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Changes in Root Architecture and Aboveground Traits of Red Clover Cultivars Driven by Breeding to Improve Persistence

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Abstract: Red clover persistence has been one of the major targets for the most breeding programs worldwide. A phenotypic characterization at above- and belowground level was performed to a set of highly persistent red clover populations. The objective was to identify phenotypic changes occurring after empirical selection for plant survival, which is the consensus criteria of persistence. Eleven red clover populations were established on mesocosms of polyvinyl chloride (PVC) tubes of 11 cm in diameter and 100 cm depth, containing as substrate a mixture (*v*/*v*) of sand, vermiculite, soil and perlite. The trial was organized in a randomized complete block design with four replicates, each replicate consisting of five mesocosms per population and one plant per mesocosm. In total, 220 mesocosms were handled. At aboveground level, growth parameters, specific leaf area (SLA) and shoot dry matter (ShootDM) were measured. At belowground level, root morphology (volume, diameter and length) and topology (altitude, external path length [EPL] and dichotomous branching index [DBI]) were measured through image analyses. Analyses of variance were performed implementing a phenotypic linear mixed model using the Restricted Maximum Likelihood method. Additionally, variance components were estimated and broad-sense heritability was calculated for each phenotypic trait. Highly persistent cultivars exhibited 30% higher ShootDM and 10% lower leaf size and SLA than the oldest low-persistent cultivar Quiñequeli. At root level, they showed 20, 50 and 50% higher crown diameter, root length density and root volume than Quiñequeli, respectively, but 20% lower DBI. Root traits exhibited medium-low values of genetic control; broad sense heritability ranged between 0.20 and 0.48. In conclusion, highly persistent red clover cultivars and experimental lines bred in Chile modified their phenotypic expression of individual plants at shoot and root levels relative to the oldest low persistent cultivar Quiñequeli. Associations among above- and belowground traits offer opportunities for designing more efficient selection strategies. For instance, the strong relationship between SLA and root traits offers tremendous potential for indirect phenotypic selection.

Keywords: root traits; root topology; forage legume; dichotomous branching index; plant survival

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1. Introduction

Red clover (*Trifolium pratense* L.) is a major forage legume species in temperate regions of the world. It is grown on approximately four million hectares worldwide and it is noted for its high-protein feed and high rate of biological nitrogen fixation [1–3]. In Chile, red clover has been a highly significant species for animal production and the seed industry, representing in the last decades nearly 15% of the sown pastures and 60% of the forage seed exports [4].

The main issue limiting red clover worldwide is its lack of persistence due to the high mortality of plants [1,4–8]. There are many biotic (pests, diseases, competition and morpho-physiology of the red clover plant itself), abiotic (pH, soil fertility and climatic conditions) and management factors (frequency and intensity of cutting and/or grazing) that are important in determining the survival of red clover plants. These factors interact, acting as a complex, and the importance of each one varies depending on the environment [4]. Persistence is an economically important trait that may be defined as sustained forage yield over several years [9]. From a pasture production point of view, its definition depends on the level of intensity of the livestock system. In high-insensitive systems is most related to biomass production and stability, whereas in extensive systems it is just related to plant survival. However, a global consensus definition involves the maintenance of adequate plant numbers across growing seasons [10].

Today, forage species are facing new challenges. They must persist and produce biomass in a range of marginal environments [11–15]. Furthermore, forage species must also confront the negative effects of water availability and environmental temperature caused of climate change [16]. Thus, currently, plant persistence is also associated with the ability of the pasture to tolerate abiotic stresses [2,11–13].

From a plant breeding point of view, persistence has been a major target in red clover [1,4–8]. In general, breeding for persistence has been successful across the world. Modern cultivars can persist 3–4 years, whereas in the past, red clover was sometimes considered a biannual species [1]. The most direct way to breed for persistence has been exposing plants to their environment and selecting for survival [4–8]. This approach has been time consuming and it may take many years before superior genotypes can be identified [8].

Despite the importance of red clover persistence, few works have attempted to identify phenotypic traits related to plant survival in a given environment. For instance, Montpetit and Coulman [17] demonstrated that red clover persistence is improved by the growth of adventitious roots from the crown. In the UK, greater red clover persistence has been associated with larger crown diameter [6]. Herrmann et al. [8] found chromosomal regions (QTLs) modulating red clover persistence. Their results showed that persistence was correlated with the length of the stem and seed yield. Additionally, they detected QTLs for all these traits in the same genome region.

In Chile, the major objective of the red clover breeding program led by INIA is to improve persistence and forage yield [4,18]. Using a geno-phenotypic breeding scheme and selecting empirically for plant survival at the end of the third or fourth growing season, new cultivars with greater persistence have been released. In a timeline of 30 years of red clover breeding, modern cultivars exhibit three to five times higher plant survival than the oldest cultivar Quiñequeli, released in 1962 [4]. In the last decade, research efforts have been done to elucidate mechanisms involved in plant survival of red clover. First, it was found that biotic stress caused by the curculionid *Hylastinus obscurus* (Marsham) is the main deleterious factor determining red clover plant survival [19]. Then, plant-insect interaction studies have demonstrated that red clover root synthetize some volatile compounds that play an important role on the insect behavior as attractive/repellent [20,21]. Currently, red clover genetic variability for the ability to synthetize different semiochemicals interacting with the curculionid that is being studied.

Morphological changes at aboveground traits have not been evaluated in the modern highly persistent red clover Chilean cultivars. An increase in the total dry matter yield associated with higher plant survival has been realized. For future breeding, it is important to reach a better comprehension of the genetic and phenotypic components of plant survival in the Chilean environmental conditions. If the main biotic factor affecting plant survival is an insect that destroys the root system [19], the root morphology and architecture likely play an important role in plant survival.

At the end of the 1980s, the red clover root system was deeply studied, because it was considering a tap-rooted model species for topological and architecture root studies [22–24]. However, all those studies were related to the role or root architecture on soil resources capture (water and nutrients). Few works have associated root traits with plant persistence. As mentioned before, Montpetit and

Coulman [17] demonstrated that red clover persistence was improved by the growth of adventitious roots from the crown. However, the methodology performed at the beginning of the 1990s was excessive time consuming and barely quantitative. In this situation, low values of narrow-sense heritability for adventitious root trait was reported (0.3). Skipp and Christensen [25] studied root diseases affecting red clover plant survival. They found that plant survival was associated with vigorous, deeply penetrating adventitious roots and also with genetic resistance to some diseases.

In recent years, interest to study plant root systems have increased given climate change (drought) and the technological advances for evaluating them in a quantitative way. It is believed that plant root system breeding can prompt a second green revolution [26,27]. In this sense, high-throughput image analysis systems have enabled to determine root morphology and architecture traits. Some root traits, such as the external path length (EPL), altitude and dichotomous branching index (DBI), describe the branching pattern of a root system. Roots can vary significantly in their topology between two extreme branching patterns, dichotomous and herringbone [22,23]. A herringbone pattern consists of root branches primarily confined to a main axis, whereas dichotomous structures are more randomly branched with each branch leading to a similar number of distal root segments. Higher values of altitude and EPL indicate that the branches conforming the root system have a high number of links. On the other hand, DBI describes the branching patterns. When DBI values tend to one or zero, the root system tends to a herringbone or dichotomous pattern, respectively [28,29].

In this work, we hypothesized that the persistence of red clover cultivars bred in Chile is associated with their phenotypic expression of some of the traits at above- and belowground level. Realizing and understanding that those phenotypic traits confer higher persistence could help to develop new and more efficient breeding methodologies. The present work aims to identify phenotypic traits associated with the improved persistence of the modern Chilean red clover cultivars and new experimental lines.

2. Materials and Methods

2.1. Plant Material

The experiment was carried out at the greenhouse facilities of the Quilamapu Research Center of INIA-Chile located in Chillán City (36°34′ S; 72°06′ O). The experimental period was extended from October 2013 to May 2014. The plant material included three cultivars released by the red clover breeding program of INIA-Chile (INIA-RCbp) in its 30 years of existence [Quiñequeli (1962), Redqueli (1997) and Superqueli (2011)], two cultivars introduced to Chile [Tuscan (New Zeeland) and StarFire (USA)] and six advanced synthetic lines (ASL) developed by the INIA-RCbp (Sel Syn IntIV, Syn IntIV, Syn IntV, Sel Syn PreI, Sel Syn PreIII and Syn PreIII). Chilean cultivars and ASL are all diploids. Persistence of the Chilean germplasm was previously characterized as the number of survival plants at the end of a growing cycle including three or four growing seasons [4]. This persistence data set was re-analyzed in order to build up a persistence ranking with the GGE biplot method [29] (Supplementary Materials Figure S1). The ranking included only Chilean populations. The persistence of the introduced cultivars was not evaluated.

The introduced cultivar was included in order to compare the phenotypic mechanisms expressed by highly persistent cultivars bred in Chile and internationally. Tuscan and StarFire are diploid cultivars bred in New Zealand and the USA, respectively. Tuscan was described as a highly persistent cultivar under grazing conditions, whereas StarFire was described as a highly persistent cultivar under cutting conditions. Improved persistence of Tuscan and Starfire was achieved through pest and disease resistance, respectively.

2.2. Plant Establishment and Growing Conditions

Seeds of similar size were sown in mesocosms consisting of PVC cylinders 11 cm in diameter and 100 cm high. The cylinders were lined inside with plastic sleeves of hi-density polyethylene film. The substrate consisted of a mixture (volume based) of 50% medium size sand (0.5–0.3 mm), 35% vermiculite, 10% volcanic ash derived soil (Andisol) and 5% perlite. Ten liters of the mixture were used in each cylinder. Mesocosms were organized in racks wrapped with a reflective insulation film. Two days before sowing, the substrate was watered with 1.6 L of a nutrient solution adjusted to pH 6.0 and consisting of (in μM): NO3 (7000), NH4 (1000), P (1000), K (3000), Ca (2000), SO4 (500), Mg (500), Cl (25), B (12.5), Mn (1), Zn (1), Cu (0.25) Mo (0.25) and EDTA-Fe (25). Three seeds per mesocosm were sown to 5 mm deep on 10 October 2013. Twenty-one days after sowing (DAS), plants were thinned to leave one plant per pot. Plants were grown in a temperature-controlled greenhouse (20 °C) with a relative humidity of 57%.

Substrate moisture was kept at field capacity with a pressurized irrigation system including 2 Lh^{-1} drip emitters (PCJ, Netafim, Israel). Mesocosms were daily irrigated and substrate water potential was recorded at 15 min interval with capacitance sensors (MPS-2, Decagon, WA, USA) located 5, 25, 45, 65 and 85 cm deep in two no-experimental unit mesocosms (Supplemental Figure S2a). During the germination period, substrate temperature at five cm deep was recorded in five random selected experimental units with 5TE capacitance sensors (Decagon, WA, USA) (Supplemental Figure S1b).

2.3. Phenotypic Characterization of Shoot System

The experiment included two periods. The first one (Period I) extended from sowing to first biomass harvest (147 DAS). This period was considered as the establishment phase, and only shoot traits were measured. The second period extended from 147 to 194 DAS, and shoot and root traits were measured.

Plant height was measured seven times during Period I (24, 34, 38, 52, 61, 101 and 147 DAS). Stem growth parameters were estimated fitting a parametric logistic growth curve in the grofit package [30] in R software. Stem elongation rate (Grate), lag phase (LagTime) and maximal plant height (Hmax) were estimated with the fitted model. Five totally expanded leaves were collected from the middle part of each plant in three sampling times (93, 147 and 194 DAS). Leaf area (LA) and dry weight (Ldw) were determined and specific leaf area was calculated (SLA = LA/Ldw). Leaf chlorophyll content was estimated in four central leaflets from the middle part of each plant (147 and 194 DAS). Chlorophyll measurements were taken with a SPAD-meter (SPAD-502Plus Konica Minolta Optics, Inc., Osaka, Japan). Shoot dry matter (DM) production was measured in two harvest times (147 and 194 DAS). Plants were cut 5 cm above ground level and dried in forced air oven at 65 °C until reach constant weight. Then, leaves (LeavesDM), stems (StemDM) and shoot DM (ShootDM = LeavesDM + StemDM) were recorded with an analytical scale (Radwag AS220/C2, Poland).

2.4. Phenotypic Characterization of Root System

At 194 DAS, roots were separated from the substrate by washing them with running tap water. Cylinders were placed on a root-washer consisting of a 50 × 150 cm tray with concave shape. The tray was built with a 3 mm sieve. The top 20 cm of each cylinder was separated from the remaining root system. Both parts of the root system were immediately stored at 4 °C in hermetic containers with 70% ethanol. In a dark room, the root system was totally extended on a black background tray with a 2-cm layer of water. Tray was illuminated with two 160 LED panel. Then, each root system was digitalized with a digital-SLR camera (Eos Rebel T5i, Canon, Tokyo, Japan) located 1.5 m above the tray. All digital images were collected under a standardized light-environment and camera set up. Average root diameter (avgD), total root volume (Vol), total root length (TrootL) and root length density (RLD = TrootL/mesocosm volume) were determined for the entire root system. For the top 20 cm section, crown diameter, altitude (a), magnitude (μ) and external path length (P_e) were determined. All root traits were obtained with image analysis software (WinRhizo TRON, Régent Instruments, Québec, QC, Canada). Dichotomous branching index (DBI) was calculated in accordance with Beidler et al. [29] as follows:

$$\mathrm{DBI} = [\mathrm{P_e} - \min(\mathrm{P_e})] / [\max(\mathrm{P_e}) - \min(\mathrm{P_e})] \quad (1)$$

$$\max(P_e) = 0.5\ (\mu^2 + 3\mu - 2) \tag{2}$$

$$\min(P_e) = \mu\ [\min(a) + 1] - 2^{\min(a)-1} \tag{3}$$

where $\max(P_e)$ and $\min(P_e)$ are the theoretical external path length (P_e) for a system of a given magnitude (μ) that has a completely herringbone or a completely dichotomous topology, respectively. The min (a) is the theoretical altitude (a) for a system of given magnitude that has a completely dichotomous topology.

Once root image analyses were performed, root biomass was dried in forced air oven at 65 °C until reaching constant weight. Then, root dry matter of the top 20 cm ($\mathrm{RootDM}_{20\ \mathrm{cm}}$), fine root DM (root < 2 mm diameter; FineRootDM) and total root DM ($\mathrm{RootDM} = \mathrm{RootDM}_{20\ \mathrm{cm}} + \mathrm{FineRootDM}$) were measured with an analytical scale (Radwag AS220/C2, Poland). Finally, the RootDM:ShootDM ratio was calculated.

2.5. Experimental Design and Statistical Analyses

Experiment was arranged in a randomized complete block design with four blocks. Each replicate included five mesocosms per red clover accessions (treatments). In total, 220 mesocosms were handled (11 treatments × 5 mesocosms per replicate × 4 replicates = 220 mesocosms). Analyses of variance were performed implementing a phenotypic linear mixed model using the Restricted Maximum Likelihood (REML) method within the ASReml-R package [31] within the R software (https://www.r-project.org/) based on the following expression:

$$Y_{ij} = \mu + acc_i + r_j + \varepsilon_{ij} \tag{4}$$

where Y_{ij} is the phenotypic value of ith plant accession (*acc*) in the jth replicate (*r*); μ is the overall population mean and ε the random experimental error~IDD $(0,\sigma^2_{\varepsilon})$. The *acc* term was considered as a fixed effect and the replicate as a random effect~IDD $(0,\sigma^2_{r})$. With this model, the *acc* effect was determined and *acc* means were compared using a least significant difference test (LSD) with the asremlPlus package [32]. A similar phenotypic model to the previously described, but considering the *acc* term as a random effect~IDD $(0,\sigma^2_{acc})$ ~it was fitted in order to estimate the variance components and broad-sense heritability (H^2) as follows:

$$H^2 = \frac{\sigma^2_{acc}}{\sigma^2_{acc} + \sigma^2_{r} + \frac{\sigma^2_{\varepsilon}}{r}} \tag{5}$$

Finally, a principal component analysis was performed with the FactorMiner [33] and factoextra [34] R-packages for visualizing the relationship among phenotypic traits and the red clover populations.

3. Results

3.1. Shoot System

Shoot growth parameters varied significantly among the red clover accessions ($p < 0.05$; Table 1). Cultivars Tuscan and Redqueli showed the lowest and highest stem elongation rate (Grate), respectively. Modern cultivar Superqueli and ASLs showed similar Grate to the oldest cultivar Quiñequeli. Lag time varied between 59.3 (Tuscan) and 77.8 (Redqueli) days. Modern cultivars and ASL showed similar lag time to the oldest cultivar. Plant height (Hmax) was 105 cm in average, ranging from 93.5 (Syn IntV) and 115.3 (Redqueli) cm. It is important to highlight that cultivars Redqueli and Superqueli did not show difference in growth parameters with regard to the oldest cultivar. However, significant difference was observed between them. Leaf traits also varied significantly among the red clover accessions ($p < 0.05$; Table 1). Leaf size ranged between 18.2 (Tuscan) and 29.9 cm^2 (Quiñequeli). Modern cultivar Superqueli and the ASL Sel Syn PreI and Syn PreIII showed significant lower leaf size than the oldest cultivar. The SLA did not show significant differences among the Chilean germplasm ($p > 0.05$), with the exception of Syn IntV (Table 1). However, modern cultivars and ASL showed, on average, 10% lower SLA than the oldest cultivar. Chlorophyll content varied between 47.4 (Syn IntV) and 53.3 SPAD units (Sel Syn PreIII and Syn PreIII). Modern cultivars and ASLs showed a similar chlorophyll content to the oldest cultivar Quiñequeli. In general, shoot traits showed medium-high level of genetic control; broad sense heritability ranged between 0.38 (SLA) and 0.61 (lag time; Table 1).

Table 1. Stem elongation rate (Grate), lag time, plant height (Hmax), leaf size, specific leaf area (SLA) and chlorophyll content of 11 red clover populations grown in mesocosms under greenhouse conditions.

Populations	Grate (cm day^{-1})	Lag Time (days)	Hmax (cm)	Leaf Size (cm^2)	SLA (cm^2 g^{-1})	Chlorophyll Content (SPAD Unit)
Quiñequeli	1.48	71.6	109.8	29.9	430.9	50.3
Redqueli	1.73	77.8	115.3	29.3	400.0	51.5
Superqueli	1.27	66.5	106.2	25.4	399.1	52.6
Starfire	1.41	67.9	109.2	27.3	380.5	51.8
Tuscan	1.17	59.3	99.6	18.2	450.5	48.0
Sel Syn IntIV	1.32	67.5	104.4	27.4	407.6	49.6
Syn IntIV	1.26	64.8	101.4	28.3	392.1	50.4
Syn IntV	1.30	73.4	93.1	22.2	505.5	47.4
Sel Syn PreI	1.37	73.6	103.9	23.0	410.7	49.3
Sel Syn PreIII	1.36	69.5	100.5	25.1	429.0	53.3
Syn PreIII	1.37	65.8	112.5	27.1	394.2	53.3
LSD value	0.280	9.15	12.48	3.9	48.76	3.25
p value	**	**	*	***	***	**
H^2	0.51 ± 0.10	0.61 ± 0.15	0.54 ± 0.12	0.51 ± 0.13	0.38 ± 0.14	0.22 ± 0.08

LSD: least significant difference; H2: broad sense heritability. *, **, *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Introduced cultivar Tuscan expressed lower values of growth parameters (Grate, Lag time and Hmax) than the Chilean populations. Otherwise, StarFire and the Chilean populations exhibited similar growth parameters because all of them were selected mainly for cutting. Tuscan showed 30% smaller leaf size than the Chilean populations with similar values of SLA, whereas StarFire showed a similar leaf size but with a 10% smaller SLA than the Chilean populations (Table 1).

3.2. Root System

Root traits varied significantly among the red clover accessions ($p < 0.05$; Table 2). Crown diameter (CrownD) was 20% higher in modern red clover accessions compared to the oldest cultivar Quiñequeli. Cultivars Tuscan and Quiñequeli and ASL Syn IntV showed the lowest CrownD values. The root system average diameter (avgD) was almost 50% higher in cultivars Redqueli and Superqueli than Quiñequeli. Among the ASL, only Syn PreIII showed significant higher avgD than Quiñequeli. The root length density (RLD) ranged between 0.19 (Tuscan) and 0.37 cm cm^{-3} (Superqueli). Cultivars Quiñequeli and Redqueli exhibited similar values of RLD; they were nearly 60% lower than Superqueli. Three ASL (Syn IntIV, Sel Syn PreI and Syn PreIII) obtained higher RLD values than Quiñequeli. The total root volume (Vol) varied between 9.5 (Syn IntV) and 21.5 cm^3 (Superqueli). Redqueli and Superqueli showed same Vol values, but they were more than 50% higher than the oldest cultivar Quiñequeli. All ASL exhibited statistically similar Vol values compared to the modern cultivar Superqueli. Topological root traits also showed significant difference among red clover accessions ($p < 0.05$; Table 2). Quiñequeli and Redqueli exhibited similar values of altitude and external path length (EPL). However, Superqueli reached nearly 20 and 50% higher altitude and EPL, respectively, than the older cultivars. Additionally, ASL Syn IntIV and Syn PreIII obtained significantly higher altitude and EPL than the older cultivars Quiñequeli and Redqueli. Dichotomous branching index (DBI) varied significantly among populations ($p < 0.001$). Modern cultivars and ASL sowed almost 20% lower DBI values than Quiñequeli. Root traits exhibited medium-low values of genetic control; broad sense heritability ranged between 0.20 (altitude) and 0.48 (RLD; Table 2).

Table 2. Crown diameter (CrownD), average root diameter (avgD), root length density (RLD), root volume (Vol), altitude, external path length (EPL) and dichotomous branching index (DBI) of 11 red clover populations grown in mesocosms under greenhouse conditions.

Populations	CrownD (cm)	avgD (cm)	RLD (cm cm^3)	Vol (cm^3)	Altitude	EPL	DBI
Quiñequeli	8.66	1.79	0.23	12.3	36.5	638.6	0.64
Redqueli	10.57	2.62	0.25	18.8	37.3	686.3	0.52
Superqueli	10.50	2.68	0.37	21.5	45.0	976.8	0.52
Starfire	10.64	2.11	0.24	18.9	37.7	658.1	0.55
Tuscan	8.47	1.67	0.19	10.4	38.4	503.2	0.67
Sel Syn IntIV	11.17	2.08	0.28	16.7	39.7	753.7	0.58
Syn IntIV	10.02	2.20	0.34	18.8	44.6	1171.6	0.47
Syn IntV	8.49	1.69	0.21	9.5	40.1	617.3	0.57
Sel Syn PreI	10.71	1.92	0.32	16.7	38.2	933.6	0.45
Sel Syn PreIII	9.56	1.86	0.28	16.0	38.0	766.8	0.55
Syn PreIII	11.63	2.32	0.32	20.0	44.2	1118.9	0.50
LSD	1.7	0.51	0.07	5.61	5.84	325.06	0.11
p value	***	***	***	***	*	***	***
H^2	0.30 ± 0.09	0.34 ± 0.08	0.48 ± 0.10	0.36 ± 0.09	0.20 ± 0.08	0.32 ± 0.08	0.37 ± 0.10

LSD: least significant difference; H2: broad sense heritability. *, *** refer to $p < 0.05$ and $p < 0.001$, respectively.

3.3. Dry Matter Partitioning and Phenotypic Relationship among Traits

Dry matter partitioning showed broad variability among red clover accessions (Table 3). ShootDM and its components (Leaves + Stems) showed significant differences among accessions ($p < 0.05$). ShootDM ranged from 25.1 (Syn IntV) to 58.7 g $plant^{-1}$ (Redqueli). Modern cultivars and ASL Syn IntIV exhibited almost 30% higher ShootDM than Quiñequeli ($p < 0.05$). Excluding ASL Syn IntV, all remaining accessions obtained similar ShootDM compared to Quiñequeli ($p > 0.05$). The fraction of biomass invested in leaves and stems components were on average 57 and 43% of the total ShootDM. Both traits showed broad phenotypic variability ranging from 17.5 (Syn IntV) to 33.6 g (Superqueli) and from 7.7 (Syn IntV) to 28.8 g (Redqueli) for LeavesDM and StemDM, respectively. Cultivars Redqueli and Superqueli showed similar ShootDM, but with different allocation. Superqueli achieved higher leaves and lower stem dry matter accumulation than Redqueli (Table 3).

Table 3. Dry matter (DM) production at above- and belowground level of 11 red clover populations grown in mesocosms under greenhouse conditions.

Populations	LeavesDM (g)	StemDM (g)	ShootDM (g)	$RootDM_{20\ cm}$ (g)	FineRootDM (g)	RootDM (g)	RootDM:ShootDM
Quiñequeli	24.8	20.2	45.0	2.97	0.66	6.47	0.12
Redqueli	29.9	28.8	58.7	4.31	1.12	7.66	0.11
Superqueli	33.6	23.3	56.9	4.25	1.04	7.35	0.13
Starfire	22.7	19.1	41.8	4.63	1.03	7.40	0.18
Tuscan	15.9	11.0	26.8	2.98	0.68	4.85	0.18
Sel Syn IntIV	31.9	19.5	51.4	3.67	1.01	6.16	0.12
Syn IntIV	30.5	27.2	57.7	4.38	1.08	7.34	0.13
Syn IntV	17.5	7.7	25.1	1.87	0.41	3.34	0.13
Sel Syn PreI	31.5	22.5	54.0	2.94	0.85	6.13	0.11
Sel Syn PreIII	23.2	17.4	40.6	3.35	1.07	6.77	0.14
Syn PreIII	26.6	22.1	48.7	4.05	0.86	6.79	0.14
LSD	6.56	5.80	11.8	0.98	0.28	1.47	0.035
p value	**	***	***	*	**	*	*
H^2	0.39 ± 0.10	0.55 ± 0.14	0.51 ± 0.11	0.21 ± 0.08	0.18 ± 0.08	0.15 ± 0.05	0.22 ± 0.04

LSD: least significant difference; H2: broad sense heritability. *, **, *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

Total RootDM ranged between 3.34 (Syn IntV) and 7.66 g (Redqueli). Chilean accessions did not show significant difference among them ($p > 0.05$), excluding Syn IntV, which showed a RootDM significantly lower than the older cultivar (Table 3). The RootDM in the top 20 cm of mesocosm ($\text{RootDM}_{20\text{ cm}}$) represented on average almost the 60% of the total RootDM. Cultivars Redqueli and Superqueli and two ASLs (Syn IntIV and Syn PreIII) showed higher $\text{RootDM}_{20\text{ cm}}$ than the oldest cultivar Quiñequeli. Dry matter production of fine roots (FineRootDM) varied significantly among red clover accessions ($p < 0.05$). The FineRootDM represented on average between 10 and 15% of total RootDM. Modern cultivars and ASL showed almost 50% higher FineRootDM than older cultivar Quiñequeli (Table 3). The Chilean accessions exhibited a RootDM:ShootDM relationship statistically similar among them. The RootDM:ShootDM relationship of the Chilean accessions was significantly lower than introduced-cultivars Starfire and Tuscan (Table 3). Biomass production and partitioning exhibited low-medium level of genetic control; H^2 values ranged between 0.15 (RootDM) and 0.51 (ShootDM) (Table 3).

Correlation analyses among phenotypic traits showed that SLA was the aboveground trait more related to the expression of belowground traits. SLA was negatively correlated with CrownD ($r = -0.79$; $p < 0.01$), RLD ($r = -0.60$; $p < 0.05$), avgD ($r = -0.68$; $p < 0.05$), rootDM ($r = -0.91$; $p < 0.001$) and Vol ($r = -0.79$; $p < 0.001$) (Figure 1A). The first two principal components (PC) accounted for 76.6% of the total variation for the 14 phenotypic traits (Figure 1B); the PC1 and PC2 captured 58.3 and 18.3%, respectively. The phenotypic relationship among traits allowed to differentiate the red clover populations. For instance, SLA was negatively correlated with several root and shoot system-related traits (Figure 1B). Among the Chilean cultivars, Quiñequeli reached the highest value of SLA, which was associated with low values of growth parameters and Shoot and Root system related traits. On the other hand, Redqueli and Superqueli were similar and showed lower values of SLA compared to Quiñequeli However, these cultivars exhibited a different phenotypic expression (Figure 1B). Superqueli and Redqueli reached similar values on PC1 but with divergent values on PC2. In fact, both cultivars accounted for similar values of root and shoot DM accumulation but with different root morphology and architecture and growth parameters (Figure 1B). For instance, Superqueli showed higher RLD and lower Lag time and Grate than Quiñequeli.

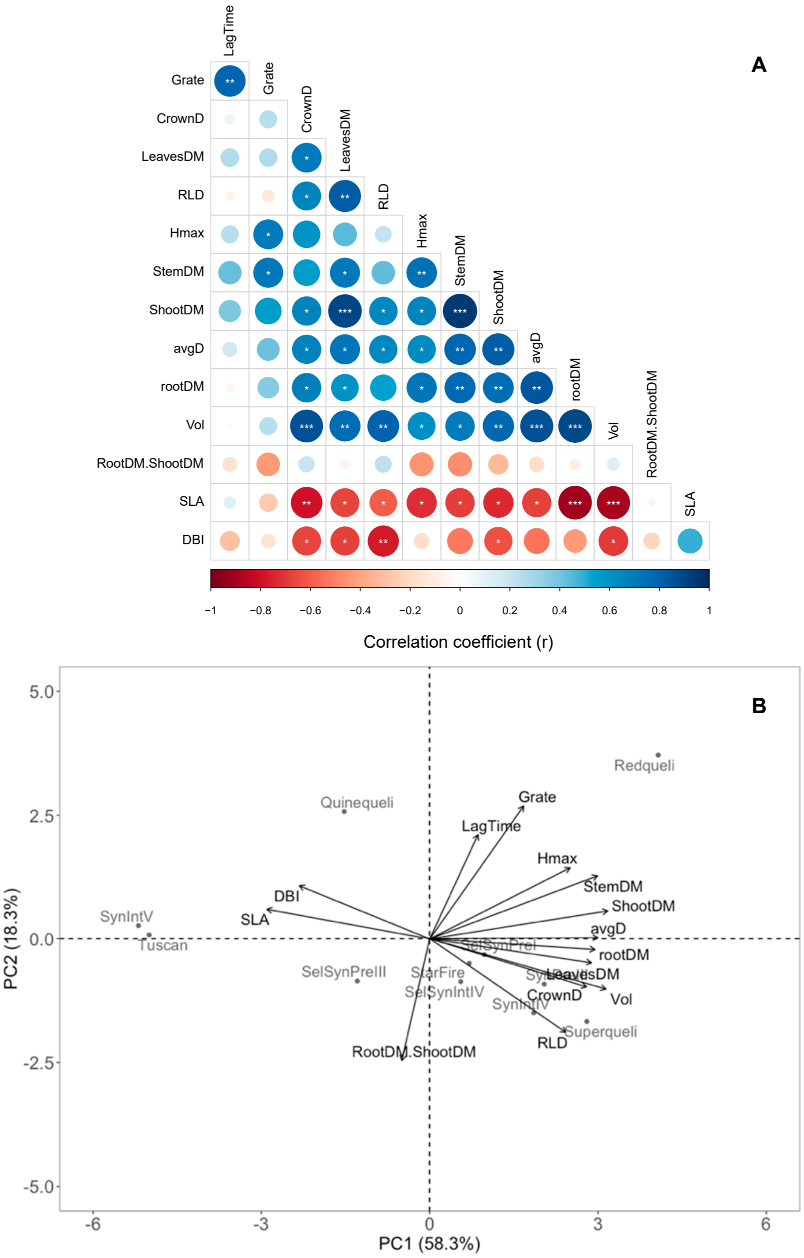

Figure 1. Correlogram (**A**) and Biplot (**B**) of the first two principal components (PC1 and PC2) for the principal component analysis of 14 traits evaluated in eleven red clover populations. The traits are: dry matter production of leaves (LeavesDM), stems (StemDM), shoot (ShootDM) and roots (rootDM). Specific leaf area (SLA), plant height (Hmax), lag time (LagTime), crown diameter (CrownD), average root diameter (avgD), root length density (RLD), total root volume (Vol), dichotomous branching index and root to shoot ratio (RootDM:ShootDM). In the correlogram *, ** and *** refer to $p < 0.05$, $p < 0.01$ and $p < 0.001$, respectively.

4. Discussion

Differences in phenotypic expression among the oldest low-persistent cultivar Quiñequeli and modern germplasm bred in Chile reveal some phenotypic traits conferring higher persistence to red clover. Aboveground, highly persistent red clover exhibited lower SLA and higher ShootDM. Belowground, they did not change total RootDM but noticeably changed root biomass allocation, morphology and architecture.

Aboveground, highly persistent red clover population (modern cultivars and ASL; Figure S1 and Table S1) showed up to 30% higher ShootDM than the oldest cultivar Quiñequeli (low persistence). Alarcon et al. [19] evaluated the ShootDM of the same set of Chilean red clover populations during two growing seasons. Their results coincide with what we observed in this work. However, at the sward level, broad genetic variability in ShootDM was also observed only from the second growing season. In perennial forage clovers, genetic investigations of the yield *per se* are scarce [35]. Genetic gains for the DM yield have been mostly related to disease and insect resistance and plant persistence [1,35]. For diploid red clover, a single work has reported genetic gains between 0.21 and 0.43% year^{-1} for DM yield per se [1], which is lower than the realized genetic gain achieved by the Chilean red clover breeding program in the last 30 years (0.4 to 2.6% year^{-1}) [4].

Modern cultivar Superqueli and ASL Sel Syn IntIV and Sel Syn PreI achieved higher ShootDM with higher LeavesDM, but achieved similar StemDM compared to the older cultivar Quiñequeli ($p < 0.05$). Since forage quality is associated positively with the leaf-to-stem ratio, owing to the greater quality of leaves relative to stems [36], it is possible to infer that the breeding of highly persistent red clover indirectly improved forage quality.

Despite some highly persistent populations (ASL) reached similar ShootDM than the older cultivar as individual plant, they surely overcome it at the sward level because of the higher survival of plants. Hoekstra et al. (2018) evaluated the agronomic performance of cultivars of two different types of European red clover (Mattenklee vs. Ackerklee). They found that during the first growing season, both types of cultivars obtained similar biomass production. However, during the third growing season, cultivars with higher persistence (Mattenklee) showed 42% higher biomass production. The relationship between plant survival and total biomass production has also been found in North American [1], European [6], Australian [7] and Chilean cultivars [4].

Growth parameters were similar among Chilean accessions; however, significant differences were found between cultivars Redqueli and Superqueli (Table 3). The latest one expressed 11 days lower Lag time than Redqueli, which might confer a higher seedling vigor [37]. Seedling vigor is an important phenotypic trait for small seeded perennials clovers because confer competitive ability during the stand establishment [2,35].

In this work, highly persistent red clover cultivars and ASLs exhibited lower values of SLA (Figure S1 and Figure 1). Additionally, SLA was negatively correlated with ShootDM and roots traits (CrownD, Vol, RLD and RootDM). Hoekstra et al. [5] measured SLA in red clover populations with divergent plant persistence. Their results were completely consistent with what it was observed in the Chilean populations. Populations with higher plant persistence also showed significantly lower SLA and this was also correlated negatively with shoot biomass production. SLA is a linear estimator of leaf thickness, which has been negative associated with photosynthetic capacity and growth rate [38]. Plants with thicker leaves (lower SLA) have been associated with increased leaves longevity but with higher construction/respiration costs [38,39].

The ASL Syn IntV, was the unique population that exhibited a phenotypic expression similar to the older cultivar Quiñequeli (Tables 1–3). Despite of that, it is one of the ASL with the higher plant persistence at field condition (Figure 1). Alarcón et al. [19] demonstrated the importance of the root borer (*Hylastinus obscurus*) in the persistence of red clover in Chile. Furthermore, Tapia et al. [40] reported that red clover can synthetize limonene, a volatile compound that plays a repellent function against root borer. Studies of plant-pest interaction, demonstrated that ASL Syn IntV was a population that achieved the lowest root borer populations in a two growing seasons field experiment [4,19]. This result may suggest that plant persistence of the ASL Syn IntV is mostly modulated by its repellency to herbivores.

In general, introduced cultivar Tuscan showed the most divergent phenotypic expression relative to the Chilean cultivars and ASLs. Tuscan expressed lower values of growth parameters (Grate, Lag time and Hmax), ShootDM and leaf size than the Chilean populations. Belowground, Tuscan showed lower CrwonD, RLD and root Vol, but higher DBI than the Chilean populations. StarFire and the

Chilean populations exhibited similar phenotypic expression because all of them were selected mainly for cutting. On the other hand, Tuscan and the ASL Syn IntV exhibited almost identical phenotypic expression (Figure 1). The persistence of both materials is mainly based on their pest resistance. Tuscan is able to synthetize high level of formononetin [7], which is an isoflavonoid with antifeedant properties [41]. As mentioned before, ASL Syn IntV could contain herbivore-repellant mechanisms associated with the synthesis of semiochemicals. The results are suggesting that the phenotypic expression of highly persistent red clover, with capacity for synthetizing chemical compounds with antifeedant/repellency properties, is associated with lower biomass productivity at an individual plant level (Figure 1).

The total RootDM and RootDM:ShootDM ratio were similar in the gradient of persistence of the Chilean red clover populations (Table 3). Maintain a stable RootDM:ShootDM ratio could be considered as an efficient metabolic strategy of DM partitioning, because the root system can lose around 30 to 50% of the total energy in respiratory processes [42]. Thilakarathna et al. [43] characterized RootDM of a set of six Canadian red clover cultivars. They found a broad difference among cultivars in RootDM, which was mainly attributed to the ploidy level and nitrogen metabolism; tetraploid cultivars reached almost twice the RootDM compared to diploids. With a soil-core sampling methodology, Bolinder et al. [44] measured root biomass at the 0–45 cm soil layer. Their results showed that, during the first growing season, red clover produces 740 g of RootDM per square meter with a RootDM:ShootDM ratio near to one, which is almost seven times higher than the values observed in this work. Skinner and Comas [45] determined the allometric coefficient (k) between the root and shoot biomass production of red clover. Their results showed that the k value tends to 1 in optimal growing condition (k = 1; relative shoot growth = relative root growth). The lower values of the Root:Shoot relationship obtained in this work was due to the ShootDM included the biomass accumulated in two harvest performed during the entire experimental period. Furthermore, the broad range of variation in the RootDM:ShootDM ratio found in different works reported in the literature also represent the high level of environmental effect on the trait, which was evident in the low value of broad sense heritability (Table 3).

Despite that the root biomass was not changed in the red clover Chilean populations, large modifications were observed in the biomass allocation within the root system. Root biomass distribution across the soil profile and fine root biomass production were significantly changed in modern populations (Table 3). The $\text{RootDM}_{20\text{ cm}}$ represented almost the 60% of total RootDM of red clover, which is consistent with others studies [45,46]. Modern cultivar Superqueli and most ASL showed 45% higher $\text{RootDM}_{20\text{ cm}}$ than the older cultivar Quiñequeli (Table 3), which indicates that highly persistent red clover populations have shallower root systems. This is the first time that shallower root systems are associated with higher persistence red clover. Several studies have associated shallower root growth with enhances topsoil foraging and higher phosphorus acquisition [26,27,47]. In the livestock area of Chile, soil originated from volcanic ash (Andisol) are predominant. They are mainly characterized by phosphorus adsorption and immobilization. Probably, P-efficiency is playing an important role in red clover persistence in the Chilean environments.

The fine root system (<2 mm diameter) is the principal pathway for water and nutrient absorption [42]. Species with high investment in the biomass in fine roots develop high root system lengths, which confer a greater soil foraging capacity [26,42]. Highly persistent red clover population increased the fine root biomass by almost 50% relative to the low-persistence cultivar Quiñequeli. Root length is expressed in relative terms as the specific root length (SRL = root length/root biomass) or the root length density (RLD = root length/soil volume). Both terms are narrowly related to the root diameter. Higher RLD or SRL genotypes tend to have greater plasticity in root growth, greater physiological capacity for water and nutrient uptake, but less root longevity and less mycorrhizal dependency than species of low root length. In this work, modern cultivar Superqueli and some modern ASLs (Syn IntIV, Sel Syn PreI and Syn PreIII) expressed up to 50% higher RLD value than Quiñequeli, which means that Superqueli and these ASLs built a root system with a longer length or higher exploratory capacity.

Few efforts have been made to interpret the role of the root system on plant persistence in red clover. Montpetit and Coulman [17], using a visual score, demonstrated that red clover persistence is improved by the growth of adventitious roots from the crown. At the same time, they reported low values of narrow sense heritability for the trait (0.3). Marshall et al. [6] demonstrated that higher crown root diameter improved plant persistence of modern cultivars of red clover bred in the UK. Both reports confirm the results obtained in this work, that is, most root traits exhibited low values of broad sense heritability and highly persistent populations exhibited 20% higher crown diameter than the lowest persistence cultivar Quiñequeli.

Topological parameters (EPL, altitud and DBI) describe the branching pattern of a root system. In this work, highly persistent cultivar Superqueli exhibited 20% and 50% higher Altitude and EPL than the lowest persistent cultivar Quiñequeli. Furthermore, higher persistent red clover populations exhibited almost 20% lower DBI values (Table 2, Figure 1). Fitter [22] pointed out that the branching pattern is modulated by soil resources, and plant modify it for increasing the root efficiency to capture water and nutrients. For instance, low-branched systems (herringbones type) are the most efficient at exploitation of the soil; however, they are the least efficient at transporting materials to the shoot system. On the other hand, highly branched systems are typically developed in soils where resources are abundant and exploitation efficiency may not be of prime importance. In this work, plants grew in abundant soil resources; thus, their potential root branching was expressed. Under this condition, modern cultivars and ASLs exhibited a more dichotomous root system compared to the oldest cultivar Quiñequeli.

5. Conclusions

Highly persistent red clover cultivars and ASLs bred in Chile modified their phenotypic expression of individual plants at shoot and root levels. At shoot level, highly persistent populations were associated with higher ShootDM, higher LeavesDM and lower SLA. At root level, highly persistent populations did not change total RootDM, but noticeably changed root biomass allocation ($\text{RootDM}_{20\text{ cm}}$ and FineRootDM), morphology (crownD, RLD) and architecture (Altitude, EPL and DBI). Highly persistent red clover exhibited lower DBI but higher RLD, CrownD and root Vol. In an applied plant breeding scenario, these results may help to design more efficient selection criteria. For instance, the strong relationship between SLA and root traits offers tremendous potential for indirect phenotypic selection.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4395/10/12/1896/s1, Figure S1: Ranking of persistence estimated according to the GGE biplot method of nine red clover populations developed by the Chilean breeding program. Persistence was evaluated in eight experiments managed under rainfed (Rn) and irrigated (Ir) conditions. Persistence was evaluated as plant survival at the end of the third and fourth growing season in Rn and Ir experiments, respectively. Number in the environments (Ir and Rn) indicate the establishment year, Figure S2: A) Daily substrate water potential (kPa) recorded at 1 h interval with capacitance sensors (MPS-2, Decagon, USA) located 5, 25, 45, 65, and 85 cm deep in two no-experimental unit mesocosms. B) Daily mean, maximum and minimum substrate temperatures recorded at 5 cm depth. Values correspond to the mean of five values recorded in five random selected experimental units. Sowing was performed on October 10th 2013, Table S1: Description of cultivars and advanced synthetic lines (ASLs) used in this study.

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