

Article

Selecting High Forage-Yielding Alfalfa Populations in a Mediterranean Drought-Prone Environment Using High-Throughput Phenotyping

Hamza Armghan Noushahi ¹ , Luis Inostroza ² , Viviana Barahona ³, Soledad Espinoza ², Carlos Ovalle ², Katherine Quitral ¹, Gustavo A. Lobos ¹ , Fernando P. Guerra ⁴ , Shawn C. Kefauver ^{5,*} , and Alejandro del Pozo ¹ 

¹ Centro de Mejoramiento Genético y Fenómica Vegetal, Facultad de Ciencias Agrarias, Universidad de Talca, Talca 3460000, Chile; hamzanaushahi143@gmail.com (H.A.N.); kquitral18@alumnos.utalca.cl (K.Q.); globosp@utalca.cl (G.A.L.); adelpozo@utalca.cl (A.d.P.)

² CRI-Quilamapu, Instituto de Investigaciones Agropecuaria, Chillán 3780000, Chile; linostroza@inia.cl (L.I.); soledad.espinoza@inia.cl (S.E.); covalle.inia@gmail.com (C.O.)

³ CRI-Raihuén, Instituto de investigaciones Agropecuaria, Cauquenes 3690000, Chile; viviana.barahona@inia.cl

⁴ Instituto de Ciencias Biológicas, Universidad de Talca, Talca 3460000, Chile; fguerra@utalca.cl

⁵ Integrative Crop Ecophysiology Group, Plant Physiology Section, Faculty of Biology, University of Barcelona, 08028 Barcelona, Spain

* Correspondence: sckefauver@ub.edu

Abstract: Alfalfa is a deep-rooted perennial forage crop with diverse drought-tolerant traits. This study evaluated 250 alfalfa half-sib populations over three growing seasons (2021–2023) under irrigated and rainfed conditions in the Mediterranean drought-prone region of Central Chile (Cauquenes), aiming to identify high-yielding, drought-tolerant populations using remote sensing. Specifically, we assessed RGB-derived indices and canopy temperature difference (CTD; $T_c - T_a$) as proxies for forage yield (FY). The results showed considerable variation in FY across populations. Under rainfed conditions, winter FY ranged from 1.4 to 6.1 Mg ha⁻¹ and total FY from 3.7 to 14.7 Mg ha⁻¹. Under irrigation, winter FY reached up to 8.2 Mg ha⁻¹ and total FY up to 25.1 Mg ha⁻¹. The AlfaL4-5 (SARDI7), AlfaL57-7 (WL903), and AlfaL62-9 (Baldrich350) populations consistently produced the highest yields across regimes. RGB indices such as hue, saturation, b^* , v^* , GA, and GGA positively correlated with FY, while intensity, lightness, a^* , and u^* correlated negatively. CTD showed a significant negative correlation with FY across all seasons and water regimes. These findings highlight the potential of RGB imaging and CTD as effective, high-throughput field phenotyping tools for selecting drought-resilient alfalfa genotypes in Mediterranean environments.

Keywords: canopy temperature; forage yield; unmanned aerial vehicles (UAV); RGB images; thermal camera



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1. Introduction

Alfalfa (*Medicago sativa* L.) is called the “queen of forages” because of its exceptional attributes, including high forage yield (FY) and quality, along with its immense nutritional value for livestock [1–4]. It is mostly used as silage, hay, and fodder for grazing animals, as well as a semi-processed protein source for non-grazing animals such as chickens and hogs. It is a significant source of vitamin A and ten other vitamins, and it provides a greater amount of digestible energy, as well as protein per hectare, than most other crops [5].

Due to its higher nutritional values, it is cultivated under a variety of climates, including the Mediterranean type, and under rainfed conditions where it is exposed to prolonged drought periods [6].

Central Chile exhibits a Mediterranean-type climate and a prolonged drought lasting five to six months, usually from middle spring to the end of summer (October–March in the southern hemisphere). Furthermore, since 2010, there has been a consistent dry year pattern in Central Chile, with deficits in mean annual rainfall ranging from 20 to 45%. There have been few events like it over the last century, and the longest drought on record is being called a megadrought [7]. Consequently, less accessibility of forage from summer to autumn severely affects livestock productivity, which depends on annual pastures. New possibilities in dryland agriculture have arisen with the introduction of deep-rooted perennial legumes, including alfalfa [8–10]. These legumes not only extend the growth period of the feed base from early summer to autumn [11] but also provide alternative approaches to farming [9].

Alfalfa has developed a variety of drought resistance mechanisms at the physiological, morphological, and molecular levels to respond and adapt to drought stress [12]. For instance, alfalfa possesses a higher root-to-shoot ratio and a larger lateral root spread when compared with those of other annual forage crops [13,14], helping to boost water absorption from the soil and minimize water consumption [14]. Furthermore, plants undergo a trade-off where they balance the need to conserve water with the requirement to accumulate carbon through photosynthesis. For instance, severe or even moderate drought stress reduces the leaf relative water content (RWC) and gas exchange of alfalfa, albeit they show signs of recovery upon re-establishment of the water supply [15,16].

The autotetraploid nature and outcrossing properties of alfalfa make it more challenging to attain genetic advancements in regard to economic features [17]. According to Muller [18], the genetic diversity of cultivated alfalfa has declined by approximately 30% compared to that of its wild populations due to domestication, making commercial cultivars less stress tolerant. Studying genetic diversity is important in plant genetic improvement programs in order to identify genetic advancement for specific population traits [19]. For this reason, it is crucial to analyze variability, comprehend productivity inheritance, and utilize descriptors at the population level to assist breeders in selecting the most effective methods [20]. Breeders that use progeny testing to select individuals, both within and between progenies, and to estimate genetic parameters have found it to be a very useful tool. In addition, selection benefits are considered when evaluating genetic variance in order to identify individuals that exhibit superior agronomic characteristics and establish a new breeding cycle [21]. To estimate genetic diversity among half-sib populations and identify the most effective discriminating descriptors, multivariate methods involving genotypic, environmental, and phenotypic associations are often employed [22].

Traditional methods for screening drought-tolerant cultivars rely on the direct evaluations of biomass and yield and handheld proximal sensors, which are both labor-intensive and time-consuming [23]. Although some plant traits, such as early vigor, ground cover, stomatal conductance, photosynthetic rate, crown reserves, root system, and leaf area index, can be used to identify drought tolerance during the growing season, measuring these traits individually in large populations is labor-intensive, time-consuming, and expensive. Moreover, most classic phenotyping techniques are destructive, measure only a few traits, and impede progress in functional genomics and plant breeding research [24,25]. Therefore, when testing a significant number of genotypes, breeders need a simple, rapid, non-destructive, and objective selection method to ease the challenges associated with selecting high-yield and drought-resistant genotypes [26]. To meet the challenges of screen-

ing a large number of accessions or genotypes, high-throughput plant phenomics is rapidly evolving, and phenotyping systems are gaining recognition [27,28].

Remote-sensing-based high-throughput plant phenotyping (HTPP) techniques have been widely employed in recent years to bridge the gap among phenotypes, genotypes, and climatic influences [29–31]. At the field level, traits with the necessary time–frequency and spatial resolution can be collected using thermal and red–green–blue (RGB) cameras installed on unmanned aerial vehicle (UAV)-based remote-sensing HTPP systems to screen alfalfa genotypes for drought tolerance and yield [32,33], drought prediction [34], and impacts of irrigation [35] in agriculture. Furthermore, greenness, leaf pigment content, including secondary metabolites in response to drought stress [36], and other traits at the canopy can be assessed using RGB images [37] processed by image analysis softwares.

Temperature is a key environmental factor affecting various physiological processes in alfalfa. Canopy temperature has emerged as a rapid and informative indicator for characterizing drought-related traits across genotypes [33,38,39]. Recent research supports the use of unmanned aerial vehicles (UAVs) as an effective tool for detecting drought tolerance in diverse crop populations [40,41], including alfalfa [33,42]. However, further studies are needed to quantitatively evaluate the potential of UAV-based multimodal imaging—particularly thermal and RGB sensors—for selecting drought-tolerant alfalfa genotypes based on forage yield (FY). This study underscores the growing importance of remote sensing technologies in alfalfa breeding and management under drought conditions. The integration of RGB and thermal indices from UAV-mounted sensors offers a robust framework for the large-scale monitoring and prediction of drought responses in alfalfa. In this study, we hypothesized that field phenotyping of an alfalfa drought-tolerant population (alfalfa-DTP) using remote sensing technologies would support the identification of high forage-yielding alfalfa populations under rainfed and irrigated conditions in a Mediterranean drought-prone environment. The objectives were to (i) develop new field phenotyping methods using remote sensing technologies, including RGB and thermal cameras, to identify high-yielding and drought-tolerant populations, and (ii) select outstanding genetic material for plant breeding. We evaluated 250 alfalfa half-sib populations containing different genetic makeups, grown for three growing seasons (2021 to 2023), under both irrigated and rainfed conditions in a Mediterranean drought-prone environment. Field phenotyping involved the use of remote sensing technology, including RGB and thermal cameras mounted on UAVs. After each harvest, the FY was calculated for each population in the panel and correlated with recorded remote sensing data.

2. Materials and Methods

2.1. Plant Material

In our earlier research, 70 alfalfa accessions—believed to possess drought tolerance traits—were introduced to Chile from various regions worldwide and evaluated over four growing seasons [43,44]. Based on their superior breeding values for dry matter production and plant persistence, 25 accessions were selected. From each of these, 10 genotypes were chosen and clonally propagated by rooting five-centimeter stem sections, producing five clones per genotype. The 250 alfalfa genotypes were placed in a randomized complete block design with five replications (clones) to perform a polycross. Inside an insect-proof cage, plants were freely intercrossed with the assistance of bumble bees (*Bombus terrestris* L.). The bees played an important role in facilitating the cross-pollination of the plants, allowing for the natural exchange of genetic material between different individuals. This controlled environment ensures that the pollination process occurs exclusively through the interaction between the plants and the bumble bees, maintaining the integrity of the experimental setup and preventing unwanted external factors from interfering with the crossbreeding

process. By allowing plants to freely intercross with the assistance of bumble bees inside an insect-proof cage, a total of 250 half-sib progenies were obtained. The experimental flowchart outlining this study is shown in Figure 1.

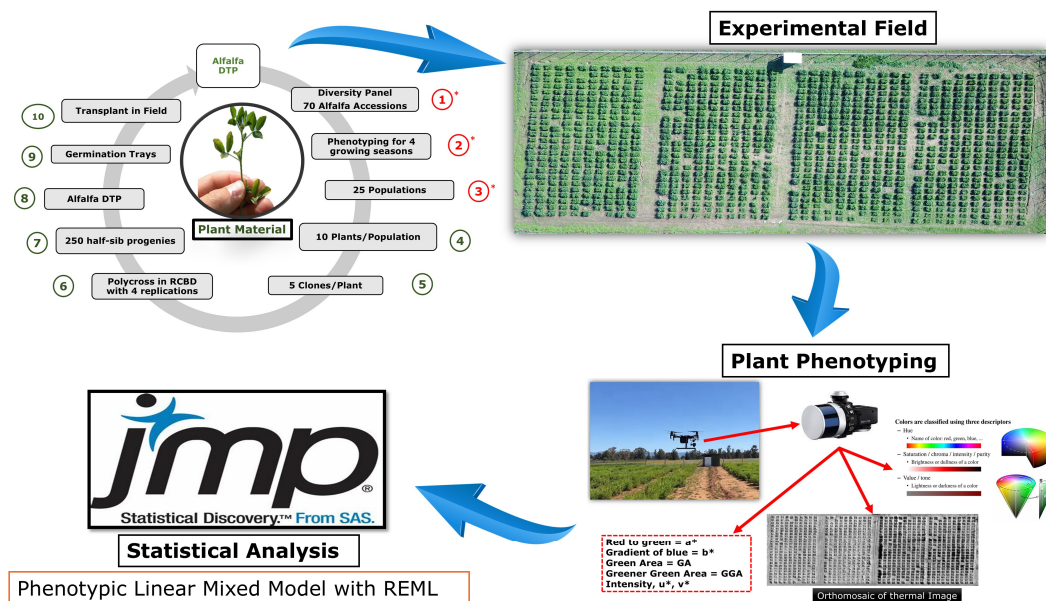


Figure 1. The experimental flowchart outlines this study process, from the creating of plant material to the statistical analysis of 250 alfalfa half-sib populations grown under both rainfed and irrigated conditions. Red stars indicate our previous study, published by Inostroza et al. (2021) [44].

These progenies were referred to as the alfalfa drought-tolerant population (Alfalfa DTP). A total of 250 half-sib progeny seeds were planted in germination trays with 200 holes each, utilizing peat moss (Kekkila, Finland) as the substrate. The substrate was irrigated daily and periodically fertilized using a solution of 1.1 gL^{-1} Phostrogen (Bayer, UK). This regular irrigation and controlled fertilization regimen provided the necessary moisture and nutrients to support the growth and development of the planted half-sib progenies. After inoculating the seedlings with a *Sinorhizobium meliloti* (strain WSM2141) suspension, they were cultivated for two months in a greenhouse. To prepare the seedlings for transplantation, they were transferred to a shelter one week before the actual transplanting process, allowing them to undergo a hardening process.

2.2. Experimental Site and Plant Establishment

The 250 half-sib seedlings were transplanted at the Cauquenes Research Station of INIA, Chile, situated at coordinates $35^{\circ}57'S$ and $72^{\circ}19'W$. This specific location was chosen as the site for further cultivation and evaluation of the alfalfa progenies. Cauquenes, being a Mediterranean drought-prone region, experiences specific climatic conditions. During the period of 2020–2023, the annual mean air temperature was 14.7°C , with minimum and maximum air temperatures in January reaching 11.2°C and 31.7°C , respectively (Table 1). In January and February, the mean maximum temperature was $>31.5^{\circ}\text{C}$, and the coldest month (July) exhibited temperatures $<2.5^{\circ}\text{C}$ in 2020–2023. The mean annual precipitation recorded in the region was 485 mm, which was below the long-term mean annual precipitation level (650 mm). After transplanting the 250 half-sib progenies in the experimental field in October 2020, the six months of drought period from November 2020–April 2021 and November 2021–April 2022 recorded 43.2 mm and 47.6 mm of precipitation, respectively. This drought condition was more severe from November 2022–April 2023, and the recorded precipitation was 30.2 mm (Table 1).

Table 1. Mean, minimum, and maximum monthly and annual temperatures, as well as monthly and annual precipitation, recorded at Cauquenes (35°57'S; 72°19'W), Central Chile.

	Year	January	February	March	April	May	June	July	August	September	October	November	December	Annual
Tmean (°C)	2020	22.4	21.5	19.9	16.4	12.8	8.7	9	9.4	11.5	14.4	17.8	19.8	15.3
	2021	21.7	20.8	19.3	15.6	11.4	9.8	8.2	9.7	11.1	14.1	17.4	20.2	14.9
	2022	20.2	20.8	18.6	14	9.6	6.6	7.1	8.8	10.9	13.2	18.4	20.5	14.1
	2023	21.5	22.6	19.9	15.9	8.4	10.2	8.9	9.8	10.8	12.6	14.4	18.1	14.4
Tmin (°C)	2020	12	10.6	9.3	6.7	4.2	3.5	3.2	2.7	3.5	4.7	7.7	9.2	6.4
	2021	11.8	10.7	9.5	7.0	4.6	3.8	1.3	3.3	3.1	4.9	7.0	9.7	6.4
	2022	9.6	10.0	8.6	5.6	2.6	0.9	1.7	1.5	2.7	4.7	8.4	10.7	5.6
	2023	11.5	11.9	9.2	7.1	-1.9	5.3	3.7	4.1	5.5	4.6	6.7	9.1	6.4
Tmax (°C)	2020	32.8	32.5	30.6	26	21.4	13.9	14.8	16.1	19.6	24.2	28	30.5	24.2
	2021	31.7	30.8	29.1	24.3	18.1	15.8	15	16	19.2	23.2	27.8	30.8	23.5
	2022	30.7	31.5	28.7	22.4	16.5	12.3	12.4	16.1	19.1	21.8	28.3	30.4	22.5
	2023	31.6	33.3	30.5	24.7	18.6	15.1	14	15.4	16.2	20.5	22.1	27.2	22.4
Precipitation (mm)	2020	2.5	0.0	0.1	47.8	38.5	177.1	14.1	55.2	29.9	18.5	0.0	0.0	383.7
	2021	19.6	0.6	0.2	22.8	127.7	58.2	45.8	69.3	45.8	7.0	0.0	0.0	397.0
	2022	0	10.6	3.2	33.8	50.2	147.2	136	57.9	15.2	9.9	16.2	0.0	480.2
	2023	1.6	0.0	0.0	12.4	53.2	148.6	123.5	165.2	122.9	28.4	21.3	1.3	678.4

The experimental plots consisted of four rows, each with a length of 1 m, placed 20 cm apart. On each row, seedlings were distributed every 10 cm (40 plants per plot). Two trials were established according to the water regime, with one subjected to rainfed conditions and the other receiving irrigation (15 mm every week). Both experiments were conducted using an α -lattice design, with two replications. Each replicate comprised 10 partial blocks, including 25 half-sib progenies. The entire experimental field contained a total of 1000 plots (Figure 2). In field trials with a high number of genotypes, the α -lattice design allows for maximizing precision, minimizing experimental error, and effectively handling spatial variation.



Figure 2. Visual of alfalfa field experimental design. The planting site is divided into two water regimes, irrigated and rainfed. (A) corresponds to rainfed alfalfa, and (B) corresponds to supplemented irrigated alfalfa. R1 and R2 represent replication 1 and replication 2, respectively.

The soil at the site is categorized as Ultic Palexeralfs and exhibits specific characteristics. It was measured to have a pH of 5.7 (measure in a water solution at a ratio of 1:2.5, within a depth of 0–20 cm). The soil also contained 2.7% organic matter, with available nitrogen (N), phosphorus (P), and potassium (K) contents in the top 20 cm measured at 45 mg kg⁻¹, 17 mg kg⁻¹, and 250 mg kg⁻¹, respectively. Prior to transplantation, the soil was adequately prepared by employing a chisel plow and disc harrows. For soil enrichment, each plot was supplemented with specific fertilizers, including 200 kg ha⁻¹ of triple superphosphate (46% P₂O₅), 2000 kg ha⁻¹ of CaCO₃, 100 kg ha⁻¹ of potassium sulphate (50% K₂O and 54% SO₄), and 20 kg ha⁻¹ of boronatrocalcite (11% B).

2.3. Plant Phenotyping

To assess FY, each plot of 0.8 m² was harvested by cutting 5 cm aboveground, and the fresh weight was measured using a scale, employing barcode scanning for accurate tracking. For dry matter analysis, the harvested samples were oven-dried at 65 °C with forced air ventilation until a constant weight was reached. A total number of 13 biomass cuts were performed over three growing seasons (2021–2023). The total annual/seasonal FY was calculated by combining the cuts from each year (season).

Just before each alfalfa cut for three growing alfalfa seasons (2020–2023), aerial RGB images for each plot were captured using an RGB 24 mm camera (featuring a 1/2.3" 12 MP sensor) mounted on a DJI Mavic Pro UAV (DJI, Shenzhen, China). The drone flights took place autonomously, with flight control provided by Pix4D software version 4.5.6 (Pix4D Lausanne, Switzerland). The flights were planned according to the following criteria: (a) flight height: 30 m; (b) image overlap: 85%; (c) flight speed: 2.3 m/s; (d) image capture frequency: 1.5 s; and (e) weather conditions: a clear and sunny day, with wind speeds

below 15 km/h. Aerial imagery orthomosaics were generated from RGB photographs using Agisoft PhotoScan Professional version 1.4.5 (Agisoft LLC, St. Petersburg, Russia), utilizing all of the images captured from the UAV. Images from each individual plot were extracted and saved using the open-source image analysis platform Fiji (ImageJ, version Java 1.8.0). Fiji is an extended version of ImageJ developed by the community and maintained by the Max Planck Institute of Molecular Cell Biology and Genetics in Dresden, Germany. Subsequently, the Mosaic tool software and Cereal-Scanner plugin, developed by Shawn Kefauver from the University of Barcelona, were utilized for further analysis (available at <https://gitlab.com/sckefauver/cerealscanner> (accessed on 6 March 2025)). This plugin extracts various color parameters from images, including hue, intensity, saturation, lightness, a^* , b^* , u^* , and v^* . CIE-LAB represents a three-dimensional color space, where L^* denotes luminosity (0 for black, 100 for white), a^* ranges from red to green (negative for greenish, positive for reddish), and b^* represents the blue gradient (negative for blue, positive for yellowish). HIS (hue, saturation, intensity) is a model commonly used in image analysis and computational vision applications. This model made it possible to detect and differentiate objects effectively, as well as provided other applications. The distance from the vertical axis (luminosity) corresponds to saturation, and the distance along the vertical axis (luminosity) represents intensity. Additionally, Fiji provided information on other parameters associated with the active photosynthetic canopy and crop senescence. These additional parameters encompassed green area (GA = pixels with $60^\circ < \text{hue} < 180^\circ$) and greener green area (GGA = pixels with $80^\circ < \text{hue} < 180^\circ$). GA and GGA are both methods used to analyze the percentage of green pixels in an image, although GGA differs from GA in that it specifically excludes pixels with yellowish-green shades. This exclusion helps provide a more accurate representation of the photosynthetically active biomass and the senescence of the leaf, enabling a more precise characterization of the plant's condition.

Canopy temperature (T_c) is a key measurement in regard to the use of thermal remote sensing for assessing crop water status. T_c was recorded using a Zenmuse XT2 13 mm (FLIR, Wilsonville, OR, USA) thermal camera placed on a Dji Matrice 200 drone (Dajiang Innovation Technology Co. Ltd., Shenzhen, China). The UAV's flight altitude was 30 m above the ground. The camera was activated every 1.5 s by an intervalometer to ensure 85% forward image overlaps, while flying at a constant speed of 1 ms^{-1} . Twenty minutes prior to each flight, the camera was turned on to regulate the internal temperature [45]. Thermal photos were captured on a sunny day prior to measuring the FY of each cut across three growing seasons. Aerial images were processed using Pix4D Enterprise's orthomosaic analysis and photogrammetry algorithms to generate high-resolution digital orthomosaics. The meteorological station in the Cauquenes experimental field (<https://agrometeorologia.cl/#> (accessed on 6 March 2025)) provided the air temperature (T_a) for each day of the drone flights. The canopy temperature difference (CTD) was determined by subtracting T_c from T_a [33] (Equation (1)).

$$\text{CTD} = T_c - T_a \quad (1)$$

2.4. Statistical Analyses

Analysis of variance (ANOVA) was conducted using JMP-Genomics version 10 (SAS Institute, Cary, NC, USA) for forage yield (FY) from 2021–2023, considering fixed factors (year, water regimen, and population), with partial and full interaction models applied to assess variance and minimize confounding effects. The variance components were estimated by using a phenotypic linear mixed model, with the restricted maximum likelihood (REML) method implemented in JMP-Genomics. The estimation was based on the following model (Equation (2)):

$$Y_{ijkl} = G_i + W_j + S_k + G \times W_{ij} + G \times S_{ik} + W \times S_{jk} + G \times W \times S_{ijk} + \varepsilon_{ijkl} \quad (2)$$

where Y_{ijkl} is the phenotypic value in the j -th water regimen (W) for the i -th population (G) in the k = th growing year (S) and the l -th replicate (r), and W is the fixed effect of water regimen, S is the fixed effect of growing years/seasons, G is the random effect of the population, $G \times W$ is the random interaction effect of water by population, $G \times S$ is the random interaction effect of growing years by population, $W \times S$ is the fixed interaction effect of growing years by water regimen, $G \times W \times S$ is the random interaction effect of growing years and population by water regimen, and ε the experimental error. The estimated variance components were utilized to calculate the broad-sense heritability (H^2) on a half-sib mean basis [46] (Equation (3)).

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \frac{\sigma_g^2 \times 1}{w} + \frac{\sigma_g^2 \times s}{s} + \frac{\sigma_g^2 \times s \times 1}{sw} + \frac{\sigma_e^2}{swr}} \quad (3)$$

Bivariate analyses, built upon the model, were conducted to estimate the covariance and correlation between FY production and RGB, as well as the thermal indices.

3. Results

3.1. Forage Yield

Winter and total FY in the years 2021, 2022, and 2023 among different half-sib population were significantly different (Supplementary Table S1; Figure 3). In the years 2021 and 2022, the winter FY exhibited comparable results under the two water regimes; however, in 2023, there was a slightly higher recorded yield under irrigated conditions (Figure 3A). The total FY in 2021 showed similar results under both water regimes, but it was significantly higher in 2022 and 2023 ($p < 0.0001$; Table 1 and Figure 3C). Also, the interaction $W \times G$ was significant for winter FY, and the $W \times S$ and $G \times W \times S$ were significant for winter and total FY (Supplementary Table S1). The broad-sense heritability (H^2) for total FY was lower at 0.16 compared to the winter FY, which was estimated at 0.40.

Among half-sib populations, a large variability was found for the average winter FY in 2021–2023, ranging from 1.4–6.1 Mg ha^{−1} (mean 3.6 ± 0.06 Mg ha^{−1}) under rainfed and 1.4–8.2 Mg ha^{−1} (mean 4.4 ± 0.06 Mg ha^{−1}) under irrigated conditions (Figure 3B). Similarly, the average total FY for the period of 2021–2023 ranged from 3.7 to 14.7 Mg ha^{−1} (mean 9.3 ± 0.46 Mg ha^{−1}) under rainfed conditions and from 6.3 to 25.1 Mg ha^{−1} (mean 15.6 ± 0.17 Mg ha^{−1}) under irrigated conditions (Figure 3D). Among the 250 alfalfa half-sib populations, the most productive populations were AlfaL4-5 (parent SARDI7), AlfaL57-7 (parent WL903) and AlfaL62-9 (parent Baldrich350), which produced the highest (>13 Mg ha^{−1} mean total FY and >4.5 Mg ha^{−1} mean winter FY during 2021–2023) FY under both water regimes (Figure 3D).

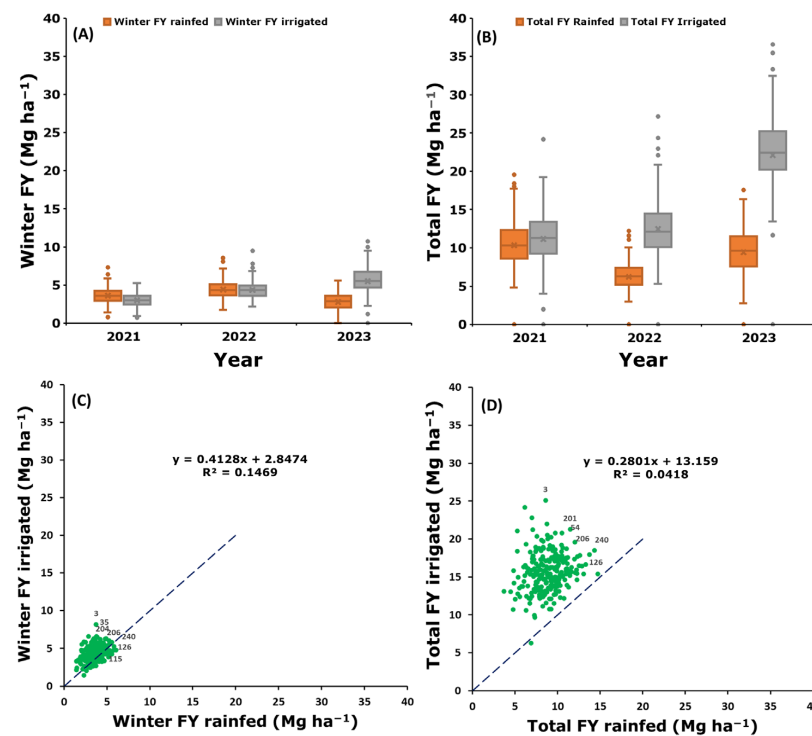


Figure 3. Winter (A) and total (B) forage yield (FY) of 250 half-sib populations grown under two water regimes, rainfed and irrigated, at Cauquenes during the period of 2021–2023, and the relationship between the mean FY (2021–2023) under rainfed and irrigated conditions during winter (C) and the entire (D) growing season. The data is based on the mean value for each alfalfa genotype and two replications, with a total of $N = 250$. In panels (C,D), each data point corresponds to a genotype, with numbers indicating high-yielding populations: 126—AlfaL4-5 (parent SARDI7), 206—AlfaL57-7 (parent WL903), and 240—AlfaL62-9 (parent Baldrich350). The dashed line signifies a one-to-one correspondence in the relationship. Box and whisker plots show population minimum; 25th percentile/median/75th percentile, and maximum; and the mean (x) of populations for each site–water regime–growing season. Circles indicate outlier data.

3.2. Red–Green–Blue (RGB) Vegetation Indices and Canopy Temperature Difference (CTD)

The RGB indices calculated per plot exhibited significant differences between half-sib populations and water regimes (Supplementary Table S1). The $WR \times G$ interaction was significant for intensity, lightness, u^* , and GA, whereas the $WR \times Y$ was significant for all RGB indices (Supplementary Table S1). RGB indices were correlated with monthly FY. Under both water regimes, hue, saturation, b^* , v^* , GA, and GGA exhibited generally positive correlations, whereas intensity, lightness, a^* , u^* , and CTD showed significant differences ($p < 0.0001$) and negative correlations with FY (Table 2). RGB indices b^* and v^* showed a higher H^2 of 0.60, whereas intensity, hue, saturation, lightness, a^* , u^* , GA, and GGA were 0.29, 0.35, 0.14, 0.34, 0.37, 0.39, 0.35, and 0.12 respectively (Supplementary Table S1).

In June 2021, all the indices were weakly correlated ($r < \pm 0.3$) and non-significant in relation to the FY under both water regimes (Table 3). In contrast, October 2021 exhibited favorable correlation values with RGB-derived indices under rainfed and irrigated regimes, particularly with u^* and a^* , followed by hue, intensity, and GGA ($p < 0.0001$) (Table 3). Similarly, during the harvests in September and November 2022, a high and significant ($p < 0.0001$) correlation was observed between FY and RGB-derived indices for 250 alfalfa half-sib populations under both water regimes (Table 3). However, in November 2022, the correlation coefficients were higher under irrigated conditions than under rainfed conditions.

Table 2. Correlation between forage yield and remote sensing indices of 250 alfalfa half-sib populations under rainfed and irrigated conditions during the 2021–2023 growing period.

Harvesting Date	Water Regime	Intensity	Hue	Saturation	Lightness	a*	b*	u*	v*	GA	GGA	CTD
Jun-21	Irrigated	−0.06 ^{NS}	−0.04 ^{NS}	−0.04 ^{NS}	−0.05 ^{NS}	0.01 ^{NS}	0.06 ^{NS}	0.03 ^{NS}	0.04 ^{NS}	0.07 ^{NS}	0.07 ^{NS}	0.03 ^{NS}
	Rainfed	−0.07 ^{NS}	0.16 *	0.27 ***	−0.05 ^{NS}	−0.11 ^{NS}	−0.06 ^{NS}	−0.17 **	−0.04 ^{NS}	0.15 *	0.16 *	0.13 *
Oct-21	Irrigated	−0.62 ***	0.66 ***	0.38 ***	−0.55 ***	−0.71 ***	0.26 ***	−0.74 ***	0.28 ***	0.57 ***	0.66 ***	−0.32 ***
	Rainfed	−0.61 ***	0.57 ***	0.47 ***	−0.58 ***	−0.61 ***	0.03 ^{NS}	−0.68 ***	0.05 ^{NS}	0.41 ***	0.57 ***	−0.28 ***
Sep-22	Irrigated	−0.56 ***	0.46 ***	0.44 ***	−0.50 ***	−0.65 ***	0.28 ***	−0.65 ***	0.25 ***	0.60 ***	0.63 ***	−0.12 ^{NS}
	Rainfed	−0.71 ***	0.57 ***	0.64 ***	−0.65 ***	−0.76 ***	0.37 ***	−0.77 ***	0.38 ***	0.70 ***	0.73 ***	−0.15 *
Nov-22	Irrigated	−0.66 ***	0.31 ***	0.70 ***	−0.61 ***	−0.69 ***	0.37 ***	−0.67 ***	0.34 ***	0.39 ***	0.45 ***	−0.76 ***
	Rainfed	−0.35 ***	0.19 **	0.34 ***	−0.28 ***	−0.51 ***	0.36 ***	−0.44 ***	0.37 ***	0.35 ***	0.38 ***	−0.57 ***
Jul-23	Irrigated	−0.05 ^{NS}	0.36 ***	0.03 ^{NS}	0.24 ***	−0.49 ***	0.21 ***	−0.52 ***	0.34 ***	0.36 ***	0.36 ***	
	Rainfed	−0.50 ***	0.67 ***	−0.25 ***	−0.47 ***	−0.45 ***	−0.22 ***	−0.51 ***	−0.17 **	0.33 ***	0.36 ***	
Sep-23	Irrigated	−0.15 *	0.02 ^{NS}	0.15 *	−0.17 **	0.08 ^{NS}	−0.20 **	0.05 ^{NS}	−0.21 **	−0.09 ^{NS}	−0.09 ^{NS}	
	Rainfed	0.02 ^{NS}	0.00 ^{NS}	0.03 ^{NS}	0.03 ^{NS}	−0.06 ^{NS}	0.06 ^{NS}	−0.06 ^{NS}	0.06 ^{NS}	0.04 ^{NS}	0.04 ^{NS}	
Nov-23	Irrigated	−0.44 ***	0.41 ***	−0.23 ***	−0.46 ***	−0.33 ***	−0.48 ***	−0.39 ***	−0.48 ***	0.44 ***	0.40 ***	−0.52 ***
	Rainfed	−0.36 ***	0.20 **	−0.26 ***	−0.39 ***	−0.17 **	−0.39 ***	−0.25 ***	−0.41 ***	0.28 ***	0.19 **	−0.34 ***

Among the indices, red color indicates a significantly negative correlation ($r = -1$), while green color indicates a highly positive correlation ($r = +1$). The values that are non-correlated ($r = 0$) or weakly correlated are shown in yellow boxes. GA, GGA, and CTD represent the green area, greener green area, and canopy temperature difference, respectively. The values marked with NS are not significant. Significant values are indicated with different levels of significance: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

Table 3. Correlation between canopy temperature difference (CTD) and red–green–blue (RGB) indices of 250 alfalfa half-sib populations under rainfed and irrigated conditions during the 2021–2023 growing period.

Harvesting Date	Water Regime	Intensity	Hue	Saturation	Lightness	a*	b*	u*	v*	GA	GGA
Jun-21	Irrigated	0.29 ***	0.04 ^{NS}	0.25 ***	0.36 ***	−0.34 ***	0.11 ^{NS}	−0.37 ***	0.18 **	0.31 ***	0.28 ***
	Rainfed	0.32 ***	−0.34 ***	−0.14 *	0.40 ***	−0.36 ***	0.42 ***	−0.29 ***	0.44 ***	0.29 ***	0.29 ***
Oct-21	Irrigated	0.38 ***	−0.30 ***	−0.31 ***	0.31 ***	0.45 ***	−0.28 ***	0.44 ***	−0.28 ***	−0.32 ***	−0.35 ***
	Rainfed	0.39 ***	−0.20 **	−0.28 ***	0.38 ***	0.30 ***	−0.06 ^{NS}	0.30 ***	−0.05 ^{NS}	−0.17 **	−0.21 ***
Sep-22	Irrigated	0.13 *	−0.20 **	−0.02 ^{NS}	0.13 *	0.16 *	0.03 ^{NS}	0.18 **	0.03 ^{NS}	−0.07 ^{NS}	−0.09 ^{NS}
	Rainfed	0.17 **	0.01 ^{NS}	−0.26 ***	0.12 ^{NS}	0.21 **	−0.29 ***	0.17 **	−0.28 ***	−0.11 ^{NS}	−0.10 ^{NS}
Nov-22	Irrigated	0.74 ***	−0.42 ***	−0.78 ***	0.68 ***	0.85 ***	−0.42 ***	0.83 ***	−0.41 ***	−0.51 ***	−0.59 ***
	Rainfed	0.29 ***	−0.25 ***	−0.36 ***	0.21 ***	0.54 ***	−0.38 ***	0.47 ***	−0.40 ***	−0.24 ***	−0.34 ***
Nov-23	Irrigated	0.71 ***	−0.79 ***	0.07 ^{NS}	0.70 ***	0.74 ***	0.52 ***	0.77 ***	0.53 ***	−0.80 ***	−0.60 ***
	Rainfed	0.69 ***	−0.65 ***	0.28 ***	0.69 ***	0.59 ***	0.53 ***	0.65 ***	0.55 ***	−0.71 ***	−0.45 ***

Among the indices, red color indicates a significantly negative correlation ($r = -1$) while green color indicates a highly positive correlation ($r = +1$). The values that are non-correlated ($r = 0$) or weakly correlated are shown in yellow boxes. GA and GGA represent the green area and greener green area, respectively. The values marked with NS are not significant. Significant values are indicated with different levels of significance: * for $p < 0.05$, ** for $p < 0.01$, and *** for $p < 0.001$.

During 2023, a notably weak correlation between FY and the RGB indices was identified (Table 3). In July 2023, only hue exhibited a strong relationship ($r > 0.6$) ($p < 0.0001$) with FY under rainfed conditions. Conversely, all other indices displayed $r < 0.5$ under both water regimes. In September 2023, a very weak correlation was observed across all RGB-derived indices, with $r < 0.5$ under both water regimes (Table 3). However, the r values were higher in November 2023 ($r = -0.5$) (Table 3). Relationships between FY and the RGB-derived indices for intensity and u^* in spring 2021–2023 are shown in Figure 4.

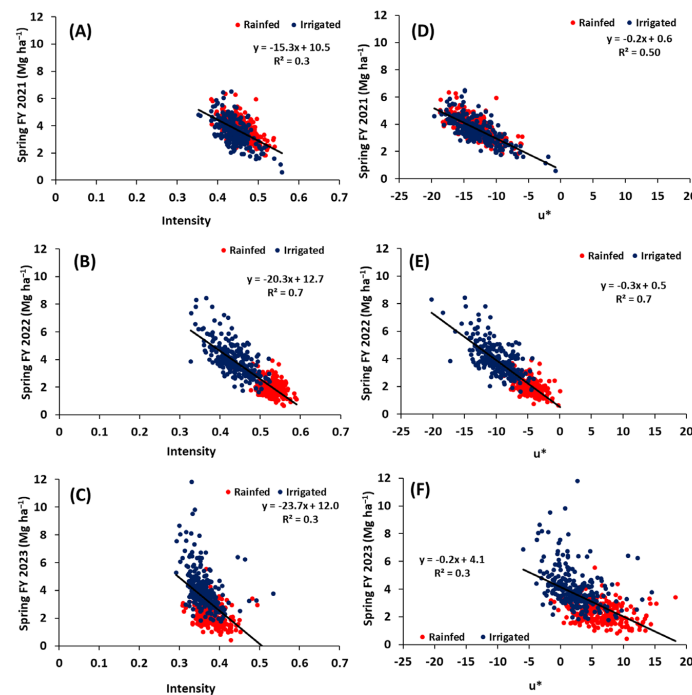


Figure 4. Relationship between the forage yield and RGB-derived index intensity in spring 2021 (A), spring 2022 (B), and spring 2023 (C) and relationship between the forage yield and RGB-derived u^* index in spring 2021 (D), spring 2022 (E), and spring 2023 (F) for the 250 half-sib populations grown under two water regimes, rainfed and irrigated, at Cauquenes during the period of 2021–2023. The data are based on the mean value for each alfalfa genotype and two replications. Each data point corresponds to a genotype. Red dots indicate populations grown under rainfed conditions, and blue dots indicating populations grown under irrigated conditions.

The CTDs among half-sib populations, seasons, and water regimes were significant, as were the interactions between $W \times G$, $W \times S$, and $W \times S$ (Supplementary Table S1). The broad-sense heritability (H^2) for CTD was relatively low ($H^2 = 0.19$) (Supplementary Table S1). The correlations between FY and CTD were, in general, negative and higher for the irrigated compared to the rainfed regime, particularly in November 2022 and November 2023 (Table 2).

3.3. Relationships Between Red–Green–Blue (RGB) Indices and Canopy Temperature Difference (CTD)

The Pearson correlations between the RGB-derived indices and the CTD for 250 alfalfa half-sib populations were calculated for three growing periods (2021–2023) (Table 3). The year 2021 exhibited a very low correlation ($r < 0.5$) between the CTD and RGB-derived indices under both water regimes across all the RGB-derived indices. In June 2021, a^* and u^* were negative, and intensity, lightness, b^* , v^* , GA, and GGA exhibited a positive correlation with the CTD under both water regimes. In October 2021, hue, saturation, b^* , v^* , GA, and GGA were negatively correlated, while intensity, lightness, a^* , and u^* were positively correlated with the CTD (Table 3). However, a negligible correlation ($r = -0.3$ to $+0.2$)

between the RGB-derived indices and CTD was identified under both water regimes during September 2022. Similarly, during the harvests in November 2022, a robust correlation ($p < 0.0001$) was observed between the CTD and RGB-derived indices for 250 alfalfa half-sib populations under both water regimes (Table 3). However, the correlation coefficients were stronger under the irrigated regime ($r = -0.8$ to $+0.9$) compared to those under the rainfed regime ($r = -0.4$ to $+0.5$). Similarly, in November 2023, a very strong correlation ($p < 0.0001$) was observed under both water regimes, while the CTD correlation values exhibited a slightly higher range ($r = -0.8$ to $+0.8$) across all RGB-derived indices under irrigated conditions compared to rainfed conditions ($r = -0.7$ to $+0.7$). Particularly, the RGB-derived indices hue, GA, and GGA showed negative correlations, while the remaining indices demonstrated positive correlations with CTD in November 2023 (Table 3). The relationships between CTD and the RGB-derived indices for intensity and u^* in spring 2021–2023 are shown in Figure 5.

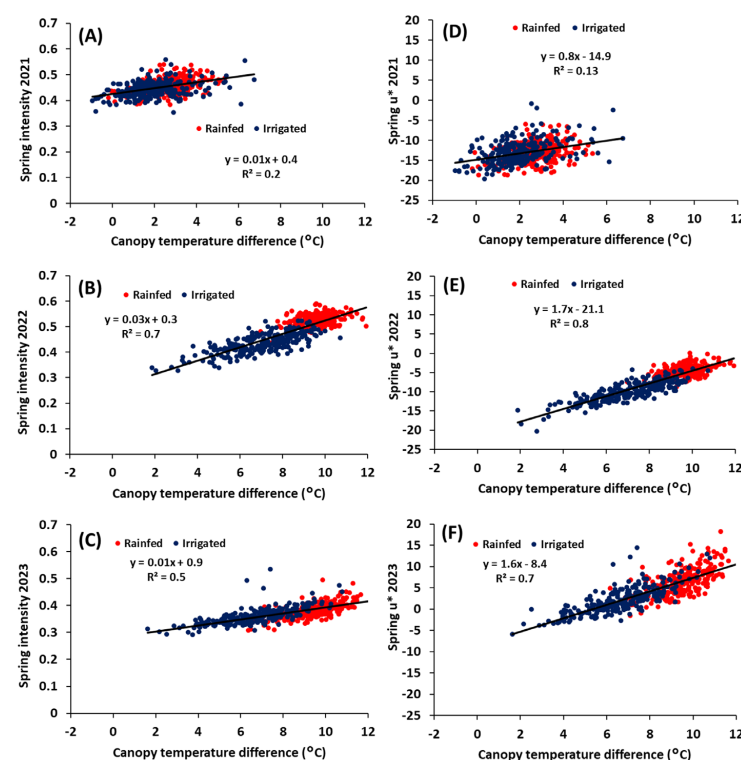


Figure 5. Relationship between the canopy temperature difference (CTD) and red, green, and blue (RGB)-derived intensity index in spring 2021 (A), spring 2022 (B), and spring 2023 (C) and relationship between the forage yield and RGB-derived u^* index in spring 2021 (D), spring 2022 (E), and spring 2023 (F) of 250 half-sib populations grown under two water regimes, rainfed and irrigated, at Cauquenes during the period of 2021–2023. The data are based on the mean value for each alfalfa genotype and two replications. Each data point corresponds to a genotype. Red dots indicate populations grown under rainfed conditions, and blue dots indicate populations grown under irrigated conditions. The CTD is the difference between the canopy temperature and the air temperature ($T_c - T_a$).

3.4. Relationships Between Forage Yield and Canopy Temperature Difference (CTD)

The CTD was correlated with the FY in all three growing seasons (2021–2023) under two water conditions (Figure 6). In spring 2021, the relationship between CTD and FY was observed to be nearly the same under both water regimes (mean CTD = 2.7°C , and mean FY = 3.7 Mg ha^{-1} for rainfed alfalfa; and mean CTD = 1.9°C , and mean FY = 3.57 Mg ha^{-1} for irrigated alfalfa), and the r values were -0.28 and -0.32 for rainfed and irrigated alfalfa, respectively (Table 2). In spring 2022, a significant negative correlation was observed

between FY and CTD, with $r = -0.76$ under irrigated and $r = -0.57$ under rainfed conditions (Table 2). The mean FY and CTD under rainfed conditions were 1.95 Mg ha^{-1} and 9.7°C , respectively. However, under irrigated conditions, the mean FY and CTD were 4 Mg ha^{-1} and 7°C , respectively (Figure 6). The correlation values differed between the two water regimes in spring 2023 ($r = -0.52$ for irrigated, which is negative, and $r = 0.11$ for rainfed, which is positive) (Table 2). The mean FY and CTD under rainfed conditions were 2.3 Mg ha^{-1} and 9.5°C , respectively. In contrast, under irrigated conditions, the mean FY and CTD were 4.1 Mg ha^{-1} and 6.6°C , respectively (Figure 6). For the rainfed alfalfa, the highly productive alfalfa populations comprised AlfaL28-5 (parent AS2), AlfaL44-10 (parent APG45675), AlfaL4-5 (parent SARDI7) and AlfaL62-9 (parent Baldrich350). Under irrigated conditions, the highly productive alfalfa populations included AlfaL28-2 (parent AS2), AlfaL35-7 (parent AS9), AlfaL35-9 (parent AS9), and AlfaL61-5 (parent Genesis).

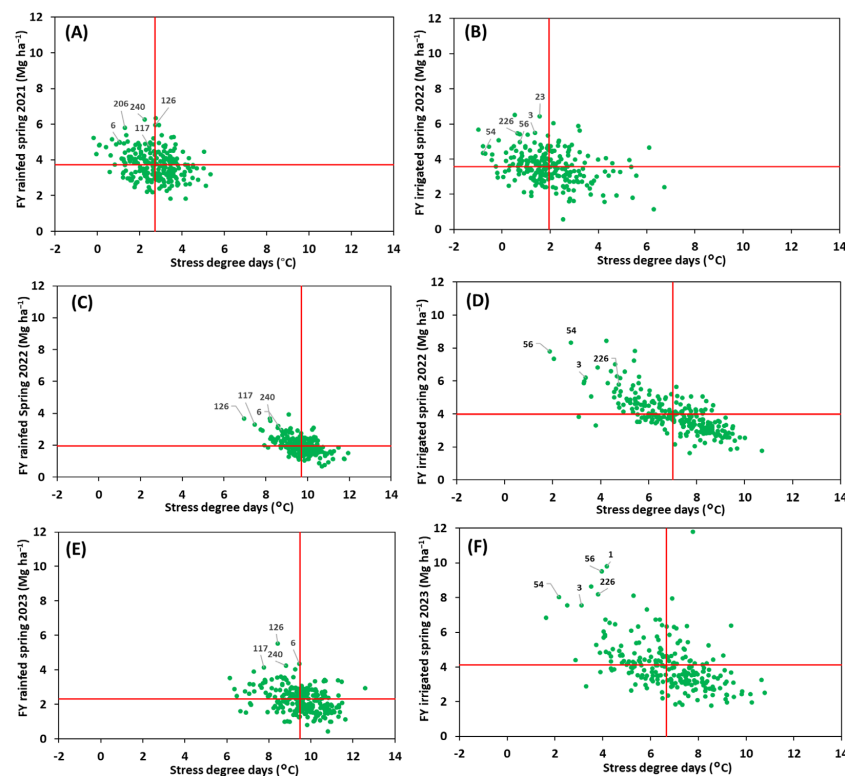


Figure 6. The relationship between the spring forage yield (FY) and canopy temperature difference (CTD) of 250 half-sib populations grown under two water regimes, rainfed (A,C,E) and irrigated (B,D,F), at Cauquenes during the period of 2021–2023. The data is based on the mean value for each alfalfa genotype and two replications, with a total of $N = 250$. Each data point corresponds to a genotype, with numbers indicating high-yielding populations. The vertical and horizontal red lines in each box scatter plot signify the mean CTD and FY, respectively, and the cross-section indicates the mean values of both variables. In rainfed alfalfa, the highly productive alfalfa populations comprised number 6—AlfaL28-5 (parent AS2), 117—AlfaL44-10 (parent APG45675), 126—AlfaL4-5 (parent SARDI7), and 240—AlfaL62-9 (parent Baldrich350). Under irrigated conditions, the highly productive alfalfa populations included number 3—AlfaL28-2 (parent AS2), 54—AlfaL35-7 (parent AS9), 56—AlfaL35-9 (parent AS9), and 226—AlfaL61-5 (parent Genesis).

4. Discussion

4.1. Forage Yield

In the drought-prone Mediterranean region of Chile, perennial forage crops are increasingly favored over annuals due to their ability to withstand 5 to 6 months of dry conditions, along with their high yield potential, environmental resilience, and long-term

survival strategies—particularly under adverse conditions such as drought [47]. According to the meteorological data recorded at Cauquenes during 2021–2023, the annual average precipitation was <26% of the long-term average precipitation (650 mm) due to a rapid change in climate. The selection of excellent populations is validated by the high FY and forage quality of certain populations that performed well under demanding conditions. In the current study, during the growing period of 2021–2023, the total FY of 250 alfalfa half-sib populations increased by 43%, and the winter FY increased by 17% when comparing the two water regimes, i.e., irrigated and rainfed, because of the application of additional water. According del Pozo [33], for 69 alfalfa accessions grown during 2018–2020 under similar climatic conditions, the FY under irrigated conditions increased by 18%. A regular and sufficient water supply guarantees sufficient moisture for healthy root development and maintains the soil structure for nutrient absorption. Most importantly for photosynthesis, this leads to increased carbohydrate production and biomass accumulation [48]. This improved photosynthetic efficiency translates into higher FYs [49] compared to those for rainfed conditions. Furthermore, upon comparing the FY data from our previous study, conducted between 2018 and 2020 [33], to those from the current study, it was noted that there was a 30% increase in mean FY under rainfed conditions and a 49% increase under irrigated conditions. The samples exhibiting increased FY have been referred to as half-sib progenies because they share one common parent and one different parent. The half-sib progenies inherit a combination of desirable traits (higher FY potential and drought tolerance) from each parent, along with increased genetic diversity and hybrid vigor, or heterosis [50,51]. Perennial forage crops like alfalfa gradually increase their yield potential over time due to specialized physiological mechanisms. These mechanisms include the proliferation of crown roots, which promote deep root formation. As a result, the plant becomes more resilient to water scarcity, particularly during the dry season lasting approximately 6 months, from November to April [52].

Alfalfa populations such as AlfaL4-5 (from parent SARDI7), AlfaL57-7 (from parent WL903), and AlfaL62-9 (from parent Baldrich350) revealed high FY and proved to be the most suitable populations in our study for both irrigated and rainfed Mediterranean climatic conditions. The three parents, SARDI7, WL903, and BALDRICH350, are commercial alfalfa cultivars [44]. The SARDI7 cultivar was specifically developed for Australian Mediterranean climates, like those found in Cauquenes, and is suitable for both rainfed and irrigated farming conditions. It exhibits key traits such as high yield, pest resistance, and winter activity, making it well-suited for cultivation under Mediterranean climatic conditions [43]. Baldrich 350 is a long-term (likely more than 15 years) adapted cultivar in Chile. It is cultivated primarily for hay and for grazing purposes in Chile, particularly during the winter season. WL903 HQ alfalfa, developed by Forage Genetics International—the leading alfalfa genetics company in the United States—is recommended for cultivation in Chile’s Central (Cauquenes) and South-Central zones, from Vallenar to Los Ángeles. This non-dormant cultivar (dormancy rating = 9) offers several outstanding characteristics, including year-round growth, high dry matter production, excellent persistence, superior nutritional quality, and an optimal leaf-to-stem ratio.

The heritability of a trait is used to predict the genetic gain from selection [53]. The difference between the broad-sense heritability of the winter FY and the total FY was determined to be 24% in the 250 alfalfa half-sib populations. A very low broad-sense heritability of the total FY (16%) indicates that different alfalfa genotypes perform variably across different environments, complicating the ability to attribute trait variation to genetic factors [54]. High environmental variability—resulting from contrasting water regimes, multi-year effects, and multiple harvests—reduced the relative contribution of genetic factors, leading to low heritability estimates. Moreover, half-sib populations share only one

parent compared to full-sibs, which share two parents; thus, the genetic similarity between individuals is lower. This results in greater genetic diversity within the half-sib population, which can dilute the expression of genetic traits [55].

4.2. Remote Sensing

Recently, remote sensing technologies—such as RGB and thermal cameras mounted on UAVs—have been introduced in plant phenotyping to predict forage crop yield under stressful environmental conditions [56]. In the present study, a range of RGB-derived indices (including intensity, saturation, hue, lightness, a , b , u^* , v^* , GA, and GGA), along with thermal indices such as canopy temperature difference, were used to evaluate forage yield in alfalfa grown under drought conditions and to identify high-yielding half-sib populations associated with drought tolerance.

RGB-derived indices play a vital role in agriculture due to their strong correlation with plant pigments, which directly influence plant growth and metabolic processes. As all RGB colors fall within the visible light spectrum (400–700 nm), each color index represents specific pigmentation and quality attributes, including plant health, phenology, stress levels, and maturity [57–59]. RGB cameras capture light within the visible spectrum. The visible light spectrum is separated into four equal segments: blue (B), from 400 to 475 nm; green (G), from 475 to 550 nm; yellow (Y), from 550 to 625 nm; and red (R), from 625 to 700 nm [60]. Each of these segments corresponds to a particular wavelength of light that the human eye interprets as blue, green, and red. RGB cameras combine these color channels to produce a composite image [61] that may be studied for a variety of purposes, including plant growth evaluation and precision agriculture.

Hue represents the basic perception of color (e.g., red, green, blue, yellow), independent of brightness or saturation. It is derived from the RGB color model and is typically expressed in degrees ($^\circ$) on a color wheel, ranging from 0° to 360° . Hue plays several important roles in plant phenotyping. For example, under water deficiency, canopy color may shift from green (120°) to yellow (60°), reflecting a reduction in chlorophyll content and resulting in lower hue angles in wilted plants compared to those in healthy samples [62]. In another study, hue was effectively used to diagnose plant diseases [63]. Strong positive correlation values between hue and FY in our study under both water regimes indicate that the plants were healthy, with a green canopy, which ultimately resulted in higher FY.

Higher and more positive saturation, b^* , and v^* values observed in 2021 and 2022 indicate healthier and more vigorous vegetation, potentially leading to higher FYs [64]. However, this trend declined in 2023. The GA and GGA indices, which estimate the percentage of green pixels (green to yellowish shades) in an image, are commonly used to assess vegetation health. Alfalfa genotypes experiencing severe wilting exhibited a lower proportion of green pixels, leading to reduced GA and GGA values, and vice versa [65].

It was observed that as the values of these RGB indices increased, the plants become more sensitive to drought, with the canopy color shifting from green to red under water-stressed conditions. These changes were associated with canopy leaf wilting [62] and ultimately led to a reduction in FY, except for in three outstanding populations—AlfaL4-5 (parent SARDI7), AlfaL57-7 (parent WL903), and AlfaL62-9 (parent Baldrich350)—which maintained strong performance under both water regimes. Similar findings regarding the relationship between RGB and FY have been reported in alfalfa [33], forage grasses [42], maize [66,67], and wheat [68], supporting our hypothesis that RGB-derived indices are valuable tools for selecting high-yielding alfalfa half-sib populations for drought-prone environments.

Other RGB-derived indices, such as intensity, lightness, a^* , and u^* , showed negative correlations with alfalfa FY. Among these, intensity and u^* shown strong correlations with

FY during the spring season. In RGB imaging, intensity refers to the overall brightness or luminance of an image, representing the total amount of light reflected or emitted by plant surfaces. It is typically calculated as the average of the red, green, and blue colors. When combined with hue and saturation, intensity offers valuable insights into plant health and drought stress, particularly in dryland agriculture. In contrast, u^* measures color variation along the red–green axis, allowing for a more precise quantification of color differences in plant imagery. Both intensity and u^* exhibited a low broad-sense heritability, estimated at 29% and 39%, respectively. This is likely due to their sensitivity to a range of factors, including pigment content, leaf structure, and physiological responses, all of which are influenced by strong genotype-by-environment ($G \times E$) interactions [69]. The genetic relationship of these traits may involve interactions between multiple genes, contributing to low heritability estimates.

Higher CTD values, accompanied by lower FY, suggest that the plants were subjected to more severe drought stress. As plants transpire, the evaporation of water from leaf surfaces absorbs heat energy, resulting in a reduction in leaf temperature and a cooling effect. However, under rainfed conditions, as soil moisture becomes depleted, the rate of evapotranspiration declines—primarily due to reduced stomatal conductance and other physiological limitations. The reduction in stomatal conductance also limits CO_2 uptake, leading to decreased photosynthetic rates, which ultimately reduces plant growth and FY [70,71]. However, when comparing CTD values with spring FY under both water regimes (Figure 6), some alfalfa populations exhibited strong performance and showed higher yields, even under stressful conditions. Under rainfed conditions, the most productive half-sib populations were AlfaL28-5 (parent AS2), AlfaL44-10 (parent APG45675), AlfaL4-5 (parent SARDI7), and AlfaL62-9 (parent Baldrich350), whereas under irrigated conditions, AlfaL28-2 (parent AS2), AlfaL35-7 (parent AS9), AlfaL35-9 (parent AS9), and AlfaL61-5 (parent Genesis) were the most productive.

Although CTD exhibits low broad-sense heritability ($H^2 = 19\%$), its correlation with forage yield (FY) remains a useful indicator for assessing alfalfa productivity under drought conditions. This is because CTD is highly influenced by environmental factors such as air temperature, wind speed, humidity, and solar radiation. These environmental factors can vary significantly between and within growing seasons, leading to high environmental and low genetic variations relative to the total phenotypic variations. Based on the results of our study, the most suitable period to measure canopy temperature is during the spring (October–November) because at this time, the FY-CTD correlation was higher ($r > -7$). The relationship between FY and the CTD has evolved as a key tool to dissect drought-tolerant genotypes [72], and similar results have been reported [33,73,74]. Thus, in addition to the u^* and intensity RGB vegetation indices, canopy temperature was determined to be a key tool in field phenotyping for identifying genotypes with higher FY and drought tolerance traits.

Although RGB- and thermal camera-derived indices represent a novel advancement in agricultural field phenotyping, there are still limitations when using them to predict FY, e.g., (i) CTD and RGB-based indices (like intensity, hue, u^* , etc.) are highly influenced by environmental conditions, such as air temperature, solar radiation, and wind speed. (ii) In the case of canopy temperature, the broad-sense heritability is general low. Also, CTD reflects transpiration and stomatal behavior, which are only partially linked to biomass production and yield. (iii) RGB indices can become saturated in dense canopies, making it difficult to distinguish among high-yielding genotypes; and (iv) RGB imagery can be affected by sun angle, shadows, and cloud cover, whereas canopy temperature readings are the most informative during peak stress hours (e.g., midday) and may be less useful early or late in the day.

5. Conclusions

Winter and total FY for alfalfa grown in the Chilean Mediterranean drought-prone environment varied widely between the three growing seasons (2021–2023) under two water regimes, rainfed and irrigated. A large variability in FY was observed among the set of 250 half-sib populations of alfalfa under rainfed and irrigated conditions. There were three alfalfa half-sib populations, AlfaL4-5 (parent SARDI7), AlfaL57-7 (parent WL903), and AlfaL62-9 (parent Baldrich350), that exhibited high FY under both water regimes, rainfed and irrigated. Moreover, the use of remote sensing tools, such as RGB and thermal camera vegetation indices, were proven to be highly valuable high-throughput phenotyping methods for evaluating the performance and phenotypic variability in alfalfa half-sib populations grown under rainfed and irrigated conditions. The RGB indices for hue, saturation, b^* , v^* , GA, and GGA exhibited positive correlations, whereas intensity, lightness, a^* , and u^* exhibited negative correlations with FY. The thermal camera-derived index CTD (Tc-Ta) showed a negative correlation with FY and appeared to be the most powerful tool for the identification of alfalfa genotypes grown under the Chilean Mediterranean drought-prone environment.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/rs17091517/s1>, Table S1: F values and probabilities (p) of ANOVA, and broad sense heritability (H^2) for total and winter forage yield, RGB and thermal camera indices of 250 half-sib populations of alfalfa grown under supplementary irrigated and rainfed water regimes at Cauquenes during the growing season 2021–2023. For RGB indices and canopy temperature difference (CTD), values of each season were the average of 5 harvest dates in 2021–22, 4 in 2022–23, and 4 in 2023–24.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors on request.

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