of the world population resides (Ramirez Barraza et al., 2019).

In recent years, prolonged droughts have been observed to cause severe damage to several vital crops in major producing areas of the world (Escalante-Magaña et al., 2019). Agriculture consumes most of the water, but in arid regions, up to 90% of the available volume is used. This is why it is necessary to look for new strategies to achieve efficient use of water. Currently, genetic engineering seeks to propose solutions where the production of food capable of satisfying the demand of the population with a smaller amount of water is achieved (Muñiz García & Capiati, 2011). However, the actual biodiversity and genetic diversity are a source of alternative crops that withstand drought stress with a better water use efficiency.

2.2 Alternative crops

The agricultural areas considered unproductive in Mexico due to their extreme degree of aridity are close to 10 million hectares (Pedroza Sandoval et al., 2014). The northern and northwest regions are the most affected. Even under this condition, agricultural activity represents one of the main activities that contribute to the economy (Salter, 2017). However, producers face a shortage of food for livestock due to the practice of extensive livestock farming, unrestricted grazing,

without management, and basically dependent on native vegetation. Extensive livestock farming is the leading cause of changes to ecosystems and loss of biodiversity in arid areas (Ramírez-Restrepo et al., 2006). One way to stop environmental deterioration and the failure of biodiversity is through options compatible with our economic activities, such as stabling animals, reducing grazing, and promoting the planting and conservation of alternative forages with new species that are drought tolerant and salinity (Diéguez et al., 2014).

Water stress is one of the main ecological factors that has a negative effect, directly impacting the pastoral ecosystems of extensive livestock farming (Olmos López, 2004). The yield of forage crops is often greatly influenced by drought. The plants that are used as an alternative forage crop are perennial legumes such as white clover (*Trifolium repens* L.), red clover (*T. pretense* L.), strawberry clover (*T. fragiferum*), and bird´s foot trefoil (*Lotus corniculatus* L.)(Álvarez-Vázquez et al., 2018). Of these, the one that has the best capacity to tolerate drought is the Lotus (Orloff et al., 2018).

2.3 Species under study *Lotus corniculatus* L. (Bird´s foot trefoil)

The *Fabaceae* family encompasses the Lotus genus, as noted by Abderrahmane et al. (2014). With over 18,000 species and approximately 720 genera globally, *Lotus*, a term introduced by Linnaeus in 1753 (Maguire *et al.,* 2020), exhibits extensive biological diversity. Within this genus, there are around 200 annual and perennial species, including the widely distributed Bird's-foot trefoil native to Europe and Western Asia and naturalized in various temperate regions such as South and North America, Australia, and New Zealand (Steiner & Garcia de los Santos, 2001).

Bird's-foot trefoil, a prevalent grass legume, thrives in poorly drained acid soils and demonstrates adaptability to sandy soils (Casler & Undersander, 2018; Clua et al., 2009). Unlike other forage legumes in eastern North America, it does not induce bloat in ruminants (Christensen et al., 2017). The species offers three distinct types—prostrate, erect, and semi-erect—with each type catering to

specific agricultural purposes. Seedling vigor is comparatively lower, and the species utilizes root reserves for early spring growth, unlike most legumes that replenish root reserves in late summer (Casler & Undersander, 2018).

Energy reserves in bird 's-foot trefoil are stored in the crown and stubble, which is crucial for respiration and subsequent plant growth (Casler & Undersander, 2018). Maintaining an appropriate cutting height, leaving the last 3 to 4 inches of grass, is recommended to preserve vital energy reserves for the following year's production (Santacoloma-Varón et al., 2017). The stored sugars, primarily located in the internodes of the stubble, amount to 85-90%, with adequate stubble essential to prevent drying out. These sugars and starches can be reactivated for respiration and new plant growth when water is available (Orloff et al., 2018; Volenec & Nelson, 2020).

Bird's-foot trefoil is valued for high-quality forage, utilized in silage, straw production, and direct green consumption (Acuña et al., 2008; Santacoloma-Varón et al., 2017). In South America, key species include *Lotus corniculatus*, *Lotus tenuis*, *Lotus pedunculatus* (synonym *L. uliginosus*), and *Lotus subbiflorus* (Ross & Jones, 1985). Notably, these species exhibit greater tolerance to soil acidity compared to other legumes like alfalfa and clover (*Trifolium* spp.) (Girardi et al., 2014). Due to its global significance as a forage legume with high nutritional value, there is a need for reproducing *L. corniculatus* with enhanced drought and salt tolerance (Díaz et al., 2005; Hunt et al., 2015; D. Wang et al., 2018). Given the varying international results and limited information in Mexico regarding its productive potential and nutritional quality, it is crucial to conduct studies on this legume to identify genotypes or accessions with favorable traits (García-Bonilla *et al.*, 2014).

2.4 Water stress

The significance of water in plant life, directly influencing growth and productivity, has been highlighted by Luna-Flores *et al.* (2012). Metabolic and photosynthetic reactions predominantly occur in an aqueous medium, making the limited

availability of water in a plant's environment lead to unfavorable responses, impacting morphology, anatomy, and physiological processes under water stress conditions (Clua et al., 2009). Water is transported within plants through three distinct routes: apoplastic (via cell walls without crossing cell membranes), transmembrane (entering cells), and symplastic (through plasmodesmata communicating between partitions) (Taiz & Zeiger, 2002). The plant's water status is often expressed through relative water content (RWC), which represents the absolute amount needed for total saturation and reflects the water balance (Mohammadkhani & Heidari, 2007).

Transpiration, the process through which plants lose a significant amount of water, mainly occurs through stomata, modified epidermal cells surrounded by guard cells (Staniak et al., 2018). Stomata, acting as hydraulically directed valves, regulate moisture loss by altering pore openings (Ammar et al., 2020). Beyond controlling transpiration, stomata serve as the entry route for CO_2 during photosynthesis (Farooq et al., 2009; Kostopoulou & Karatassiou, 2017). Various factors influence transpiration rates and stomatal activity, including vapor pressure deficit, light quality and quantity, environmental CO_2, air contaminants, leaf temperature, and soil water content (Carter et al., 1997; Flexas et al., 2004; Sharma et al., 2020). The initiation of stomatal closure, according to Hernández et al. (2013), is signaled by the roots and dependent on soil water content.

Transpiration becomes a necessary secondary process for photosynthesis during the day when the vapor pressure deficit is higher (Ammar et al., 2020; Hasanuzzaman, 2020). The combination of transpiration and low soil water availability significantly affects plants, with stomatal closure mediated by abscisic acid to prevent water loss. Each plant species has a specific response threshold to water potential and factors influencing stomatal opening (Flexas et al., 2004; Taiz & Zeiger, 2002).

Water stress, induced by global climate change and intense drought periods, adversely affects plant development, crop growth, and productivity. Understanding genetic and physiological mechanisms that enable plants to

tolerate water deficit becomes crucial for designing efficient cropping systems in water-limited regions (Inostroza et al., 2015). Plants employ adaptive strategies during water stress, with C4 and CAM plants exhibiting anatomical and metabolic differences to reduce water loss, while C3 plants may experience reduced photosynthetic advantages in drought conditions (Sharma et al., 2020). Symptoms of water deficit in plants include delayed growth, restricted CO_2 diffusion, decreased photosynthesis, and accelerated leaf senescence (Mohammadkhani & Heidari, 2007).

2.5 Efficiency in water use

The presence of water in the soil plays a crucial role in the water balance, serving as the primary source from which plants extract the necessary amount for metabolic processes and compensate for water lost during transpiration, as highlighted by Browne et al. (2020). Soil characteristics, including its moisture and nutrient retention capacity, are determined by its texture, which, in turn, influences factors such as aeration through the number of pores and capillaries supporting root respiration (Farooq et al., 2009; Mohammadkhani & Heidari, 2007). Additionally, soil texture determines the cation exchange capacity responsible for retaining and exchanging available ions (Valentine et al., 2011). The field capacity (FC) of soil, varying with soil type, describes the point when the soil drains water by gravity after irrigation or rain, remaining humid with a water potential close to zero. In contrast, the permanent wilting point (PWP) denotes the soil water content at which most plants remain wilted, even during nighttime (Browne et al., 2020; Karatassiou et al., 2014; Talbi et al., 2020).

The concept of available water pertains to the amount of water within the soil falling within an acceptable range between the field capacity and the permanent wilting point. The closer the soil is to the PWP, the more challenging it becomes for the plant to access water (Browne et al., 2020). Efficient water use (EWU) is considered a physiological parameter that demonstrates tolerance to water deficit in various productive species, thereby aiding in the development of plant improvement programs. It is crucial to note that plant dry matter production is

closely linked to soil water availability (Luna-Flores et al., 2012). Consequently, there is a pressing need to identify new genotypes with higher EWU, as it signifies the relationship between accumulated plant dry matter and transpired water, as emphasized by the Inter-American Institute for Cooperation on Agriculture (IICA, 2010).

2.6 Antioxidant System

The primary role of the antioxidant system is to regulate reactive oxygen species (ROS) through the utilization of specific ROS as substrates or cofactors. Ultimately, this process results in the production of non-reactive or less reactive species, thereby controlling oxidation (Xie et al., 2019). ROS, arising from normal metabolic processes, necessitates elaborate mechanisms in plants to manage them at sustainable levels, preventing damage caused by excess ROS (Peralta-Pérez & Volke-Sepúlveda, 2012). Oxygen (O_2), a relatively stable molecule, can generate highly reactive species by electron transfer (superoxide radical anion, hydrogen peroxide, hydroxyl radical) or altering spin state (singlet oxygen)(Moreno et al., 2011).

Living organisms possess antioxidant defense systems comprising enzymatic and non-enzymatic antioxidants to eliminate these radicals (Moattar et al., 2016). Two response mechanisms in oxidation-reduction situations exist the non-enzymatic system (ascorbic acid, polyphenols, chalcones, tocopherols, anthocyanins, carotenoids, glutathione, etc.) and the enzymatic system (catalase, peroxidase, ascorbate peroxidase, glutathione reductase, superoxide dismutase, etc.) (Moreno et al., 2011).

Antioxidant enzymes play a crucial role in stabilizing or deactivating free radicals, preventing the oxidation of cellular components and subsequent damage. Due to their single pair of electrons, free radicals are highly reactive and readily interact with proteins, lipids, carbohydrates, and nucleic acids, causing denaturation (Alici & Arabaci, 2016). Various mechanisms, such as energy reduction or electron donation, contribute to the conservation of antioxidant enzymes. The enzymatic

response during periods of stress provides an adaptive advantage to oxidative stress in plants (Borsani et al., 2001).

Spectrophotometric measurement of enzymatic activities offers researchers a precise method to study a crucial aspect of defense against oxidative stress (Elavarthi & Martin, 2010). *In vitro*, determinations of antioxidant capacity provide only an approximation of the complex phenomena occurring *in vivo* (Kuskoski et al., 2005). The overall antioxidant capacity of a combination of different systems is not solely determined by the sum of individual antioxidant capacities (Kuskoski et al., 2005; Morales et al., 2008).

2.7 Non-Enzymatic Antioxidant Systems

2.7.1 Secondary metabolism

Encompassing diverse chemical substances, it contrasts with primary metabolism products and includes phenolic compounds, nitrogenous toxins, terpenes, polyacetylene hydrocarbons, and oxalates (Ramos et al., 1998). Ecological properties attributed to secondary metabolites vary and are influenced by environmental factors such as light, water, temperature, and climate changes. These compounds act as UV filters, reducing damage caused by UV-B radiation, and their concentration can be affected by climate change, impacting plant defense responses against pathogens (Morris et al., 2021).

2.7.2 Phenolic compounds

The extensive array of secondary metabolites in plants holds significance due to their involvement in various cellular processes, including morphology, growth, reproduction, defense mechanisms, and germination (Jurado et al., 2016). Characterized by at least one aromatic ring with one or more hydroxyl substituents, these compounds encompass flavonoids, phenolic acids, tannins, stilbenes, coumarins, and lignans (Chohan et al., 2019). Their biosynthesis and concentration are influenced by factors such as genotype, biotic elements (pests,

pathogen infections), and abiotic stressors (light, temperature, nutrient availability, water conditions, growth parameters, and UV radiation) (Yábar et al., 2019).

2.7.3 Flavonoids

Recognized for their potent antioxidant properties, they play a crucial role in eliminating hydroxyl radicals, superoxide anions, and peroxyl radicals (Bulugahapitiya, 2018). While their primary function involves the coloration of plant organs, flavonoids also contribute to physiological activities related to light perception and impact the flavor and aroma of plants (Liu et al., 2021). Additionally, flavonoids serve as signaling molecules in certain leguminous plants, influencing the interaction between atmospheric nitrogen-fixing bacteria (Garzón, 2010).

2.7.4 Tannins

Tannins are high-molecular-weight secondary phenolic compounds present in various fruits, trees, and temperate forage species (Naikoo et al., 2019). Classified as condensed (CT) and hydrolysable (HT), their chemical structure and concentration depend on factors like species, crop, tissue, development stage, and environmental conditions (Acuña et al., 2008; García, 2004; Otero & Hidalgo, 2004).

2.7.5 Saponins

These are glycosides primarily found in plants and exhibit diverse properties, including foaming in water (Quillay-Davila *et al.,* 2018). Despite potential harmful effects, saponins are present in various forages and cover crops, such as clover *L. corniculatus*, where they are found in smaller amounts (Price et al., 1987). This comprehensive exploration sheds light on the multifaceted roles of non-enzymatic antioxidant systems and their diverse components in cellular processes and environmental adaptations (Naikoo et al., 2019).

2.7.6 Photosynthetic Pigments

Plants experience biotic and abiotic stress simultaneously or in combinations, affecting growth and development. Osmond et al. (1987) mention that stress caused by high radiation, UV radiation, water deficit, salinity, pathogens, heavy metals, or high temperatures can affect the photosynthesis process. Chloroplasts are mainly considered to be the target of environmental stress since they manifest the primary and typical response during photodamage (Hasanuzzaman, 2020). The damage caused by an excessive increase in reactive oxygen species (ROS) because of the rise in the conversion of energy to O_2 molecules forms complexes that disturb the redox status of both the stroma and the thylakoid membrane (Flexas & Medrano, 2002; Jimenez et al., 2012). As a consequence of the previous process, the efficiency in the photosystems is low, CO_2 fixation decreases, and loss of efficient use of water, among others (Mickky et al., 2018).

The study of pigments is essential from an ecophysiological point of view because it provides information on productivity and stress events, among others. Leaf concentrations of photosynthetic pigments (chlorophyll a and b, carotenoids) generally decrease in response to stress or disease (Kume et al., 2018; Maoka, 2020). Although they may also be related to photo acclimatization (Reigosa, 2001). These changes produce significant differences in leaf reflectance and transmittance spectra and allow for screening monitoring of plant health (Casierra-Posada et al., 2012; España-Boquera et al., 2010).

Chlorophyll (*chl*) and carotenoid molecules are the antenna pigments in the two photosystems (Sánchez et al., 2018). Flexas et al. (2004) mention that the complexes that make photosynthesis possible are distributed in the chloroplast: PSII in the grana, PSI in the lamellae, ATPase in the lamellae, and the cytochrome complex in the grana and its margins (Manrique Reol, 2003). Concepts related to the second reaction involve the reduction and fixation of CO_2 to organic molecules using byproducts of the first reaction (Signorelli et al., 2019)

The Calvin cycle (C3 pathway) is the central pathway of CO_2 reduction (Reigosa, 2001). There are some other adaptive processes of different types than those of plants with the C3 cycle. An adaptation in several grasses included an additional

pathway for CO_2 concentration as well as a special anatomical modification of the leaf known as Kranz Anatomy, where two different types of photosynthetic cells were shown, which form a separation of the fixation and reduction processes of CO_2, in the C4 pathway (Taiz & Zeiger, 2002). Another adaptation was found in succulent plants called CAM (crassulaceae acid metabolism). These plants assimilate atmospheric CO_2 during the night, and it is stored as malate in vacuoles; most of it is exhausted during the day with the production of CO_2 (Azcón-Bieto & Talón, 2003). This adaptation is a response to the need to conserve water during the day in desert areas (Moreno et al., 2011).

The determination of chlorophylls and carotenoids is frequently essential data. Their quantification generally uses equations involving extinction coefficients and absorbances measured in the leaf extract at specific wavelengths (España-Boquera et al., 2010; Val et al., 1985). Moreover, recently, quantification through fluorescence emission through the use of portable equipment can provide quantitative and qualitative information on photosynthesis (IICA, 2010).

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CHAPTER III: Secondary Metabolites and Their Antioxidant Activity Enhance the Tolerance to Water Deficit on Clover *Lotus corniculatus* L. through Different Seasonal Times

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Secondary Metabolites and Their Antioxidant Activity Enhance the Tolerance to Water Deficit on Clover *Lotus corniculatus* L. through Different Seasonal Times

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Abstract: This study aimed to evaluate the effects of a water limitation in different ecotypes and one variety of *Lotus corniculatus* L. on the production of secondary metabolites and their antioxidant activity in response to a water deficit (WD) through other seasonal times. A randomized block experimental design with three replicates was used. Two levels of soil water content and five genotypes were arranged in a factorial way (2 × 5) with ten treatments for replication. The 255301 ecotype showed significantly higher ($p \leq 0.05$) concentrations of total phenols, with a concentration of 86.6 mg Gallic Acid Equivalent (GAE)/gram of fresh weight (gFW); total flavonoids, with a concentration of 63.2 mg Quercetin Equivalent (QE)/gFW); total tannins (71.7 mg GAE/gFW); and radical scavenging activity, with an average of 200 mg Trolox Equivalent Antioxidant Capacity (TEAC)/gFW) in winter under a WD. The 255305 ecotype showed an increase in radical scavenging activity of 230 mg (TEAC)/gFW) and a total tannin concentration of 65.3 mg GAE/gFW in winter and spring, respectively, under a WD. The 255301 ecotype showed an increase in the concentration of total saponins (254.8 mg saponins/gFW) in summer under a WD. All these responses were triggered to mitigate a water deficit and extreme temperatures.

Keywords: stress physiology; biochemical processes; drought; forage crops; soil moisture

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1. Introduction

Climate change has modified weather patterns with increasingly extreme events. Rainfall is one of the climatic components with an environmental impact causing extreme events in the world [1]. In addition, the world's rising population, now pegged at more than 8 billion, is putting intense pressure on using natural resources for agri-food production [2]. Livestock is a supplier of agri-food products and an essential protein source in the family diet. Consumers' high demand for meat, milk, and other derivatives has increased animal production and led to a greater need for forage [3]. Forage production consumes 76% of the freshwater available for productive activities on the planet [4], which is a significant problem for regions due to the scarcity of this natural resource.

Drought and intensive agri-food production activity cause environmental deterioration in arid areas [5] where the productivity is with a high-water demand. This makes it necessary to perform strategies to produce forage through alternative crops with a lower water consumption and the ability to compete with other crops in quality and quantity [6,7].

Alfalfa (*Medicago sativa* L.) is the main forage crop, with the best nutritional quality and quantity of fresh and dry biomass produced as food for livestock. Paradoxically, however, it is one of the crops with the highest water consumption [8].

Several species are studied as alternative forage crops in the leading livestock areas of the world, with studies related to the evaluation of water efficiency, environmental adaptation, and productivity. Among these species are white clover (*Trifolium repens* L.), red clover (*T. pretense* L.), pink clover (*T. fragiferum*), and birdsfoot treefoil (*Lotus corniculatus* Lam.) [9]. The *Lotus* genus has more than two hundred annual and perennial species with different growth habits, such as prostrate, erect, and semi-erect forms [10]. Most ecotypes and varieties of *L. corniculatus* are grown in regions with a humid temperate climate [11]. Few studies have been conducted under extreme conditions, such as those of arid lands [10]. However, *Lotus* has considerable genetic diversity, which is an opportunity to explore the response capacity of the genetic resources of this genus, which could adapt to conditions of low water availability and extreme temperatures.

The plants have several morphological, physiological, and chemical mechanisms to tolerate extreme environments [12]. Some physiological indicators, such as phytochemicals produced through secondary metabolism, named secondary metabolites, are highly reactive in plants under environmental stress, such as a water deficit, soil salinity, extreme temperatures, and a nutrient deficiency [13,14]. They play a crucial role in the survival of plants in marginal conditions since they are activated in response to environmental stress and function as agents and physiological regulators [15]. Secondary metabolites, such as phenolic compounds, alkaloids, and terpenoids, have been shown to have antioxidant and antimicrobial properties, which help mitigate oxidative damage caused by water scarcity [16,17].

Furthermore, reactive oxygen species (ROS) are increased under water stress as molecular signals that activate adaptive responses in plants [18]. ROS and secondary metabolites' activity interaction is a complex process that allows plants to survive in extreme environments [19]. This study aimed to evaluate different ecotypes and one variety of *Lotus corniculatus* L. in terms of its ability to produce secondary metabolites with antioxidant activity under a water deficit through other seasonal times.

2. Materials and Methods

2.1. Geographic Location

The experiment was conducted at the experimental field of the Unidad Regional Universitaria de Zonas Aridas of the Universidad Autonoma Chapingo at Bermejillo Mapimí, Durango, Mexico. The region is located at 25.8° NL and 103.6° WL and an elevation of 1130 m. The area has a desert climate with rain in summer and cool winters, with an average annual rainfall of 258 mm, average potential evaporation of 2000 mm, and an average temperature of 21 °C, with a maximum of 33.7 °C and a minimum of 7.5 °C [20]. Temperatures recorded inside the shade mesh were extremely high in summer, low in winter, and moderate during spring and autumn. The experimental area within the shade mesh was covered in advance with a plastic cover to avoid alterations in the soil moisture content in the pots during rainy periods.

2.2. Experimental Setup

A randomized block experimental design with three replicates was used under shade mesh conditions. The treatments of soil water content were as follows: (1) no water deficit (NWD) with 100% of field capacity (FC), and (2) water deficit (WD) with 89% of FC in four ecotypes identified with ID numbers 255301, 255305, 202700, and 226792, and one variety named Estanzuela Ganador of *L. corniculatus*, which was obtained from different areas, such as Europe, North America, and South America, with a temperate humid climate (Table 1).

Table 1. Identification, country of origin, and growth habits of different plant genetic resources of *Lotus corniculatus* L.

Ecotype Identification/Variety *	Country of Origin	Growth Habit
255301	France	Semi-erect
255305	Italy	Semi-erect
202700	Uruguay	Erect
226792	Canada	Semi-erect
Estanzuela Ganador	Uruguay	Erect

* Donated by the Institute of Natural Resources of the Postgraduate College at Montecillo, Mexico.

The Lotus genetic materials evaluated in this study were selected based on their ability to survive the environmental conditions in the study area, from a total of 12 original genetic materials: 4 varieties and 8 different accessions.

Ten treatments were established of the factorial 2 × 5. The experimental unit was a plant in a pot with three replicates. Seedlings were previously grown in 1 kg black plastic bags in soil mixed with compost at a 3:1 ratio for two months. After this time, plants of approximately 20 cm in height were transplanted into rigid 18 kg plastic pots containing a substrate prepared to have a ratio of 50:30:20 of soil, compost, and sand, respectively. According to a physical and chemical analysis of the substrate, it was 26% silt, 22% clay, and 52% sand, with a pH of 7.73, electric conductivity (EC) of 7.47 dS/m, and bulk density of 1.46 g/cm^3 (Figure 1) The experiment was carried out from March 2021 to May 2022.

Figure 1. A view of part the experiment of *Lotus corniculatus* L. under shade mesh. Bermejillo, Dgo. México.

According to the determination using the membrane pot method [21], the substrate used in the pots, the soil moisture content at the field capacity (FC), and the permanent wilting point (PWP) were 27.5% and 17.5%, respectively (Figure 2).

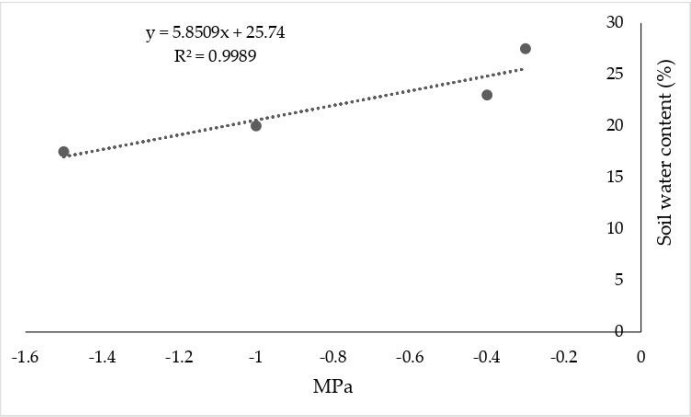

Figure 2. The membrane pot method calculates the down curve of soil moisture (10).

2.3. Irrigation

Irrigation was performed manually, and during the first 15 days of the experiment, the soil moisture was maintained at FC (27.5%) for all treatments. Subsequently, the water content in the soil was restricted until maintaining the differentiated ranges of 26.5% ± 1 and 23.5% ± 1. The upper limit of soil water content in the first range was 27.5%, corresponding to 100% of FC, and the upper limit of the second range was 24.5%, corresponding to 89% of FC. This water decrease in the soil was decided since *L. corniculatus* is a plant with a C3 photosynthetic pathway, which is sensitive to water stress [22].

The different genetic resources of *L. corniculatus* were homogenized in the plant canopy by cutting the biomass at 62 days after transplanting (DAT). Since the plant grows by forming a rhizome with several new shoots, the cuts of fresh matter on the different sampling dates were made with pruning shears 6 cm above soil level, using a plastic ring that allowed the cutting height to be uniform [23]. From this date, seasonal cutting intervals were defined: two cuts at intervals of 42 days in each seasonal period (summer, autumn, and spring) and 92 days in winter (a single cut), the latter interval defined by the slow growth of the plant due to low temperatures.

The soil water content was measured regularly in real-time using a digital tensiometer (Model: MO750, Extech Instruments Co., Laredo, TX, USA). When the soil moisture content reached the lowest limit of a treatment, irrigation was resumed until the upper limit of each irrigation treatment. During the experimental time, the environmental temperature and humidity inside the shade were recorded daily using a digital thermometer-hygrometer (Model OUS-WA62, ORIA, Shanghai, China).

2.4. Measured Variables

Fresh tissue of *L. corniculatus* samples was used to quantify secondary metabolites according to the method reported by Melgarejo et al. [24]. All samples were stored at −40 °C until they were used. The total phenols concentration (TPC) and total tannins concentration (TTC) were measured using Folin–Cioucalteu reagent [25]; the extract was prepared with 1 mg of plant tissue in 1000 µL of methanol, which was shaken for 1 h and subsequently centrifuged at 4000 rpm for 15 min. These variables were measured in mg Gallic Acid Equivalents per gram of fresh weight (GAE/gFW) [26]. The total flavonoid concentration (TFC) was quantified using the method described by Maksimovíc et al. (2005) [27] with modifications. Quercetin was the standard, so the result was expressed in mg Quercetin Equivalents (QE/gFW). At the same time, total saponins concentration (TSC) was calculated using colorimetry with Lieberman–Burchard reagent [28] in mg

of saponins/GFW. Radical Scavenging Activity (RSA) was measured using absorbance of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical [29] reported in Trolox Equivalent Antioxidant Capacity (TEAC)/gFW.

2.5. Data Analysis

Data were analyzed with normality tests, one-way ANOVA, Tukey's range test, and simple linear regression using the PASW Statistics statistical program for Windows 18.0.0 Chicago, IL, USA, SPSS Inc.

3. Results

3.1. Temperature and Relative Humidity

The temperature was recorded within the shade mesh from June 2021 to May 2022. An average maximum temperature of 30 °C, an average minimum of 20 °C, a maximum of 46.9 °C, and a minimum of −4.6 °C were recorded. The middle and maximum temperatures per day were 16.6 and 40.1, 19.8 and 32.7, 9.8 and 37, and 4.5 and 30 °C in spring, summer, autumn, and winter, respectively. Along with the experiment's relative humidity, the average was 57.5%, with a minimum mean of 42% and a maximum mean of 57.5% (Figure 3). The experimental area within the shade mesh was covered in advance with a plastic cover to avoid alterations in the soil moisture content in the pots during rainy periods. July and September had the highest rainfall, with 44 and 54 mm averages, respectively.

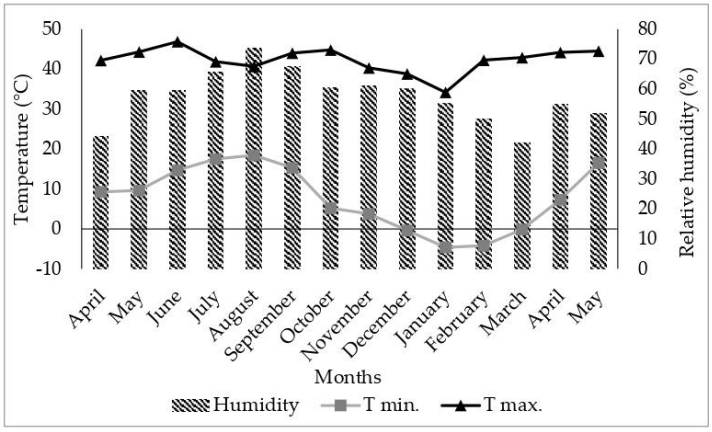

Figure 3. The performance of maximum, minimum, and mean temperatures (°C) and mean relative humidity (%) inside the shade mesh from April 2021 to May 2022.

3.2. Phenols

The total phenols concentration (TPC) in the tested plant resources was significantly different ($p \leq 0.05$). In winter 2021–2022, the 255301 and 226792 ecotypes showed a higher concentration with values of 86.6 and 87.2 GAE/gFW under a WD. At the same time, the lowest concentration was found in 226792 ecotype under NWD (Table 2).

Table 2. Total phenols concentration in different ecotypes and one *L. corniculatus* variety under water deficit throughout the seasons.

Ecotypes/Variety	SWC	Total Phenols Concentration (mg GAE/gFW)			
		Summer 2021	Autumn 2021	Winter 2021–2022	Spring 2022
255301	100% FC (NWD)	38.793 ± 4.5 ^{a}	20.134 ± 3.4 ^{a}	61.325 ± 4.5 ^{b}	25.359 ± 3.2 ^{a}
	89% FC (WD)	47.301 ± 0.5 ^{a}	33.275 ± 2.6 ^{a}	86.622 ± 3.8 ^{a}	31.116 ± 1.3 ^{a}
255305	100% FC (NWD)	50.670 ± 1.5 ^{a}	27.374 ± 5.3 ^{a}	73.453 ± 1.8 ^{ab}	29.323 ± 0.7 ^{a}
	89% FC (WD)	45.181 ± 7.7 ^{a}	26.850 ± 1.9 ^{a}	81.437 ± 5.7 ^{ab}	30.906 ± 1.4 ^{a}
202700	100% FC (NWD)	41.521 ± 8.8 ^{a}	20.848 ± 3.4 ^{a}	62.495 ± 5.2 ^{b}	23.894 ± 3.5 ^{a}
	89% FC (WD)	40.451 ± 1.4 ^{a}	29.363 ± 2.8 ^{a}	76.156 ± 6.2 ^{ab}	30.715 ± 1.0 ^{a}
226792	100% FC (NWD)	38.975 ± 1.9 ^{a}	28.591 ± 1.2 ^{a}	61.450 ± 0.7 ^{b}	25.035 ± 0.2 ^{a}
	89% FC (WD)	39.017 ± 3.4 ^{a}	31.117 ± 4.9 ^{a}	87.248 ± 1.4 ^{a}	36.392 ± 3.0 ^{a}
Estanzuela Ganador	100% FC (NWD)	49.593 ± 5.4 ^{a}	30.404 ± 3.8 ^{a}	66.796 ± 2.0 ^{ab}	25.353 ± 3.8 ^{a}
	89% FC (WD)	38.962 ± 1.9 ^{a}	27.781 ± 1.8 ^{a}	73.926 ± 6.7 ^{ab}	33.561 ± 3.0 ^{a}

Mean ± standard deviation. Tukey test ($p \leq 0.05$). Figures with the same letter within the same column are statistically equal. SWC = soil water content; GAE = Gallic Acid Equivalent; FC = field capacity; NWD = no water deficit; WD = water deficit.

3.3. Flavonoids

The total flavonoid concentration (TFC) was remarkably similar to the TPC performance with a statistical difference ($p \leq 0.05$). Only in the winter was the Estanzuela Ganador variety significantly higher, with a concentration of 63.2 mg QE/gFW in a WD. In comparison, the 226792 ecotype had the lowest value with 27.1 mg QE/gfw under NWD. The TFC was quantified as 12.8–63.2 mg QE/gFW within the evaluated time (Table 3).

Table 3. The total flavonoid concentration in different ecotypes and one *L. corniculatus* variety under water deficit throughout the seasons.

Ecotypes/Variety	SWC	Total Flavonoid Concentration (mg QE/gFW)			
		Summer 2021	Autumn 2021	Winter 2021–2022	Spring 2022
255301	100% FC (NWD)	16.592 ± 1.4 ^{a}	18.440 ± 1.6 ^{a}	34.557 ± 6.8 ^{cd}	12.898 ± 1.6 ^{a}
	89% FC (WD)	19.084 ± 2.1 ^{a}	17.478 ± 1.1 ^{a}	57.965 ± 0.4 ^{ab}	20.454 ± 1.0 ^{a}
255305	100% FC (NWD)	19.919 ± 1.9 ^{a}	19.452 ± 1.4 ^{a}	42.826 ± 0.9 ^{bcd}	16.385 ± 0.2 ^{a}
	89% FC (WD)	17.084 ± 2.5 ^{a}	19.253 ± 1.3 ^{a}	51.887 ± 3.8 ^{abc}	18.975 ± 2.2 ^{a}
202700	100% FC (NWD)	20.731 ± 0.5 ^{a}	17.512 ± 2.8 ^{a}	43.294 ± 5.3 ^{bcd}	13.975 ± 1.2 ^{a}
	89% FC (WD)	16.220 ± 0.5 ^{a}	16.517 ± 1.2 ^{a}	56.008 ± 3.1 ^{ab}	18.703 ± 0.6 ^{a}
226792	100% FC (NWD)	16.594 ± 0.5 ^{a}	15.385 ± 0.8 ^{a}	27.187 ± 1.4 ^{d}	13.393 ± 1.7 ^{a}
	89% FC (WD)	16.415 ± 1.6 ^{a}	16.345 ± 2.6 ^{a}	53.210 ± 0.4 ^{ab}	19.485 ± 0.3 ^{a}
Estanzuela Ganador	100% FC (NWD)	19.016 ± 2.3 ^{a}	15.070 ± 1.5 ^{a}	48.435 ± 3.7 ^{abc}	13.076 ± 1.8 ^{a}
	89% FC (WD)	16.377 ± 0.2 ^{a}	18.839 ± 0.7 ^{a}	63.291 ± 4.3 ^{a}	19.668 ± 1.8 ^{a}

Mean ± standard deviation. Tukey test ($p \leq 0.05$). Figures with the same letter within the same column are statistically equal. SWC = soil water content; QE = Quercetin equivalent; FC = field capacity; NWD = no water deficit; WD = water deficit.

3.4. Tannins

The total tannins concentration (TTC) also performed similarly to the TPC and the TFC in the plants evaluated. In winter, there was a significantly greater response ($p \leq 0.05$) in the 255301 and 202700 ecotypes, with 71.7 and 65.3 mg GAE/gFW under a WD. The 202700 ecotype showed the lowest response, with 44.5 mg GAE/gFW under NWD. In addition, the 253301 ecotype presented a significant difference among plant genetic resources in spring 2022, with the highest response of 32.3 mg GAE/gFW under a WD. On the other hand, the 255301 ecotype and the Estanzuela Ganador variety had the lowest response with concentrations of 20.6 and 20.4 mg GAE/gFW under NWD. This means that for these genetic resources, the production of tannins was not required to mitigate the environmental stress associated with low temperatures and the water deficit (Table 4).

Table 4. Total tannins concentration in different ecotypes and a *L. corniculatus* variety under water deficit throughout the seasons.

Ecotypes/Variety	SWC	Total Tannins Concentration (mg GAE/gFW)			
		Summer 2021	Autumn 2021	Winter 2021–2022	Spring 2022
255301	100% FC (NWD)	34.331 ± 3.5 ^{a}	34.543 ± 3.6 ^{a}	48.345 ± 1.3 ^{bc}	20.656 ± 3.2 ^{b}
	89% FC (WD)	36.300 ± 2.2 ^{a}	43.844 ± 3.0 ^{a}	71.710 ± 3.4 ^{a}	32.353 ± 1.0 ^{a}
255305	100% FC (NWD)	41.277 ± 3.7 ^{a}	37.889 ± 5.6 ^{a}	59.382 ± 3.3 ^{abc}	23.502 ± 0.7 ^{ab}
	89% FC (WD)	38.756 ± 2.6 ^{a}	40.720 ± 2.9 ^{a}	65.365 ± 4.7 ^{a}	27.314 ± 4.1 ^{ab}
202700	100% FC (NWD)	30.542 ± 5.0 ^{a}	33.179 ± 2.7 ^{a}	49.949 ± 3.0 ^{bc}	22.043 ± 2.7 ^{ab}
	89% FC (WD)	32.745 ± 2.3 ^{a}	37.277 ± 1.7 ^{a}	62.004 ± 2.9 ^{ab}	26.880 ± 2.0 ^{ab}
226792	100% FC (NWD)	36.121 ± 0.7 ^{a}	38.794 ± 0.8 ^{a}	44.550 ± 1.6 ^{c}	23.777 ± 0.7 ^{ab}
	89% FC (WD)	30.868 ± 1.8 ^{a}	37.666 ± 3.8 ^{a}	60.701 ± 3.0 ^{ab}	28.751 ± 1.8 ^{ab}
Estanzuela Ganador	100% FC (NWD)	41.653 ± 0.5 ^{a}	35.806 ± 3.4 ^{a}	49.798 ± 3.0 ^{bc}	20.471 ± 1.7 ^{b}
	89% FC (WD)	26.447 ± 0.1 ^{a}	41.340 ± 0.8 ^{a}	60.463 ± 1.9 ^{ab}	32.006 ± 2.0 ^{a}

Mean ± standard deviation. Tukey test ($p \leq 0.05$). Figures with the same letter within the same column are statistically equal. SWC = soil water content; GAE = Gallic Acid Equivalent; FC = field capacity; NWD = no water deficit; WD = water deficit.

3.5. Saponins

The total saponins concentration (TSC) had the highest variation throughout the seasons. It had values from 31.2 to 247.3 GAE/gFW in spring and summer, respectively. There was no statistical difference among *Lotus* genetic resources, except in summer 2021 and winter 2021–2022. The 255301 ecotype in a WD was the most heat-sensitive, producing 254.8 mg saponins/gFW, compared to 226792, which showed the lowest concentration with 138.7 mg saponins/gFW (Table 5).

Table 5. The total saponins concentration of in different ecotypes and one *L. corniculatus* variety under water deficit throughout the seasons.

Ecotypes/Variety	SWC	Total Saponins Concentration (mg Saponin/gFW)			
		Summer 2021	Autumn 2021	Winter 2021–2022	Spring 2022
255301	100% FC (NWD)	180.460 ± 14.2 ^{ab}	161.762 ± 8.9 ^{a}	92.652 ± 9.4 ^{b}	31.211 ± 3.9 ^{a}
	89% FC (WD)	254.869 ± 14.3 ^{a}	186.385 ± 1.1 ^{a}	145.342 ± 7.0 ^{a}	45.301 ± 7.7 ^{a}
255305	100% FC (NWD)	222.720 ± 11.6 ^{ab}	138.888 ± 2.8 ^{a}	131.771 ± 3.6 ^{ab}	34.169 ± 9.5 ^{a}
	89% FC (WD)	247.373 ± 14.6 ^{ab}	186.005 ± 2.2 ^{a}	107.550 ± 7.6 ^{ab}	38.773 ± 8.1 ^{a}
202700	100% FC (NWD)	176.378 ± 16.5 ^{ab}	146.048 ± 1.9 ^{a}	140.397 ± 14.3 ^{ab}	30.338 ± 6.3 ^{a}
	89% FC (WD)	187.382 ± 8.0 ^{ab}	171.172 ± 6.9 ^{a}	128.749 ± 5.8 ^{ab}	42.577 ± 8.4 ^{a}
226792	100% FC (NWD)	142.177 ± 18.6 ^{b}	174.395 ± 2.1 ^{a}	122.021 ± 13.2 ^{ab}	34.887 ± 7.8 ^{a}
	89% FC (WD)	138.890 ± 17.4 ^{b}	143.549 ± 9.9 ^{a}	120.065 ± 3.9 ^{a}	42.595 ± 1.1 ^{a}
Estanzuela Ganador	100% FC (NWD)	195.905 ± 3.9 ^{ab}	169.869 ± 3.8 ^{a}	106.424 ± 7.4 ^{ab}	28.424 ± 2.8 ^{a}
	89% FC (WD)	183.737 ± 6.9 ^{ab}	195.811 ± 3.2 ^{a}	136.781 ± 3.4 ^{ab}	35.935 ± 2.0 ^{a}

Mean ± standard deviation. Tukey test ($p \leq 0.05$). Figures with the same letter within the same column are statistically equal. SWC = soil water content; FC = field capacity; NWD = no water deficit; WD = water deficit.

3.6. Radical Scavenging Activity

The DPPH technique detailed the highest radical scavenging activity (RSA). In the 255301 ecotype, the summer, autumn, and winter had values of 78.6, 114.2, and 244.9 mg TEAC/gFW, respectively. In the summer, the 255305 ecotype had a value of 79.8 mg TEAC/gFW, and the 226792 ecotype in winter had a value of 179.4 mg TEAC/gFW, all under a WD. On the other hand, those with the lowest response were the 226792 ecotype in summer, with 27.9 mg TEAC/gFW, and the Estanzuela Ganador variety in autumn and winter (18.7 and 114.7 mg TEAC/gFW) under a WD, but also under NWD with 93.6 mg TEAC/gFW (Table 6).

Table 6. Radical scavenging activity in ecotypes and one variety of *L. corniculatus* under water deficit in different seasons.

Ecotypes/Variety	SWC	Radical Scavenging Activity (mg of TEAC/gFW)			
		Summer 2021	Autumn 2021	Winter 2021–2022	Spring 2022
255301	100% FC (NWD)	42.528 ± 9.4 ^{cd}	51.819 ± 5.9 ^{abc}	133.138 ± 12.0 ^{bcd}	47.110 ± 4.8 ^{a}
	89% FC (WD)	78.613 ± 3.6 ^{a}	114.253 ± 10.9 ^{a}	244.910 ± 10.6 ^{a}	70.941 ± 6.7 ^{a}
255305	100% FC (NWD)	73.382 ± 3.9 ^{ab}	105.165 ± 10.7 ^{a}	209.139 ± 11.2 ^{abc}	75.290 ± 10.3 ^{a}
	89% FC (WD)	79.895 ± 3.2 ^{a}	87.647 ± 12.8 ^{ab}	221.492 ± 16.3 ^{ab}	64.810 ± 3.1 ^{a}
202700	100% FC (NWD)	60.7661 ± 2.2 ^{bc}	85.630 ± 8.2 ^{ab}	165.731 ± 8.9 $^{a–d}$	58.623 ± 5.8 ^{a}
	89% FC (WD)	66.822 ± 2.7 ^{abc}	23.665 ± 1.4 ^{bc}	187.358 ± 14.1 $^{a–d}$	72.837 ± 2.6 ^{a}
226792	100% FC (NWD)	27.992 ± 7.5 ^{d}	24.782 ± 1.1 ^{bc}	141.540 ± 17.0 ^{bcd}	47.339 ± 2.6 ^{a}
	89% FC (WD)	47.137 ± 5.5 ^{bcd}	80.657 ± 4.1 ^{abc}	179.409 ± 8.9 ^{a}	85.027 ± 1.4 ^{a}
Estanzuela Ganador	100% FC (NWD)	43.686 ± 8.9 ^{cd}	62.400 ± 6.6 ^{abc}	93.630 ± 6.1 ^{d}	62.555 ± 7.2 ^{a}
	89% FC (WD)	49.230 ± 3.1 ^{bcd}	15.478 ± 1.6 ^{c}	114.749 ± 9.4 ^{cd}	67.850 ± 10.9 ^{a}

Mean ± standard deviation. Tukey test ($p \leq 0.05$). Figures with the same letter within the same column are statistically equal. SWC = soil water content; TEAC = Trolox Equivalent Antioxidant Capacity; FC = field capacity; NWD = no water deficit; WD = water deficit.

A direct proportional relationship among TAA and CTP (R^2 = 0.58), CTF (R^2 = 0.59), and CTT (R^2 = 0.53) was confirmed (Figure 4a–c) using regression technique, which is congruent, since TAA is an accumulated expression of the different metabolites reactive to the oxidation process due to the effect of environmental stress (Figure 4).

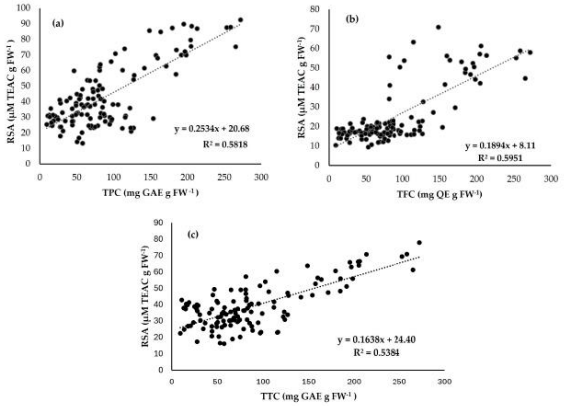

Figure 4. Relationship of radical scavenging activity (RSA) with the total phenols concentration (TPC) (**a**), the total flavonoid concentration (TFC) (**b**), and total tannins concentration (TTC) (**c**) in different ecotypes and one *L. corniculatus* variety.

4. Discussion

All the evaluated plant genetic resources maintained adequate phenolic compound production in the different seasons. However, the low temperatures in winter increased the output of this secondary metabolite, with the 255301 and 226792 ecotypes being the most susceptible as they produced more phenols to mitigate the cold stress in a WD. Kowalczewski et al. [30] quantified the TPC from 5.00 to 8.16 mg GAE/gFW in *Hordeum vulgare* L. var. KWS Olof, and although the water stress decreased the TPC, the photosynthetically active radiation (PAR) increased. Wong-Paz et al. [16] pointed out that plants in semi-arid areas have high antioxidant capacity due to a high content of total phenols as a defense mechanism against plant stress. Baali et al. [18] reported that in *L. corniculatus* plants

collected in March, the CTP was 87.1 ± 14.5 mg GAE/gFW, and the radical scavenging activity (RSA) was 26.9–79.7 mg TEAC/gFW.

The Estanzuela Ganador variety was susceptible to low winter temperatures, increasing the concentration of flavonoids as a defense mechanism against low temperatures and a water deficit. The 226792 ecotype was the most tolerant to winter conditions, with the most deficient production of this metabolite under NWD and without requiring the production of this metabolite to mitigate the WD. The TFC increased in response to low temperatures and a water deficit, with values higher than those -reported by Baali et al. (2019) [18], who documented values of 36.5 ± 2.1 mg QE/gFW in TFC in *L. corniculatus* extracts in response to environmental stress intensity. Additionally, the TFC in *L. corniculatus* varies depending on the environmental temperature; for example, it increases at 10 °C and decreases at 30 °C [31]. The decrease in TFC in summer, autumn, and spring could be due to the natural tolerance of most plant genetic resources evaluated in this study to the extreme temperatures in these seasons, as they do not require the antioxidant activity that promotes flavonoids. Cheng et al. [32] reported that only low temperatures, and not high temperatures, encourage the expression of genes fitted to produce flavonoids and, therefore, their accumulation.

The response of the 255301 and 255792 ecotypes and the Estanzuela Ganador variety had high tannin concentrations in winter and spring, except for the 255792 ecotype, which only reacted this way in winter. All the mentioned responses were under a water deficit. This secondary metabolite signifies the importance of mitigating the abiotic stress associated with low temperatures and a water deficit. Previous studies in three *Lotus* species (*L. glaber*, *L. uliginosus*, and *L. corniculatus*) found a high tannin content in the leaves, stems, and flowers, which varied in the different seasons of the year, except for *L. glaber*, for which there were no differences in spring, summer, and autumn [33,34]. The increase in tannins observed in winter could be due to the maturity of the plants and the increase in lignin, thereby decreasing the forage digestibility [35].

Saponins were the most immediate secondary metabolite to the elevated temperatures reported in summer. At the same time, the TPC, TFC, and TTC were more sensitive to low temperatures in winter and high temperatures in summer under a water deficit, with different responses among the plant genetic resources of *Lotus*. Szakiel et al. [36] described that saponins could be involved in adapting plants to survive in adverse climatic conditions, with a positive correlation between the saponin content and the physiological phase throughout the year. Baali et al. [18] detected the presence of saponins in *L. corniculatus* using the qualitative foam test. Santacoloma [37] reported the absence of saponins in the leaves, petioles, and stems of *L. corniculatus*, which may be associated with the favorable environmental conditions of the study area.

The antioxidant activity results were correlated to the production of some secondary metabolites in the content of phenols, flavonoids, and tannins in winter under a water deficit, particularly in the 255301 and 255305 ecotypes, as well as the Estanzuela Ganador variety, but only in terms of flavonoid production. On the other hand, saponins did not contribute to the antioxidant activity in the winter phase. Still, they did contribute in the summer, which is the time of highest temperatures, in the 255301 and 266792 ecotypes, which were under a water deficit since they were the phylogenetic materials with the highest RSA in that season. This suggests that saponin production is activated as a defense mechanism against heat and a water deficit. Indeed, antioxidant compounds are produced as a defense mechanism to prevent growth inhibition and damage to the photosynthetic apparatus, cell membranes, and proteins, with a specific response to a water deficit dependent on the genotypes used [38].

Finally, it is essential to point out that this study was under semi-controlled shade mesh conditions, so the results presented here require validation in the field.

5. Conclusions

The 255301 ecotype increased the concentration of phenols, flavonoids, and tannins under a water deficit. In contrast, the 255305 ecotype improved the tannin concentration and total antioxidant activity under both no water deficit and water deficit conditions, respectively, in winter when the temperatures were lower. The 202700 and 226792 ecotypes and the Estanzuela Ganador variety showed similar performance in soil moisture content, with lower values in secondary metabolite concentrations and antioxidant activity throughout the year. The 255301 ecotype showed an increase in the concentration of saponins as a mitigation response to stress due to the high temperatures and water deficit in summer. This diversity of responses indicates a high potential for *L. corniculatus* as an alternative forage crop in arid lands.

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CHAPTER IV: RELATIVE WATER CONTENT, CHLOROPHYLL INDEX, AND PHOTOSYNTHETIC PIGMENTS ON *Lotus corniculatus* L. IN RESPONSE TO WATER DEFICIT

plants

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Article

Relative Water Content, Chlorophyll Index, and Photosynthetic Pigments on *Lotus corniculatus* L. in Response to Water Deficit

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Abstract: This study aimed to evaluate different *L. corniculatus* L. ecotypes under water-deficit conditions to identify changes in relative water content and photosynthetic pigments as indicators of physiological responses during different years' seasons. The experiment was conducted in a randomized block design with three replicates. Ten treatments were performed as a factorial of 2 × 5, where the first variation factor was the soil water content—no water deficit (NDW) with 100% field capacity (FC), and water deficit (DW) corresponding to 85.4% of the FC—and the second variation factor comprised four ecotypes and one variety of *L. corniculatus*. A significant effect was identified on the concentration of photosynthetic pigments, mainly total chlorophyll, with chlorophyll a in the 255301 ecotype with records of 187.8, 167.5, and 194.6 mg g^{-1} FW in WD, corresponding to an increase of 86.0%, 172.6%, and 16.6%, respectively, in relation the lower values obtained in the ecotype 202700 under NWD. In carotenoids, higher concentrations were observed in the 255301 and 202700 ecotypes and the Estanzuela Ganador variety under WD in most seasonal periods, except summer; a similar response was found in the 202700 ecotype and the Estanzuela Ganador variety during the winter season, also in WD. The results showed that the first two principal components accounted for 71.8% of the total variation, with PC1 representing chlorophyll *a*, chlorophyll *b*, and total chlorophyll, and PC2 representing carotenoids, temperature, relative chlorophyll index, and relative water content. The observations were grouped based on soil moisture content, with the optimal moisture group exhibiting higher chlorophyll and carotenoid concentrations. The findings suggest that soil moisture content significantly affects the performance of *L. corniculatus* ecotypes, and the plant shows seasonal variations in response to water-deficit conditions. This research contributes to understanding the physiological responses of *L. corniculatus* and its potential as a water-efficient forage crop for promoting sustainable agriculture and enhancing food security.

Keywords: plant physiology; drought; plant water stress; plant performance; forage

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1. Introduction

Agriculture worldwide depends undoubtedly on the availability of water, which is scarce, putting food security at risk [1]. Additionally, the increase in population demands significant quantities of food, meat, and milk, which come from livestock farming [2]. This means high demand for forages, grasses, and legumes, which are essential food sources for livestock activity [3]. Also, this production system demands large volumes of water in irrigation areas, which are increasingly affected by the greater intensity of droughts due to climate change [4]. Furthermore, global climate change shows its impact through

extreme temperature and precipitation events that adversely affect agri-food production in different regions of the planet [5,6]. Arid areas have recorded temperatures above or below the historical average during the summer and winter seasons. Droughts have worsened in time and space, affecting rainfed and irrigated agriculture and livestock [7,8].

Irrigated agricultural areas of northern Mexico are the leading suppliers of forage for stabled livestock—mainly alfalfa, maize, and sorghum forage. The production of these crops is affected by the low availability of water, the aquifer's low recharge, and the water resource's overexploitation. Notably, alfalfa is widely recognized for its high nutritional value as a forage food; however, it has a high rate of water demand, estimated at an average of 2 m of annual irrigation depth or greater [4,9]. The production of crops with high water demand in regions with aquifer depletion and intensive agricultural production is the main challenge to be overcome through technology that allows for mitigating social and environmental impacts [10,11].

Several studies have been concentrated on developing and analyzing diverse crop species that exhibit tolerance to various abiotic stressors, notably drought, salinity, light intensity, extreme temperatures, and heavy metals, either individually or in combination [12]. These intensive agricultural production systems with low availability and poor-quality water are being contaminated with salts and heavy metals (arsenic, cadmium, and lead) due to being extracted from great depths of the subsoil [13]. Therefore, the importance of options that reduce the environmental impact caused by the intensive use of water–soil–plant resources is essential in managing agri-food production systems sustainably. From this perspective, alternative crops with lower water demand and adequate productive competitiveness with other crops, such as alfalfa, are viable alternatives for agricultural areas with low availability of water resources [14]. Hence, exploring native or exotic crops with the potential to adapt to different environments is required. Generally, research is focused on studying tolerance to water stress, extreme temperatures, and biodiversity in agroecosystems [15].

In recent research, the legume *Lotus corniculatus* L., commonly known as birdsfoot trefoil, is being studied as an alternative forage crop [16]. This plant is a perennial native to Europe and has spread to different parts of the world due to its adaptative capability to different environments and desirable characteristics as a forage [17–19]. *L. corniculatus* is a leguminous plant that can fix nitrogen, thus improving soil fertility [20], and its large root system allows it to access water from deeper into the soil with the efficient use of hydric resources [21]. In addition, *L. corniculatus* has high nutritional value since its foliage is abundant in protein and is very tasty for livestock. Although this species of forage crop comes from temperate–humid environments, its great genetic diversity enables it to grow and develop adequately in conditions of extreme environments, such as arid zones [22]. Drought stress is the primary abiotic factor constraining plant growth, distribution, and survival, significantly limiting forest productivity in different ways such as the inhibition of photosynthesis and osmoregulatory processes [23].

Different researchers report that some physiological indicators, such as relative water content and photosynthetic pigment content, play an essential role in tolerance to water deficit in plants [24,25] and other adverse factors, such as extreme temperatures [26]. A reduction in chlorophyll content in the leaves could lead to harm to the photosynthetic system, consequently impacting the operation of both photosystems II and I [27]. This underscores the importance of studies on adaptive mechanisms of the photosynthetic system to gain strong insights into plant stress responses [12]. Investigations at a biochemical level of the role played by photosynthetic pigments and other variables related to physiology, such as the water status of the plant and chlorophyll content in *Lotus corniculatus*, still need to be explored. The objective of this study was to evaluate the performance of different ecotypes and a variety of *Lotus corniculatus* in terms of relative water content, concentration of photosynthetic pigments (chlorophyll *a*, chlorophyll *b*, and total chlorophyll), as well as relative chlorophyll index in response to water deficit during different seasonal times.

2. Results

2.1. Experimental Climatic Conditions

Temperature variations (T) usually affect most physiological processes of all organisms. The average temperatures recorded under shade mesh were 28.5, 23.4, 17.5, and 28.3 °C during summer (2021), autumn (2021), winter (2021–2022), and spring (2022), while the variations in relative humidity were 58.8, 47.5, 41.5 and 32.2%, respectively (Table 1). Additionally, the different ecotypes tolerated maximum temperatures of 46.9 °C and minimum temperatures of −4.6 °C, which denotes the ability to survive extreme climatic conditions.

Table 1. The average seasonal maximum and minimum temperatures and relative humidity values inside the shade mesh during the experimental period.

Season	Temperature (°C)			Relative Humidity (%)		
	Maximum	Minimum	Mean	Maximum	Minimum	Mean
Summer (2021)	37.20	19.87	28.5	86.55	31.08	58.8
Autumn (2021)	37.02	9.85	23.4	73.29	21.83	47.5
Winter (2021–2022)	30.50	4.55	17.5	61.13	21.91	41.5
Spring (2022)	40.13	16.66	28.3	53.58	10.85	32.2

2.2. Relative Water Content

The moisture content in the soil did not produce changes in the RWC among the ecotypes or the *L. corniculatus* variety that was evaluated ($p \leq 0.05$). In general, the highest values of this variable were observed in the autumn season (Figure 1).

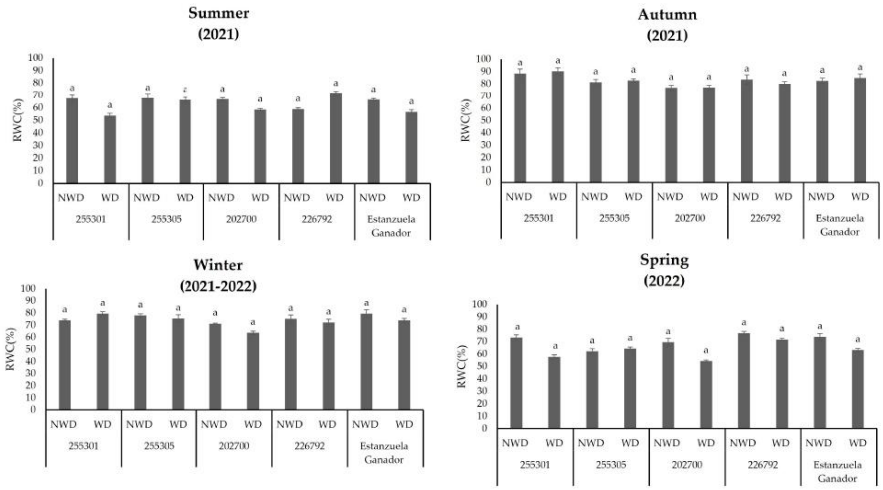

Figure 1. Relative water content (RWC) in different *L. corniculatus* ecotypes and the evaluated variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

2.3. Photosynthetic Pigments

The moisture content in the soil affected the RCI among the evaluated *L. corniculatus* plants. In this study, the RCIs of 255305 and 226792 ecotypes were highest ($p \leq 0.05$) in the

autumn season with values of 244.6 and 236.5. At the same time, the Estanzuela Ganador variety registered the lowest index (147.3) under NWD conditions. The RCI did not vary in the ecotypes as a result of the moisture content in the soil during summer, winter, and spring (Figure 2).

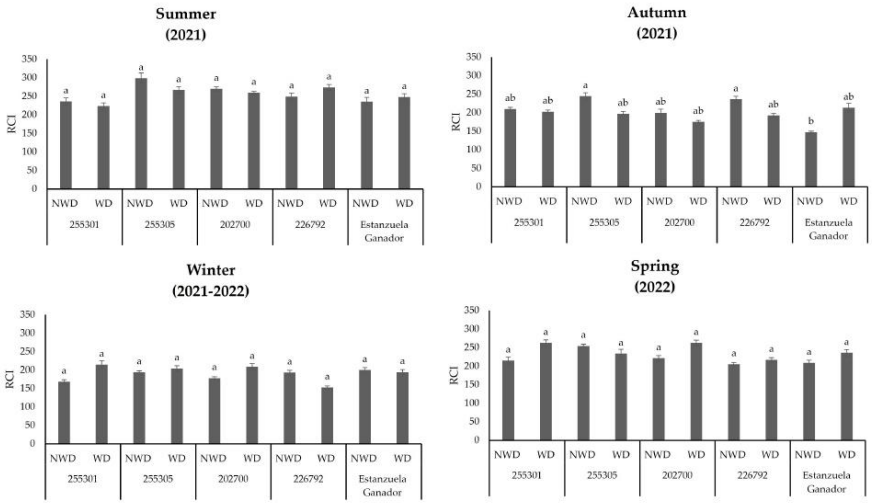

Figure 2. Relative chlorophyll index (RCI) in different *L. corniculatus* ecotypes and the evaluated variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

The moisture content in the soil produced changes in *Chl a* concentration among the *L. corniculatus* ecotypes and the studied variety in the autumn, winter, and spring seasons ($p \leq 0.05$). The 255301 ecotype recorded 187.8, 167.5, and 194.6 mg g^{-1} FW in WD, corresponding to increases of 86.0%, 172.6%, and 16.6%, respectively, in relation to the lower values obtained in the ecotype 202700 under NWD. The ecotype 226792 had the lowest response with values of 100.6, 61.4, and 81 mg g^{-1} FW in NWD. However, in the summer, no variations were observed in this pigment as a result of the effect of soil moisture content (Figure 3).

The plant genetic resources of *L. corniculatus* evaluated in this study did not show changes in chlorophyll b (*Chl b*) content ($p \leq 0.05$) as a result of the effect of soil moisture content, which means that all ecotypes and the Estanzuela Ganador variety were not affected in terms of chlorophyll b as a result of the soil moisture content throughout the seasons (Figure 4).

The 255301 ecotype was outstanding in terms of total chlorophyll content ($p \leq 0.05$) in WD during the autumn, winter, and spring seasons, with values of 279.4, 198.9, and 241.2 mg g^{-1} FW. In winter, the 202700 ecotype and Estanzuela variety showed high *Chl t* concentrations in NWD, with 186.9 and 192 mg g^{-1} FW. The 226792 ecotype had a lower *Chl t* concentration in NWD during the autumn, winter, and spring seasons (150.9, 72.3, and 107.3 mg g^{-1} FW), and a similar response under the same soil moisture content was observed in the 202700 ecotype during the winter (Figure 5).

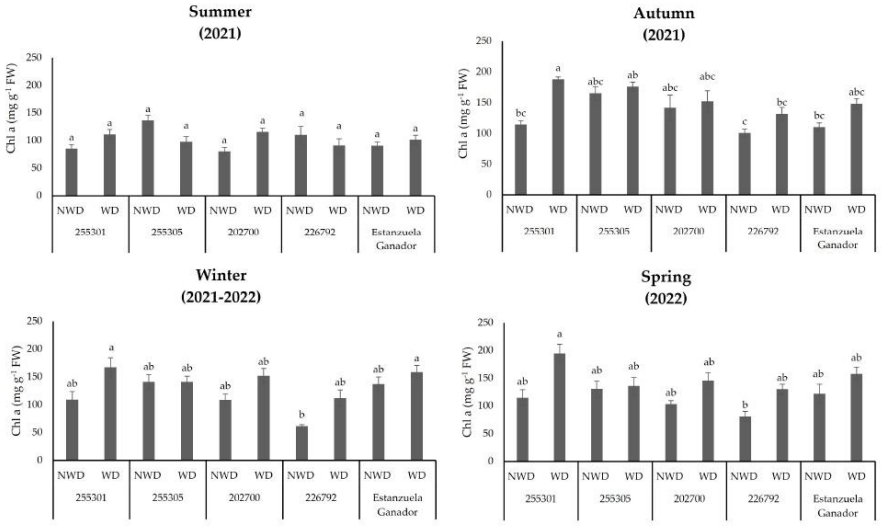

Figure 3. Chlorophyll a (*Chl a*) content in *L. corniculatus* ecotypes and the studied variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

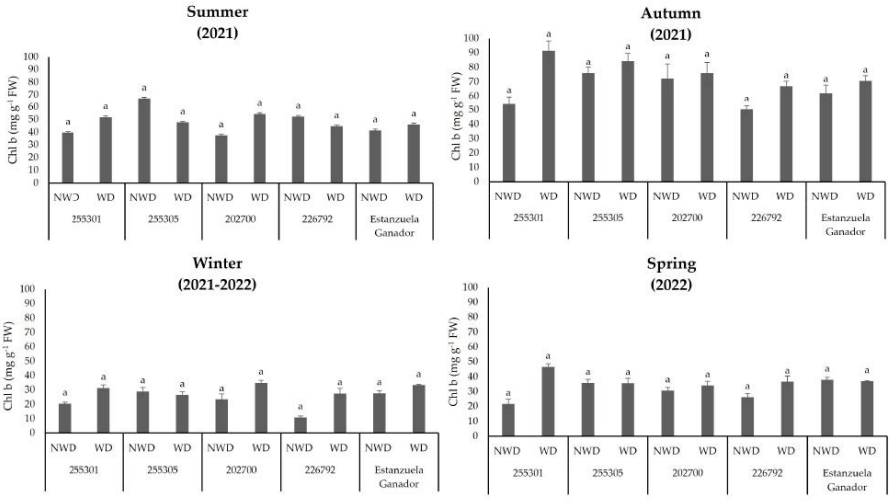

Figure 4. Chlorophyll b (*Chl b*) content in *L. corniculatus* ecotypes and the studied variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

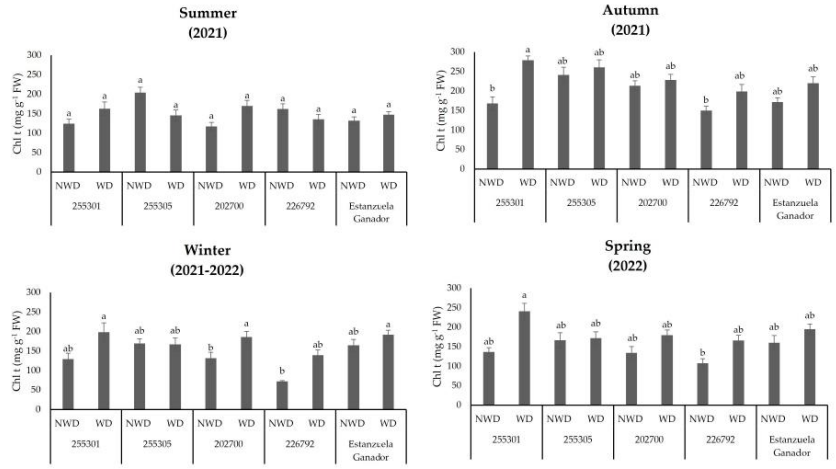

Figure 5. Total chlorophyll (*Chl t*) content in *L. corniculatus* ecotypes and the studied variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

The concentration of carotenoids showed significant effects ($p \leq 0.05$) in the spring season. The highest concentration was in the 255301 ecotype under WD conditions with 53 mg g^{-1} FW. Meanwhile, the 202700 and 226792 ecotypes were lower in NWD with values of 23 and 18.7 m g^{-1} FW, respectively (Figure 6).

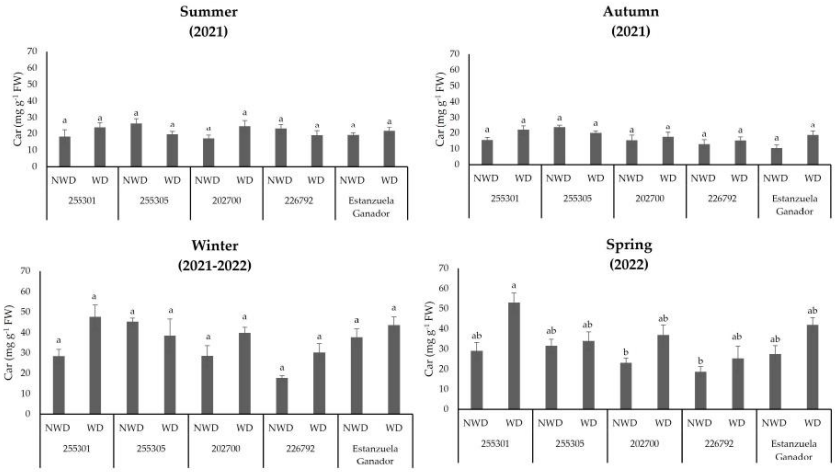

Figure 6. Carotenoid (*Car*) content in *L. corniculatus* ecotypes and the studied variety throughout the seasons in two soil moisture content levels. Values indicate the mean ± standard deviation. Common lowercase letters per column are not statistically significant (Tukey HSD, $p \leq 0.05$). NWD = no water deficit; WD = water deficit.

2.4. Multivariate Analysis

Principal Component Analysis (PCA) shows that the first two Principal Components (PCs) explain 71.8% of the total variance, or at least seven variables measured in the study. The first Principal Component (PC1) explained 42.1%, and the second (PC2) explained 29.7% of the variance. The structure of PC1 is integrated with the contents of *Chl a*, *Chl b*, and *Chl t* in a positive correlation; meanwhile, PC2 is made up of the variables *Car*, T, RCI, and RWC. As mentioned above, the first three variables correlate positively, and the RWC negatively connects with the group (Figure 7).

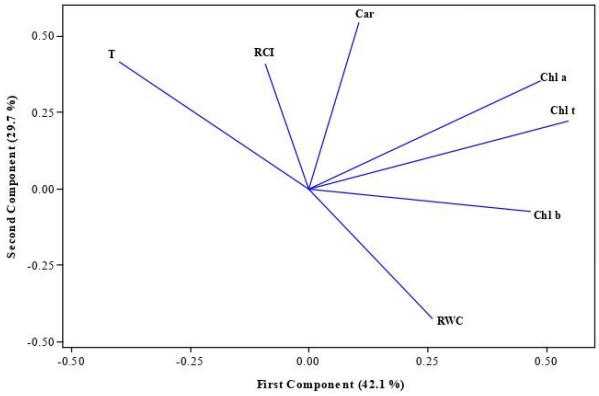

Figure 7. Distribution of variables—temperature (T), relative chlorophyll index (RCI), carotenoids (*Car*), chlorophyll a (*Chl a*), chlorophyll b (*Chl b*), total chlorophyll (*Chl t*), and relative water content (RWC)—in the orthogonal plane defined by the first two principal components (PCs) extracted from 120 observations as part of the experimental pots of four ecotypes and one variety of *L. corniculatus*.

Based on Figure 8, the high cumulative percentage of the total variations observed in PC1 and PC2 is considered the evaluation factor, referring to soil water content (SWC), ecotypes, and the variety of *L. corniculatus* during the evaluation time. The 120 observations in Figure 8 are not grouped in a regular pattern according to ecotypes; however, two large groups of SWC were observed. The first group corresponds to NWD, located in the right part of Figure 8. High concentrations of *Chl a*, *Chl t*, and *Car* characterize this group. In addition, the second group is in the lower left part of the figure, and these observations correspond mainly to WD. In the groups mentioned above, the variables registered low concentrations of *Chl a*, *Chl t*, and *Car*, and healthy concentrations of RWC.

Figure 9 explains the first two PCAs, i.e., how the distribution of 120 observations of the seven variables mentioned above study correspond to the SWC during the different seasons of the year in four ecotypes and one variety of *L. corniculatus* assessed in this study. Six large groups are identified. The black circles, the upper blue triangles, the green rhombuses, and the red squares are predominantly found in the lower left part of Figure 9. These results present low levels of *Chl a*, *Chl b*, *Car*, and *Chl t*, as well as the red squares of RCI and RWC. On the other hand, three groups of observations were identified on the right side of the graph: black sum marks, inverted purple triangles, and pink triangles. A high concentration of *Car* and RCI is characterized by additional black marks. The inverted purple triangles are also remarkably high in *Chl a* and *Chl t*. Meanwhile, the pink triangles are mainly related to low *Chl b* and RWC. All these groups are associated with water deficit.

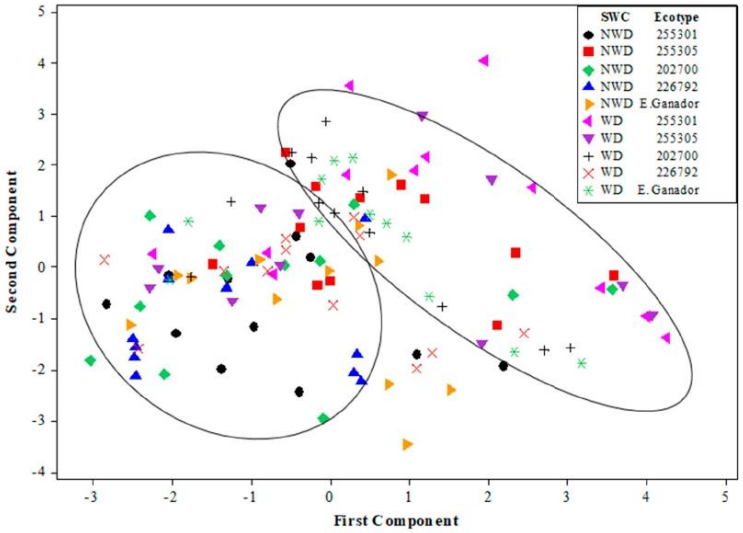

Figure 8. Distribution of 120 observations as part of experimental pots of four ecotypes (255301, 255305, 202700, and 226792) and one variety (Estanzuela Ganador) of *L. corniculatus* in the orthogonal plane defined by the two first principal components (PCs). The experiment was carried out over 413 days under two soil water contents (SWCs): without a water-deficit condition (NWD) of 26 ± 1.5%, and a water-deficit condition (WD) of 22 ± 1.5%.

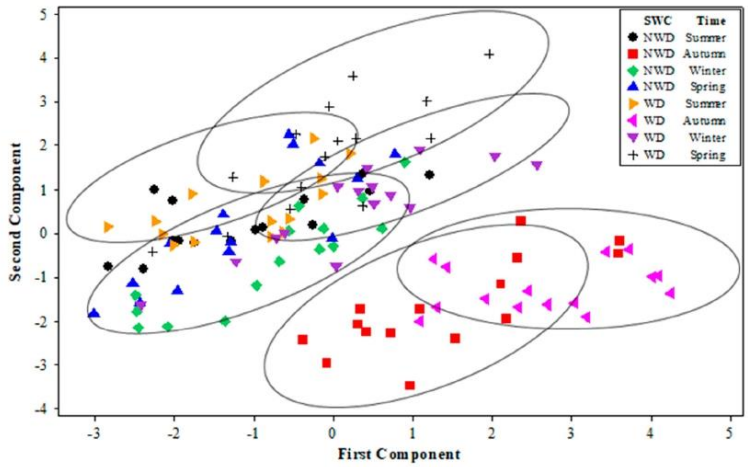

Figure 9. Distribution of 120 observations as part of experimental pots of four ecotypes (255301, 255305, 202700, and 226792) and one variety (Estanzuela Ganador) of *L. corniculatus* in the orthogonal plane defined by the two first principal components (PCs). The experiment was conducted over 413 days under two soil water contents (SWCs). The evaluations were completed four times, corresponding with the seasons (summer, autumn, winter, and spring).

3. Discussion

Even though the study was carried out in shade mesh conditions, the temperature variation was extreme, with values recorded from −4.6 °C in winter to 46.9 °C in summer. This shows the survival capacity of *Lotus*'s genetic materials in extreme environments typical of the northern region of Mexico.

Developing research on the response of photosynthetic pigments in *L. corniculatus* ecotypes under water deficit is vital. Understanding this relationship provides valuable information about the possible adaptive mechanisms of plants in adverse environments, such as water-deficient conditions and extreme temperatures [28]. The genetic materials of *L. corniculatus* evaluated in this study tolerated extreme minimum and maximum temperatures. This shows the plant's resilience to harsh environmental conditions, which indicates the potential adaptive ability of this species of *Lotus* to extreme environments [29]. The evaluation of ecotypes for tolerance to environmental stress, such as water deficit and extreme temperatures, is complex due to the integral and interaction responses in the plants [30].

Environmental temperature influences several physiological processes in plants, including pigment production and its accumulation [31]. Higher temperatures can accelerate metabolic processes, sometimes increasing pigment synthesis [32]. In this study, *L. corniculatus* showed higher concentrations of pigments at higher temperatures (Table 1) during the spring season in soil with optimal soil moisture content, although the responses to this environmental factor in plants are different depending on the plant species [31]. The 255301 and 255305 ecotypes and the Estanzuela Ganador variety presented higher concentrations of pigments when the plant was not in water-deficit conditions (Figure 8). However, excessively high temperatures can cause heat stress and negatively affect pigment production [28].

Responses at the morphological, physiological, biochemical, and genetic levels vary depending on the ecotypes used, the environment, and the ecotype–environment interactions [33]. From a physiological point of view, the flow rate of matter and energy, such as photosynthesis and transpiration, as well as stomatal conductance and relative water content (RWC), are variables frequently explored in response to water deficit [34]. The RWC is directly related to the water status of the plant [35]. In this study, the ecotypes and the studied variety of *L. corniculatus* showed a low response to RWC in both soil water content levels and showed suitable stability when the plant moved from favorable to unfavorable water conditions.

Water stress can decrease chlorophyll content [36]. This reduction in photosynthetic pigment content can be observed as yellowish or brown foliage [37]. However, in this study, the concentrations of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids in water deficit were higher than in no water deficit in most of the genetic plant materials evaluated through autumn, winter, and spring, suggesting an interesting seasonal response of adjustment. The response of an increase in pigment content was shown in the 255301 ecotype, which had a higher range of *Chl a*. Chl was shown in the 255301 ecotype, which had a higher content of *Chl a*, *Chl b*, and *Car* as a tolerance response to water deficit in all seasons except summer. These results are consistent with those reported by Mafakheri et al. [38], who found that photosynthetic pigments are highly sensitive to water deficit and low temperatures.

Other important biochemical indicators in plants' responses to environmental stress are the concentrations of solutes and photosynthetic pigments [39]. In this study, *L. corniculatus* plants showed changes in pigment concentration in RWC with soil water content. The concentrations of these pigments decreased without stress due to the water deficit in the genetic materials evaluated. This suggests that under optimal water conditions in the soil, the tolerant activity promoted by the concentration of photosynthetic pigments is not required without water stress. However, these compounds play an essential role through the osmotic adjustment process [40] in water deficit to allow a state of turgor in the tissues in the face of the risk of plant dehydration [41].

Relative water content (RWC) is an indicator of the water status and hydration level of plants, and based on this information, the capacity to retain water in the tissues can be inferred [42]. Additionally, the influence of RWC on pigment concentration in plants can vary depending on the specific pigment, the plant species, and the adaptation strategies that the plant uses [43]. This study showed that when RWC decreased, *Car*, *Chl a*, and *Chl t* were increased. Some plants can increase their *Car* concentration as a protective mechanism against water deficit [44]. In this study, *Car* concentrations rose notably in the spring and winter seasons in the 255301 ecotype and the Estanzuela Ganador variety with no water deficit. However, a decrease in RWC can generally lead to changes in pigment concentration [42]. These changes were observed in *L. corniculatus* ecotypes; the concentration of *Chl b* decreased when RWC was low.

The RCI measures a plant's relative concentration of chlorophyll [45,46]. During the spring, without a water deficit, the RCI is higher than in other seasons of the year. A higher RCI value indicates that higher chlorophyll content is associated with healthier and more vigorous plants [45]. RCI can monitor plant stress and track physiological changes over time.

In general, *Chl a* content increases during winter in certain plants as a physiological response to low light conditions; this phenomenon is known as winter chlorophyll increase or winter chlorophyll accumulation [47]. *L. corniculatus* ecotypes showed a high concentration of this pigment during the winter season under conditions of water deficit. The highest concentration of *Chl a* was observed in the 255301 ecotype and the Estanzuela Ganador variety under water deficit. The above suggests that the shorter days during winter and the lower angle of radiation incident on the plant result in reduced light intensity and limited exposure to sunlight. Increasing *Chl a* content in plants can maximize and improve their weak absorption capacity and photosynthetic efficiency even under low light conditions [48]. This increase in chlorophyll in winter allows perennial leaf plants such as *L. corniculatus* to continue carrying out essential metabolic processes, including photosynthesis and carbon assimilation, under limited conditions during winter [49].

On the other hand, *Chl b* is one of the primary pigments responsible for capturing light energy during photosynthesis, and can protect plants from excess light energy [50]. In this experimental trial, *L. corniculatus* ecotypes showed the highest pigment concentrations in autumn under water-deficit conditions. This could be associated with a water deficit because plants reduce water loss by closing their stomata, reducing the uptake of carbon dioxide for photosynthesis. This process can cause an imbalance between light absorption and carbon fixation, which could cause photodamage to chlorophyll molecules [51]. However, *Chl b* can help dissipate excess light energy, minimizing the potential for photodamage [50]. According to Figure 3, *Chl b* concentration decreases when T increases. These changes are part of the plant's adaptive responses to maintaining water stress and physiological balance.

When plants are exposed to limited water availability, several changes occur in their photosynthetic pigments. Chlorophyll synthesis can be inhibited, decreasing chlorophyll content, including *Chl a* and *Chl b* [52]. This reduction in pigments can cause visible symptoms of stress (chlorosis or wilting). Despite these changes, photosynthetic pigments remain essential for capturing light energy and protecting the plant from excess light [48].

According to the multivariate analysis (Figure 1), the structure of the first two PCs showed notable bivariate associations, such that when T and RCI increase, RWC and Chl b decrease. There is an interesting case where *Car* also increased *Chl a* and *Chl t*. On the contrary, when *Car* decreased, RWC increased. Furthermore, when *Chl a* was increased, *Chl b* decreased. These results are mainly observed under conditions of optimal soil moisture content.

4. Materials and Methods

4.1. Geographic Location of the Study Area

The experiment was conducted under shade mesh conditions in the Unidad Regional Universitaria de Zonas Aridas experimental field of the Universidad Autonoma Chapingo in Bermejillo Durango, Mexico. The region is located at 25.8° NL and 103.6° WL and an altitude of 1130 m. The climate is arid, with rainfall in the summer and a cool winter, an average potential evaporation of 2000 mm, an average temperature of 21 °C, maximum of 33.7 °C and minimum of 7.5 °C, and an average annual precipitation of 258 mm [53].

4.2. Experimental Design

A randomized block experimental design with three repetitions was used. Ten treatments were established as a product of a 2 × 5 factorial, and thirty treatments were carried out in the experimental area. The first variation factor was the water content in the soil: no water deficit (NWD) corresponding to 100% field capacity (CC), and water deficit (WD) at 85.4% CC. The second variation factor comprised four ecotypes of *L. corniculatus* with identification codes 255301, 255305, 202700, and 226792, and the Estanzuela Ganador variety, which were procured from France, Italy, Uruguay, Canada, and Uruguay, respectively.

The experimental unit (EU) comprised one tiller of *L. corniculatus* per pot with three replicates. Previously, the seedlings were vegetatively reproduced in black plastic bags of 1 Kg capacity in soil mixed with compost. Subsequently, after two months, one plant—a rhizome with an average height of 30 cm and a tiller radius of 5 cm—was transplanted into a rigid plastic pot with 18 kg of soil capacity. The soil was a substrate prepared with a 50:30:20 soil, compost, and sand ratio. According to the physical and chemical analysis of the substrate, it had a sandy loam texture with 26% silt, 22% clay, and 52% sand, with a pH of 7.73, electrical conductivity (EC) of 7.47 dS m^{-1}, and apparent density of 1.46 g cm^{-3}. The experiment was carried out from March 2021 to May 2022.

The calculations of the field capacity (FC) and the permanent wilting point (PWP) were carried out using the membrane pot method using the soil moisture drawdown curve expressed in energy tension (MPa) according to Richards [54]. The field capacity (FC) was 27.5% and the permanent wilting point (PMP) was 17.5%.

4.3. Experimental Monitoring

The climatic conditions of temperature and relative humidity within the shade mesh were measured using a digital thermometer–hydrometer (Model OUS-WA62, ORIA, China). Irrigation was applied to each pot based on soil moisture content, which varied between 26.5% ± 1 and 23.5% ± 1 for the NWD and WD treatments. Since the field capacity was 27.5%, 23.5% of the NWD treatment average corresponded to 85.4% of FC. The soil moisture content in each pot was monitored with a real-time meter (Model MO750, Extech Instruments Co., Laredo, TX, USA).

In the first 15 days after transplanting into the pots, irrigation was at CC (27.5%) for all treatments. Subsequently, soil moisture content was restricted until the differentiated ranges of 26.5% ± 1 and 23.5% ± 1 were maintained. The irrigation intervals were defined by applying water up to the upper limit of each soil moisture treatment, corresponding to 27.5% for the WD treatment and 24.5% for the NWD treatment, and applying recovery irrigation when the lower limits for each soil moisture content were reached, corresponding to 25.5% and 22.5%, respectively.

The different genetic materials of *L. corniculatus* were homogenized. The plant canopy was cut 62 days after transplanting (DAT) with the use of pruning shears at 6 cm above the level of the potting substrate; for that, a plastic ring was used that allowed the cutting height to be standardized [55]. From that date, seasonal cutting intervals were defined: two cuts at intervals of 42 days in each seasonal period of summer, autumn, and spring, and 92 days in winter (a single cut). The latter was defined by the slow growth of the plant.

4.4. Measured Variables

4.4.1. Relative Water Content (RWC)

The relative water content of the leaves of *L. corniculatus* was calculated using the formula proposed by Browne et al. [56], for which the saturated weight was recorded after immersing the tissue sample in distilled water for 24 h. The dry biomass was obtained by drying the plant in an oven with air circulation at 65 °C until its constant weight was reached. The results are expressed as a percentage (%) using the following formula:

$$RWC = [((FWB - DWB))/(DWB - WSB)] \times 100 \quad (1)$$

where RWC = relative water content, FWB = fresh weight of biomass, DWB = dry weight of biomass, and DSB = weight of saturated biomass.

4.4.2. Relative Chlorophyll Index (RCI)

The measurement of this variable was carried out between the fourth and fifth leaves from the flag leaf, as suggested by De Lima Vasconcelos et al. [57], using the CM 1000 chlorophyll meter (Mod 29950, Brand Spectrum Technologies Inc., Aurora, IL 60504, USA). The measurement was performed every ten days from the cut-off between 9:00 and 10:00.

4.4.3. Photosynthetic Pigments

Photosynthetic pigments were determined using the Wellburn method [58] described, in which ten fresh leaves were cut into circles of 5 mm in diameter. The leaf samples were placed in a test tube; then, 10 mL of methanol (CH_3OH) was added and incubated in the dark at room temperature for 24 h. Absorbance (A) was measured with a spectrophotometer at 470 nm (carotenoids), 653 nm (chlorophyll b, *Chl b*), and 666 nm (chlorophyll a, *Chl a*). The calculation of the pigment concentration was carried out with the following formulas [58]:

$$Chl\ a\text{: } [15.65 \cdot (A_{666}) - 7.34 \cdot (A_{653})]\ [V/1000 \times W] \quad (2)$$

$$Chl\ b\text{: } [27.05 \cdot (A_{653}) - 11.21 \cdot (A_{666})]\ [V/1000 \times W] \quad (3)$$

$$Car\text{: } [[1000 \cdot (A_{470}) - 2.86 \cdot (\text{Chl } a) - 129.2\ (\text{Chl } b)]/221]\ [V/1000 \times W] \quad (4)$$

$$Chl\ t\text{: Chl } a + \text{Chl } b \quad (5)$$

where V = Extraction volume and W = fresh weight of leaf sample. The concentrations of Chl *a*, Chl *b*, and *Car* are expressed as mg g^{-1} fresh weight (FW).

4.5. Data Analysis

The data were analyzed with a test of means (Tukey) and Principal Component Analysis (PCA) using the statistical programs PASW Statistics for Windows, version 18.0 (SPSS Inc., Chicago, IL, USA), and Minitab Version 16.2.4 (Minitab, LLC, State College, PA, USA).

5. Conclusions

L. corniculatus ecotypes showed potential for adaptation to extreme environments of high and low temperatures throughout the seasons, with statistical differences depending on the season. The relative water content was almost stable among the evaluated ecotypes, which denotes response stability to water-deficit conditions. Total chlorophyll and carotenoid content were the leading indicators of response to water deficit in the 25301 ecotype in most seasons, except during the summer. In contrast, the 202700 ecotype and the Estanzuela Ganador variety responded during the winter, also in water deficit. The photosynthetic pigments formed a first response cluster, and the carotenoids in water deficit formed another cluster, which explains 71.8% of the variance. Some bivariate associations were identified, the first being an inversely proportional relationship between

environmental temperature and the chlorophyll index with the relative content of water and chlorophyll *b*. In contrast, the content of chlorophyll *a* and total chlorophyll with carotenoid content was an inversely proportional relationship. This study provides important, crucial, essential information on the response of *Lotus corniculatus* to water deficit, which is valuable for future study programs applied to tolerance to water deficit in extreme environments.

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V. GENERAL CONCLUSIONS

In conclusion, this research contributes to the ongoing efforts aimed at addressing water scarcity in agriculture by highlighting the potential of alternative crops like *Lotus corniculatus* L. and elucidating their adaptive mechanisms. By advancing our understanding of plant responses to water deficit, this study lays the foundation for future initiatives focused on sustainable agriculture and environmental resilience.

The comprehensive evaluation of *Lotus corniculatus* ecotypes presented in this study offers valuable insights into the plant's adaptive mechanisms to extreme environmental conditions, particularly water deficit. The findings highlight the remarkable potential of certain ecotypes, such as 255301 and 255305, in adapting to high and low temperatures across different seasons. Notably, these ecotypes exhibited stability in relative water content, indicating their resilience to water-deficit conditions.

Photosynthetic pigments, notably chlorophyll, and carotenoids, emerged as primary indicators of response to water deficit, forming distinct clusters that explain a significant portion of the variance. Bivariate associations further underscored the intricate relationships between environmental factors and pigment concentrations, providing deeper insights into the physiological responses of *L.corniculatus* to water stress.

Moreover, the study elucidated the ecotype-specific responses in secondary metabolite concentrations and antioxidant activity under water deficit conditions. While the 255301 ecotype demonstrated an increase in phenols, flavonoids, and tannins, the 255305 ecotype exhibited enhanced tannin concentration and antioxidant activity, particularly during winter when temperatures were lower. This variability in response underscores the adaptability and versatility of *L. corniculatus* as a potential alternative forage crop in arid environments.

Additionally, the study identified the 255301 ecotype's unique response in increasing saponin concentration as a mitigation strategy against stress induced by high temperatures and water deficit in summer. Such diversity in responses further emphasizes the potential of *L. corniculatus* to contribute to sustainable agriculture in water-limited regions.

Overall, this research provides crucial insights into the physiological and biochemical responses of *Lotus corniculatus* to water deficit, laying the groundwork for future studies aimed at enhancing tolerance to water scarcity and other abiotic stress in extreme environments. By harnessing the adaptive traits of resilient ecotypes, there is immense potential to advance sustainable agricultural practices, ensure food security, and promote environmental resilience amidst escalating climate change challenges.