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Caracterización fenotípica de una población de trébol blanco (*Trifolium repens* L.) para tolerancia a frío

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CARACTERIZACIÓN FENÓTIPICA DE UNA POBLACIÓN DE TRÉBOL BLANCO (*Trifolium repens* L.) PARA TOLERANCIA A FRÍO

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CARACTERIZACIÓN FENOTÍPICA DE UNA POBLACIÓN DE TRÉBOL BLANCO (*Trifolium repens* L.) PARA TOLERANCIA A FRÍO

PHENOTYPICAL CHARACTERIZATION OF A WHITE CLOVER POPULATION
(*Trifolium repens* L.) FOR COLD TOLERANCE

Palabras adicionales: adaptación a frío, desarrollo de estolones, morfología de plantas, concentración de carbohidratos, índices espectrales de vegetación

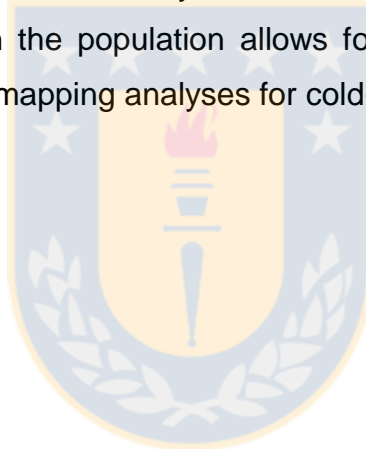
RESUMEN

El trébol blanco (*Trifolium repens* L.) es la principal leguminosa forrajera en las praderas de la zona centro y sur de Chile, y las regiones templadas del mundo. Nuevos genotipos con tolerancia a frío permitirían el desarrollo de cultivares con mayor productividad y temporadas de crecimiento prolongadas hacia finales de otoño e inicio de primavera. El objetivo del ensayo fue caracterizar fenotípicamente una población 192 genotipos de trébol blanco para tolerancia a frío, en condiciones contrastantes de frío invernal. Se utilizó un diseño Alpha-Lattice, con 24 bloques incompletos y dos repeticiones. Los rasgos morfológicos, fisiológicos y bioquímicos se evaluaron en condiciones de campo durante tres temporadas de crecimiento entre 2013 y 2016. Se detectó una alta variabilidad entre los genotipos en todos los caracteres estudiados. A consecuencia de las bajas temperaturas disminuyó la longitud y el diámetro de los estolones, longitud de pecíolos y acumulación de materia seca, mientras que aumentó la tasa de elongación de estolones y área foliar. Los índices NDVI y MTCI junto con la concentración de carbohidratos solubles en agua variaron entre los genotipos y se vieron influenciados por el frío. La alta variabilidad fenotípica de la población permitiría utilizarla como una fuente de variabilidad genética en estudios de mapeo asociativos para trébol blanco con tolerancia a frío.

SUMMARY

White clover (*Trifolium repens* L.) is the main forage legume species in the more productive grassland areas of the central and southern zones of Chile, as well as in all the temperate regions of the world. New cold-tolerant genotypes would allow to

develop cultivars of high productivity with the ability to extend the growing season in early spring and late autumn. The objectives of this experiment was to characterize phenotypically a population of 192 white clover genotypes for cold-tolerance under contrasting winter cold conditions. An Alfa Lattice experimental design of 24 incomplete blocks and two replicates was used. Morphological, physiological and biochemical traits were evaluated under field conditions, during three growing seasons from 2013 to 2016. High variability among genotypes was observed in all the characters studied. The length and diameter of the stolon internodes, the petiole length, and dry matter accumulation, decreased in response to low temperatures, while the stolon elongation rate and the leaf area increased. The NDVI and MTCI indices and the stolon water-soluble carbohydrates concentration varied significantly among the genotypes as affected by the low temperatures. The high phenotypic variation observed within the population allows for using it as a source of genetic variability in association–mapping analyses for cold-tolerance in white clover.



INTRODUCCION GENERAL

El trébol blanco (*Trifolium repens* L.) es la principal leguminosa forrajera en climas templados. Está distribuido en Chile desde la zona central hasta el extremo sur (30 to 55°S). La amplia utilización del trébol se debe a que es fijador de nitrógeno atmosférico, mejora la calidad nutricional del forraje y aumenta el consumo y utilización de la pradera por parte de los animales. Sin embargo, debido a su alto poder meteorizante, las praderas de trébol blanco son establecidas en asociación con gramíneas como ballica perenne (*Lolium perenne* L.), formando praderas permanentes destinadas a pastoreo (Goulas *et al.*, 2003).

Los beneficios económicos del trébol blanco son reales cuando su presencia en las praderas supera el 30%, ya que disminuyen las necesidades de una fertilización nitrogenada. Sin embargo, en la actualidad la participación del trébol blanco en las praderas permanentes por lo general, no va más allá del 10% y presenta alta variación estacional y entre años, estando fuertemente influenciada por la forma de utilización de la pradera (Goulas *et al.*, 2003; Nolan *et al.*, 2001). Condiciones de suelo y clima también afectan la persistencia y proporción del trébol en praderas mixtas (Wachendorf *et al.*, 2001a), ya que temperaturas entre 5 a 7°C disminuyen la producción de fitomasa aérea del trébol como consecuencia de los cambios morfológicos y fisiológicos en las plantas por la aclimatación a condiciones de inviernos fríos (Helgadóttir *et al.*, 2001; Wachendorf *et al.*, 2001a; Goulas *et al.*, 2003).

Después del invierno, el rebrote de la ballica comienza antes que el rebrote del trébol blanco, por sus menores requerimientos de temperatura y mayor capacidad competitiva relacionada con rasgos morfológicos y fisiológicos, que retardan y deprimen el crecimiento del trébol ya que los estolones se ubican sobre la superficie del suelo o enterrados al final del período invernal (Davidson and Robson, 1986; Collins *et al.*, 1991; Wachendorf *et al.*, 2001b). De esta forma, el hábito de crecimiento de ambas especies, ballica y trébol, disminuyen la calidad de radiación a la superficie del suelo, donde se encuentran los puntos de crecimiento del trébol blanco y estolones. Como consecuencia de una menor radiación, los brotes axilares del estolón permanecen latentes o mueren y la aparición de nuevos estolones secundarios y puntos de crecimiento disminuyen (Teuber y Laidlaw, 1996;

Wachendorf *et al.*, 2001a).

Cultivares de trébol blanco con menores requerimientos de temperaturas mejorarían el rebrote temprano en primavera y extenderían la estación de crecimiento del trébol hasta finales del otoño/inicio de invierno, mejorando la producción del trébol, la calidad nutricional de la mezcla ballica-trébol y la eficiencia económica de los sistemas de producción animal asociados (Svenning *et al.*, 1997), debido a una reducción del forraje conservado (heno o ensilaje) necesario durante el período invernal.

El trébol blanco se habría adaptado a los ambientes chilenos tras su introducción hace más de cien años a través de un proceso de selección natural, que permite el desarrollo de poblaciones naturalizadas caracterizadas por cambios fenotípicos como respuestas a los efectos ambientales, según Helgadóttir *et al.*, (2001). Estas adaptaciones morfológicas y fisiológicas observados en ambientes fríos afectan las dimensiones del estolón, la ramificación y el número de puntos de crecimiento del trébol y el tamaño de las hojas, la longitud de los peciolo y el área foliar (Rhodes *et al.*, 1994; Collins *et al.*, 1991; Helgadóttir *et al.*, 2001). Dichos cambios han sido estudiados en Europa para la obtención de nuevos cultivares de trébol blanco con un alto grado de resistencia a inviernos fríos y prolongados, combinado con una mayor capacidad de crecimiento a bajas temperaturas durante la primavera (Murray *et al.*, 2000). Plantas con pérdidas de estolones y menor producción de puntos de crecimiento después de períodos de fríos serían más sensibles al frío, mientras que las plantas con mayor densidad de puntos de crecimiento a finales de otoño/principios de primavera serían más tolerantes al frío. Por lo tanto, el último tipo de adaptación morfofisiológica, según Harris *et al.* (1983) y Collins *et al.* (1991), favorecer el rebrote del trébol temprano en primavera y la aparición y desarrollo de nuevos estolones, lo que también mejoraría con una mayor interceptación de radiación solar por parte de las hojas (Wachendorf *et al.*, 2001a, b).

Los carbohidratos no estructurales, reservas de nitrógeno y aminoácidos como prolina y arginina, se han relacionado con la tolerancia a frío en trébol blanco y se han considerado agentes criopreservadores (Svenning *et al.*, 1997; Frankow-Linderberg, 2001, Wachendorf *et al.*, 2001a, b). Estos han sido descritos por Goulas *et al.* (2003), quienes señalan a las raíces y estolones como órganos de acumulación

de los agentes criopreservadores y donde se observa la rápida disminución de su concentración luego del rebrote. Los cultivares de trébol tolerantes a frío presentan mayor concentración de carbohidratos no estructurales y sacarosa, mientras que el contenido de prolina en los cultivares sensibles a frío solo alcanza la mitad del contenido de los cultivares tolerantes a frío, sin embargo no difieren después de períodos de inviernos (Svenning *et al.*, 1997).

En los últimos años se han desarrollado nuevas estrategias de mejoramiento para optimizar la eficiencia en la selección de rasgos genéticos complejos relacionados con la tolerancia a frío, sin embargo, la caracterización fenotípica esencial para los programas de mejoramiento, no ha perfeccionado las técnicas de registro y análisis de datos las que continúan siendo lentas y engorrosas, dificultando la asociación de la variación genética con la expresión fenotípica de las plantas (Cobb *et al.*, 2013). Los recientes avances en técnicas de caracterización genotípica han permitido aumentar el número de plantas en las poblaciones estudiadas y así cubrir un mayor rango de diversidad genotípica, respecto a técnicas anteriores. Como consecuencia se dispone más eficientes para el fenotipado (Araus and Cairns, 2014). En la actualidad se están desarrollando nuevas técnicas para generar datos precisos en poco tiempo, disminuir la interacción genotipo x ambiente y evitar el daño de la planta mediante muestreos invasivos (Cobb *et al.*, 2013; Araus and Cairns, 2014). La caracterización fenotípica de alto rendimiento, desarrollado en los últimos años, incluye la mecanización del registro de datos con el uso de equipos semiautomáticos, análisis de imágenes y softwares específicos para cada carácter (Araus and Cairns, 2014; Inostroza *et al.*, 2016). Estos últimos han permitido el desarrollo de índices espectrales o de vegetación, los que mediante el registro de la reflectancia del espectro electromagnético de las plantas permite estimar propiedades de estas y, al mismo tiempo corregir factores de confusión como la reflectancia del suelo (Clevers and Kooistra, 2012). Características relacionadas con el desarrollo y capacidad fotosintética de las plantas pueden ser estimados a través de índices como el de vegetación de diferencia normalizada (NDVI), el índices de clorofila terrestre MERIS (MTCI), Punto de inflexión del borde rojo (REIP) y Índice relación modificada 2 (MCARI 2), entre otros (Dash and Curran, 2004; Barati *et al.*, 2011; Clevers and Kooistra, 2012).

Esta investigación está enmarcada dentro de un proyecto para la selección de genotipos de trébol blanco tolerante al frío (FONDECYT 1130340), cuyo objetivo principal es la identificación de regiones genómicas (QTLs) implicadas en la expresión de caracteres fenotípicos que potencia la aclimatación y la tolerancia de las plantas al frío, para un análisis de mapeo asociativo (Acuña and Inostroza, 2013). Se utilizó como fuente de diversidad genética para la tolerancia al frío, una colección de germoplasma de trébol blanco de poblaciones naturalizadas en las regiones frías de la Patagonia argentina y chilena (Zappe *et al.*, 1994; Zappe and Acuña, 1996; Tabla 1). La caracterización fenotípica en condiciones de campo, en ambientes contrastantes de frío invernal, contribuirá a identificar genotipos tolerantes a frío para futuros programas de mejoramiento genético para liberar nuevas variedades de trébol blanco con mejores tasas de crecimiento a inicios de primavera y finales de otoño, permitiendo extender la temporada de pastoreo.

HIPOTESIS

Existen diferencias fenotípicas (morfológicas, fisiológicas y bioquímicas) entre los genotipos de una población de trébol blanco para mapeo asociativo, constituida por 192 genotipos obtenidos a partir de 28 poblaciones naturalizadas en la zona fría del cono sur de América (Patagonia de Argentina y Chile) y dos cultivares de la especie, mediante exposición a bajas temperaturas de plántulas por 48 h en cámara de frío.

OBJETIVO GENERAL

Caracterizar fenotípicamente una población de 192 genotipos de trébol blanco para tolerancia frío, en tres ambientes contrastantes en relación con la temperatura invernal.

OBJETIVOS ESPECÍFICOS

- Determinar las características morfológicas de los genotipos de trébol blanco en ambientes con condiciones contrastantes de frío, evaluando la longitud y diámetro de los estolones y longitud de los entrenudos; el área foliar específica y largo de pecíolos, y materia seca acumulada.
- Evaluar la variabilidad fisiológica de los genotipos de trébol blanco en ambientes

contrastantes de temperatura mediante el uso de índices de vegetación.

- Evaluar la variabilidad bioquímica de los genotipos de trébol blanco en ambientes contrastantes de temperatura determinando la concentración de carbohidratos solubles en agua en los estolones.

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CAPÍTULO 1

PHENOTYPIC VARIATION OF COLD TOLERANCE RELATED TRAITS OF A WHITE CLOVER (*Trifolium repens* L.) ASSOCIATION-MAPPING POPULATION

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ABSTRACT

White clover (*Trifolium repens* L.) is the main forage legume species in grazing pastures in central, central-southern and southern Chile, as well as in all the temperate regions of the world. The selection of cold-tolerant genotypes is an important step for breeding cultivars to improve the clover growth curve and extend the grazing season in early spring and autumn. The objective of this work was to phenotypically characterize a 192 white clover genotypes association-mapping population for cold tolerance under contrasting cold conditions (colder month mean minimal air temperatures: 2.5, 1.6, -1.4 °C). An Alpha Lattice experimental design was used, with 24 incomplete blocks, and two replicates. Morphological, physiological and biochemical traits were evaluated in a field experiment at different dates over three growing seasons from 2013 to 2016. A high degree of variability was found among the genotypes for all the characters studied. There was no effect of environment when the experimental sites were considered as a source of variation, but comparison of measurements taken in autumn and early spring showed that in response to winter conditions there was a decrease in stolon length and diameter, petiole length, leaflet size, and dry matter accumulation, while the rate of stolon elongation and specific leaf area increased. The NVDI and MTCI vegetation indices, and water-soluble carbohydrate concentrations in stolon also varied among genotypes and were affected by winter cold. The high degree of phenotypic variability allows for using this association-mapping population as a genetic source for cold-tolerant white clover.

ABBREVIATIONS

DMA, Dry matter accumulation

LA, Leaf area

MTCI, MERIS terrestrial chlorophyll index

NDVI, Normalized difference vegetation index

PL, Petiole length

REP, Red Edge Position Index

SER, Stolon elongation rate

SID, Stolon internode diameter

SIL, Stolon internode length

SLA, Specific leaf area

SR-AT-PM, Santa Rosa- Atacalco- Puente Marchant experimental sites, respectively

SRIs, Spectral reflectance indices

WCAM, White clover association-mapping population

WSC, Water-soluble carbohydrates

WCN, White clover naturalized population



INTRODUCTION

The white clover (*Trifolium repens* L.) is the main forage legume species in temperate climate regions around the world. The species is found in Chile from the central region to the far south (30 to 55°S). It is widely used because of its capacity to fix N from the atmosphere, as well as its nutritional quality for animal feed. However, white clover is a bloating legume that should be grown associated with grass species, usually perennial ryegrass (*Lolium perenne* L.), for grazing by ruminants (Goulas et al., 2003).

The economic advantages of including white clover in a grass-legume mixture arise from its N contribution and a notable reduction in N fertilizer requirements when the clover proportion in the pasture represents at least 30%. But, in practice, the level of clover participation only reach around 10%, with a high seasonal and annual variation that is strongly affected by grassland management and use (Nolan et al., 2001; Goulas et al., 2003). The soil and climatic conditions also affect the clover persistence and proportion in grass-clover mixtures. Pasture productivity and persistence decrease under low air humidity and periods of cold (Wachendorf et al., 2001a) as a consequence of morphological and physiological changes to acclimate to winter cold (Helgadóttir et al., 2001; Wachendorf et al., 2001a).

Perennial ryegrass spring regrowth start before clover regrowth, due to the lower temperature requirements. This delays and depresses clover growth because the stolons are located close to the soil surface or buried at the end of the winter (Collins et al., 1991; Wachendorf et al., 2001b). The growth habits of the two species, grass and clover, reduce light quality at the soil level where the stolon and the white clover growing points are located. At low levels of radiation, the stolon axillary buds remain latent or die and fewer secondary stolons and growing points appear (Teuber and Laidlaw, 1996; Wachendorf et al., 2001a).

More cold-tolerant white clover cultivars can enhance early spring regrowth and extend the clover growing season until late autumn/early winter, thus improving clover production, the nutritional quality of the grass-clover mixture, and the economic efficiency of associated animal production systems (Svenning et al., 1997), the latter because of less need for more expensive conserved forage (hay or silage) in winter.

Since its introduction a hundred years ago, the white clover has adapted to

Chilean environments through natural selection that has resulted in naturalized populations characterized by phenotypical changes in response to local conditions (Helgadóttir et al., 2001). Morphological and physiological changes in response to cold conditions affect stolon dimensions, branching and the number of clover growing points, leaf size and area, and petiole length (Collins et al., 1991; Svenning et al., 1997; Helgadóttir et al., 2001). These changes have been studied in white clover cultivars in Europe. The plants which lose stolons and produce less growing points after a cold period would be more cold sensitive, while plants with higher growing point density in late autumn/early spring would be more cold tolerant. According to Harris et al., (1983) and Collins et al., (1991), the latter type of morpho-physiological adaptation favors early spring clover regrowth and the appearance and development of new stolons, which also improves if the leaf area intercepts sufficient radiation for growth (Wachendorf et al., 2001a, 2001b).

Non-structural carbohydrates, N reserves, and amino acids like proline and arginine are considered cryopreservers related to white clover cold tolerance (Svenning et al., 1997; Frankow-Lindberg, 2001, Wachendorf et al., 2001a, 2001b). Cold tolerant white clover cultivars have higher concentrations of non-structural carbohydrates and saccharose, and twice the proline content of cold sensitive cultivars. However, they do not differ after the winter cold period (Svenning et al., 1997).

New breeding strategies have been developed in recent years to improve the selection efficiency of genetic-complex traits like cold tolerance. However, phenotypic characterization, which is essential for the breeding programs, has not improved the techniques for data registration and analysis and continues to be highly time-consuming, which makes it difficult to associate genetic variation with phenotypic expression (Cobb et al., 2013). Recent advances in genotype characterization have allowed that the populations under study reach higher plant numbers, and cover higher diversity ranges, than in the past. As a consequence, phenotyping procedures are more efficient (Araus and Cairns, 2014). Researchers are developing techniques to rapidly generate precise data, decrease genotype/environment interactions, and avoid plant damage by invasive sampling (Cobb et al., 2013; Araus and Cairns, 2014). High-throughput phenotyping includes mechanization of data registration with

use of semi-automatic equipment, image analyses, and specific software for each character (Araus and Cairns, 2014; Inostroza et al., 2016).

This research is in the framework of a project to select cold tolerant white clover genotypes (FONDECYT 1130340), the main objective of which is to identify genomic regions (QTLs) by association mapping analysis that are involved in the expression of phenotypic characters that enhance plant acclimation and cold tolerance (Acuña and Inostroza, 2013). A germplasm collection of naturalized white clover populations in cold regions of Argentinean and Chilean Patagonia (Zappe et al., 1994; Zappe and Acuña, 1996; Table 1) was used as a source for genetic diversity for cold tolerance. Phenotypic characterization under field conditions in contrasting winter environments can contribute to identify cold tolerant genotypes for programs to breed white clover cultivars with better growth rates in early spring and late autumn, thus extending the grazing season. This experiment was conducted at three sites with contrasting winter conditions for identifying morphological, physiological and biochemical phenotypic differences among 192 genotypes selected from 28 naturalized populations collected in the coldest regions of the South American southern cone, as well as two cultivars of the species. The objective was to determine variability in stolon and leaf dimensions, plant growth and dry matter accumulation, vegetation indexes, and water-soluble carbohydrate concentrations in stolon. This phenotypic information will be associated with molecular data obtained in a parallel process of molecular characterization.

MATERIALS AND METHODS

The association-mapping population

A white clover association-mapping population (WCAM) of 192 genotypes was selected from 28 naturalized white clover populations (WCN) from Argentinean and Chilean Patagonia (Zappe et al., 1999), and from two cultivars, Weka and Haifa (Table 1), according to the procedure described by Acuña et al. (2014). First, the three most cold sensitive (94-55, 94-54 and 94-22) and the three most cold tolerant NWC (94-30, 94-19 and 94-65) were selected. The 96 most cold sensitive and the 96 most cold tolerant genotypes were selected from the sensitive and tolerant NWC, respectively. These genotypes were cloned to transplant them in the field.

Experimental sites

The experiment was conducted at three sites: Santa Rosa (SR) Experimental Field (36°32'S, 71°55'O), Atacalco (AT) (36°53'S, 71°37'O) and Puente Marchant (PM) (36°54'S, 71°32'O). These sites are located in a transect rising to the Andean mountains, around 36°S, and have a winter temperature gradient related to the altitude (140, 659 and 1054 m.a.s.l., respectively). Temperature (°C), air humidity (%), radiation ($W\ m^{-2}$), wind speed ($km\ h^{-1}$), and soil temperature (°C) at 10 cm depth were recorded during the experiment at 60-minute intervals by automatic meteorological stations (WatchDog 2900ET, Spectrum Technologies, IL, USA) positioned at the experimental sites, except at SR, where the meteorological data were obtained from the station of the Experimental Field (AGROMET, 2016). Figure 1 shows the air and soil temperature, and rainfall during the experiment.

The soils at all sites originate from volcanic ash (Andisols). The soil at SR is shallow and belongs to the Pueblo Seco series (Stolpe, 2006); the soil at AT is deep and from the Santa Barbara series; and the soil at PM is shallow and has not been described in the literature. Table 2 shows the nutrient availability and other chemical characteristics of the soils for the three sites.

Setting up and managing the experimental plots

The soil was ploughed and rolled, and glyphosate ($3\ L\ ha^{-1}$) was applied 20 days before planting. Fertilizer was applied at planting in an area of $0.01\ m^2$ ($10 \times 10\ cm$) per plant at a rate of $0.4\ g\ plant^{-1}$ of triple superphosphate ($79.1\ kg\ ha^{-1}$ of P), mixed with the 0-10 cm soil layer; $0.2\ g\ plant^{-1}$ of potassium chloride ($99.6\ kg\ ha^{-1}$ of K), on the soil surface; and $0.1\ g\ plant^{-1}$ of urea ($46\ kg\ ha^{-1}$ of N) broadcast on the soil surface. Experiments were set up in the spring (October-November 2013), with plant spacing of $1 \times 1\ m$. Plants at all sites were irrigated with a pressurized irrigation system with $2\ L\ h^{-1}$ drip emitters. During the growing season (October-April) plants were irrigated twice a day for an hour, except at PM, where the same amount of water was applied every 48 h ($4\ L\ h^{-1}$ for 2 h). Broadleaf weeds and grasses were periodically removed manually or with Cletodima $1\ L\ ha^{-1}$.

Unripe flowers were cut to prevent seed production and the appearance of new plants. A maintenance fertilizer containing P, K, Mg and S was applied on the surface in December 2014, in an area of $0.25\ m^2$ ($0.5 \times 0.5\ m$) around each plant $7.5\ g\ plant^{-1}$

of triple superphosphate (59.3 kg ha⁻¹ P), 2.5 g plant⁻¹ of potassium sulfate (41.5 kg ha⁻¹ de K) and 5 g plant⁻¹ of sulphomag (21.6 kg ha⁻¹ of Mg; 44 kg S ha⁻¹).

Experimental design and statistical analyses

An Alpha Lattice experimental design was used, with 24 incomplete blocks and two replicates. An ANOVA was applied to test the genotype variations (G) of the studied characters, date of measurements, and experimental sites. The ANOVA was carried out with SAS (The Mixed Procedure) according to the following linear model.

$$Y_{ijl} = \mu + \tau_i + \gamma_j + \rho_{l(j)} + \varepsilon_{ijl}$$

A combined ANOVA was also applied to test the effect of environment (E) and the G x E interaction. The standard error to compare genotype means was calculated (Gomez and Gomez, 1984).

Plant morphology and growth

Stolon dimensions were measured weekly from January to May 2014 (1st growing season) and from December 2014 to February 2015 (2^d growing season). Stolon internode, between the second and third fully expanded leaf, of two randomly selected stolons per plant were marked to measure: stolon length (from the node of third fully expanded leaf to the stolon growing point) to determine the elongation rate (SER, mm day⁻¹); stolon internode diameter (SID, mm) at the midpoint; and stolon internode length (SIL, mm).

Mean leaflet area (LA, cm²), petiole length (PL, cm) and specific leaf area (SLA cm² g⁻¹) were measured before the first cutting of the first growing season (February 2014) and before the last cutting of the second growing season (April 2015). Because of the late start of clover growth at the PM experimental site, these variables were measured before the first cutting after winter in November 2014 and 2015. Five trifoliate fully expanded leaves were randomly sampled for all characters. An area meter (MK2) was used to measure LA, after which the leaflets were dried in a forced air oven at 65°C.

Physiological characters

Spectral reflectance of the plant canopy was measured with a portable spectroradiometer (FieldSpec®, HandHeld 2TM, ASD Inc. CO, USA). Measurements

were taken before the November 2014 and April 2015 cuttings (2nd growing season) and in December and March of 2015-2016 (3rd growing season). The spectroradiometer was mounted on a tripod to measure at a height of 25 cm. The sensor covered a circular area 12.5 cm in diameter. Three spectra per plant were taken and averaged. A full spectrum consisted of 725 narrow channels with 1 nm interval between 350 and 1075 nm. Measurements were made on clear days from 1100 to 1600 h. Periodic radiometric calibrations were made against a field reference panel (Spectralon, ASD). Reflectance information was used to calculate two spectral reflectance indices (SRIs): the normalized difference vegetation index (NDVI) (Rouse et al., 1974), and MERIS terrestrial chlorophyll index (MTCI) (Dash and Curran, 2004).

Biochemical characters

Stolon water soluble carbohydrates (WSC) were measured three times during the 2014 cold season using the anthrone reactive method (Yemm and Willis, 1954). The sampling dates were chosen to cover the entire cold period, at the beginning (June 10), middle (August 6), and end (September 9) of the cold season. Four stolon sections (> 4 cm length) per plant were sampled. They were immediately washed with water and dried in a forced-air oven at 105°C for one hour and then at 40°C for 16 h (Frankow-Lindberg, 2001). In total, 3072 samples were collected (192 genotypes x 2 replications x SR and AT x 3 times, and PM x 2 times), dried and grounded (0.1 g sample⁻¹). Three hundred and sixty samples were selected (approximately 10% of the samples) for conventional WSC anthrone determination. The results were used to predict WSC concentrations of all the samples by NIR spectroscopy and chemometric analysis, scanning over a spectral wavelength range of 12000-4000 cm⁻¹ with an MPA-FT NIR analyzer (Bruker Optik GmbH, Ettlingen Germany), according to the procedures described by Monzon (2017). The stolon-WSC was expressed as mg of WSC per unit of stolon dry weight (g).

Dry matter accumulation

Herbage dry matter accumulation (DMA, g plant⁻¹) was determined by cutting whole plants at a height of two cm with an electric shearer (Oster, SHOWMASTERTM, USA). Cuttings were taken at seven dates during the experimental period (April, October and November, 2014; February, May and December, 2015; March, 2016).

The fresh material was dried in a forced-air oven at 65°C until reaching a constant weight.

RESULTS

Plant morphology and growth

There were significant differences ($P < 0.05$) among genotypes (G) in stolon and leaf morphology (Table 3). Environment (E) and the G x E interaction did not significantly ($P > 0.05$) affect these characters. Within date of measurements and sites, the genotype distribution tended to follow a similar pattern, which is why Figures 2 and 3 only show selected results from representative dates and sites to compare two dates separated by a cold period. There was broader genotype variation for SID in autumn 2014 (1.2 to 2.75 mm) than in summer 2015 (0.94 to 2.03 mm) (Figure 2). Genotypes 45 and 51 of the WCN population 94_19 had SID of around 2 mm at both evaluation dates.

The SIL of 148 genotypes (77%) in autumn 2014 ranged between 6.2 and 12.6 mm, while only two genotypes among the rest of the WCAM population, both from a tolerant WCN population (94_19), had SIL of more than 20 mm (Figure 2). During the second growing season (summer 2015) SIL were no longer than 13.5 mm, with broader distribution than in the first season, as can be seen in Figure 2. A high proportion of genotypes (69%) had SIL between 6.1 and 8.9 mm. These results were not consistent when comparing autumn with summer, because genotype 50 from a sensitive WCN population (94_22) had the highest SIL in summer.

SER was slower in the first period of measurement (2014) than in the second (2015), as can be seen in Figure 2. In 2014, 59% of WCAM genotypes had elongation rates between 0.27 and 0.82 mm day⁻¹, but only 16 genotypes had rates of over 1.09 mm day⁻¹. In the second period (summer 2015), 81% of the genotypes had daily stolon elongation rates lower than 1.08 mm day⁻¹, and two of the rest of the genotypes had rates higher than 2 mm day⁻¹.

Regarding leaf morphology, during the first growing season at the PM site, the petiole lengths (PL) of 127 genotypes were between 5.3 and 9.2 cm (Figure 3), and at a higher range in 11 genotypes (11.2 to 17.0 cm). During the second growing season (April 2015) the petioles were shorter than in February 2014 (Figure 3); 148

genotypes (77%) did not reach PL of more 5.6 cm. Nine genotypes presented petioles longer than 9.4 cm, and the highest PL value was 15.2 cm, which was reached by genotype 41 from the tolerant WCN population 94_19.

During the first period of evaluation (Figure 3), LA presented values between 0.50 and 3.07 cm². Nevertheless, a high number of genotypes (45%) were grouped in the range 0.87 – 1.23 cm². In April 2015 (Figure 3) leaflet size (LA) was less than in the first measurement period, following a similar tendency to that of petiole length (PL), and ranged from 0.27 to 2.47 cm². The genotypes with LA of less than 0.90 cm² represented 72% of the WCAM population, while only 10% had LA larger than 1.21 cm².

Unlike the other measured characters, the genotype SLA distribution was more homogeneous. SLA varied from 200 to 363 cm² g⁻¹ at the first measurement date (Figure 3), however, the measurements from April 2015 found that a higher percentage of WCAM population genotypes; 89% of the genotypes were between 166 and 320 cm² g⁻¹, while the highest value was 524 cm² g (Figure 3).

Physiological characters

The ANOVA of the spectral indexes, NDVI and MTCI, found significant differences ($P < 0.05$) among genotypes at the three experimental sites in November 2014 and at SR and PM in March 2016 (Table 3). The MTCI index was also significantly affected at AT and PM in April 2015, and at AT in December 2015. A combined ANOVA was carried out to determine G x E interaction and confirmed a high degree of variability within genotypes ($P < 0.001$), the lack of environmental effects ($P > 0.05$), and a weak effect of the interaction ($P < 0.05$), particularly for NDVI. These results allowed for averaging the site values from the measurement dates. The averaged NDVI for the three sites at the first three measurement dates featured a high percentage of genotypes with values over 0.80 (78% in November 2014, 79% in April 2015, and 65% in December 2015) (Figure 4). In contrast, the NDVI values in March 2016 were lower and the genotypes were grouped mainly in the higher classes of the histogram (Figure 4); 75% of the genotypes were distributed between 0.58 and 0.81. The measurements taken in spring, November 2014 and December 2015, showed that 60 and 63% of the genotypes had MTCI values of 2.11 and 2.63, respectively. However, in autumn, April 2015 and March 2016, the genotypes were concentrated mainly in

central classes of the histograms. Additionally, these genotypes had lower MTCI values than in the spring evaluations; 75 and 70% of the genotypes presented MCTI values below 2.03 and 2.12 in April 2015 and March 2016, respectively.

Biochemical characters

The analyses of variance found significant differences among genotypes for WSC concentration in stolons (Table 3) at all dates of measurement ($P < 0.05$). Most of the genotype WSC concentration values ranked around medium levels, with 72% between 158 and 207 mg g⁻¹ for the late autumn (June) sampling at AT (Figure 5). WSC concentrations in early spring (September) were lower than at the previous measurement dates, and 75% of the genotypes did not reach values higher than 135 mg g⁻¹. There was no effect of environment ($P > 0.05$), but the G x E interaction was significant for the three dates (combined ANOVA), which means that the same individual genotypes had different performances as a response to the specific environmental condition of the experimental sites. Figure 6 shows that when temperature increased in August (Figure 1), WSC stolon concentrations decreased due to the early spring growth initiation at SR and PM, but this was not the case at AT.

Dry matter accumulation

Dry matter accumulation varied significantly among genotypes ($P < 0.05$) at all experimental sites on first and second evaluation dates in November 2014 and April 2015. However, only the results from the AT site continued this trend in December 2015, and by March 2016 the genotypes at all the sites were statistically the same (Table 3).

The histograms in Figure 7 group the genotypes based on the DMA averages of the three sites. As can be seen, the genotypes are classed mainly in the lower DMA categories. The range of DMA variation and the distribution within that range were similar in November 2014 and December 2015 (spring cuttings). In 2014, 88% of the genotypes had values under 80 g plant⁻¹, and in 2015, 95% accumulated less than 89 g of dry matter plant⁻¹. The autumn cuttings presented a contrasting performance of the WCAM genotypes. In April 2015, 15% of genotypes had DMA between 19 and 47 g plant⁻¹, while in March 2016, 89% of genotypes had DMA lower than 43 g plant⁻¹. Only 3 to 5% of genotypes are in the higher classes of the histograms for the first

three cutting dates.

DISCUSSION

Plant morphology and growth

Boller and Nösberger (1983), Murray et al. (2000), and others have studied the effects of low temperatures on white clover leaf and stolon morphology and growth (elongation rates and growth habits). Working with white clover cultivars, they have described how stolon elongation rates, stolon internode length, and leaf area decrease in response to winter cold, thus representing cold tolerance mechanisms (Helgadóttir et al., 2001). The genotypes characterized in this experiment showed similar responses when evaluated in the field after the first winter (2014). In summer 2015, the diameter (SID) and length (SIL) of stolon internodes decreased compared with dimensions recorded at autumn 2014 (Figure 2). Before winter, 70% of the genotypes reached SID values between 1.35 and 1.82 mm, but after winter, 58% was distributed in the range of 1.25 – 1.56 mm. The SIL decreased from 77% of genotypes between 6.2 to 12.6 mm at autumn 2014, to 69% between 6.1 to 8.9 mm at summer 2015. However, these results are partially consistent with Frankow-Linderberg (1999) and Murray et al. (2000), who did not find differences between white clover cultivars for internode length.

The SER was higher in summer 2015 than in autumn 2014 (Figure 2) and 81% of genotypes exhibited SER of less than 1.08 mm day^{-1} , showing a similar distribution to autumn 2014 where 89% were between 0.0 and 1.09 mm day^{-1} . Helgadóttir et al. (2001) found that genotypes of the white clover cultivar AberHerald that survived a winter in Iceland had lower stolon extension rates, shorter internode lengths, smaller leaves and higher specific leaf area than the original population. This finding would confirm the presence of cold tolerant genotypes in the WCAM population, because in all evaluations after cold periods some genotypes followed a similar tendency, even though in this case all the genotypes survived. Helgadóttir et al. (2001) differences in the performances of the cultivars AberHerald and Undrom, the natural selection had favored plants with higher stolon extension rates, longer internodes and higher specific stolon lengths (mg mm^{-1}). This result was attributed to the long period of Undrom under the Icelandic cold conditions (three years) compared with only one

year of AberHerald. Undrom was considered to have already adapted to cold. The higher SER and SIL of some WCAM genotypes could be inherited from the original populations (WCN).

Leaf morphology evaluations showed that petioles were shorter (PL) and LA was less in April 2015 than in February 2014 (Figure 3), which may be the result of the 2014 winter cold of the colder experimental site (PM), where the plants were under snow for about one month. This reduction in PL and leaf size (LA) could be an adaptation to low temperatures, which other researchers have also observed. Boller and Nösberger (1983) compared two white clover ecotypes, Valley (560 m.a.s.l.) and Alpine (1910 m.a.s.l.), and found the Alpine ecotype had shorter petioles and less LA. PL was the character more affected in that study.

The SLA of the WCAM genotypes was also affected by low temperatures (Figure 3). A high percentage (89%) did not reach values of more than $320 \text{ cm}^2 \text{ g}^{-1}$, although four genotypes had larger SLA than before the cold period. The latter genotypes express characters associated with plants adapted to low temperatures (Boller and Nösberger, 1983; Helgadóttir et al., 2001).

Davidson and Robson (1986) studied the effect of two temperature regimes (20°C day/ 15°C night, 10°C day/ 8°C night) on S100 white clover cultivar grown under controlled conditions. They found that the plants subjected to the lower temperatures had lower SLA ($340 \text{ cm}^2 \text{ g}^{-1}$) than the plants under the higher temperature regime ($377 \text{ cm}^2 \text{ g}^{-1}$). These results should to be considered with caution, if compared with the results of our experiment, because both temperature regimes are in the upper temperature range for the growth of this species, which would be appropriate for selecting heat tolerant germplasm, while the temperature regimes of the experimental sites compared in our study (Figure 1) are at the lower end of the range for growth, which is suitable for selecting cold tolerant genotypes. However, at the PM site, where plants were under at least 15 cm of snow in June 2014, the ground area covered by plants, which was estimated measuring their diameter, was smaller at this site than at SR and AT, along with shorter stolon and larger leaf area. At the SR experimental site, with the highest air and soil temperatures of the three sites (Figure 1), plants had the largest ground cover, which could reflect higher number and greater length of stolons. These results concur with evaluations carried out in January

and February 2014 by Inostroza et al. (2016) in an earlier paper of this experiment related to the use of multispectral aerial images, in which the area of plant ground cover was determined. The mean area covered by plants was 3421.7 cm² at SR, while plant size at AT and PM was less sensitive, with means of 2011.1 and 2354.7 cm², respectively. Additional measurements by soil core extraction in September-October 2014 determined the means for total stolon length according to the site environments (323.1, 367.9 and 428.7 cm cm⁻² for PM, SR and AT, respectively), and significant ($P < 0.01$) G x E interaction (combined ANOVA, not shown) for this trait.

Physiological characters

The NVDI and MTCI indices are estimations of the physiological status of plants when they are measured. Although neither index gives direct DMA or chlorophyll content values of DMA, they provide values associated with these variables (Barati et al., 2011; Hughes et al., 2017). The NVDI is derived from physical plant properties, such as the leaf area index (LAI, leaf area/ground area), the development stage, and accumulated biomass (Carlson and Ripley, 1997). The NVDI of most of the genotypes on the first three measurement dates was over 0.8, indicating that the majority of genotypes had high status of growth and development (whole ground cover), and vigor. This was particularly the case in November 2014 and December 2015 (spring regrowth), when only 3 and 6% of genotypes, respectively, had values under 0.7. Carlson and Ripley (1997) reported that plants with indices between 0.5 and 0.8 achieve total ground cover, while indices near 0.0 are related to bare soil. In March 2016, the genotypes were concentrated in the middle and lower histogram categories (Figure 4). Some 9% of the genotypes had indices between 0.34 and 0.5, which indicates low aerial development or the presence of senescent material. These results and the differences between the first three measurements and that of March 2016 could be related to the growth rate. Carlson et al. (1990) asserted that the NVDI increases linearly when LAI increases during the first regrowth phase, then the growth curve enters an asymptotic phase and NVDI increases more slowly in relation to increases in LAI. The measurements in November 2014 and April 2015 were made during the regrowth period a month after the cuttings, as also occurred with the measurements in December 2015, while, the measurements in March 2016 were made three months after the last cutting at the end of a summer with high

temperatures (Figure 1), which may explain the lower NVDI index, given the presence of senescent material, less biomass and barer soil.

Plant development was checked visually during the regrowth period in December 2015. The plants at AT and SR were generally larger (more ground area covered), taller, and more vigorous (dark green color) than those at PM. This evaluation is clearly reflected in the NVDI and MTCI indices, which reflect the dominant effects of extreme cold at PM during the 2015 winter (Figure 1).

According to Dash and Curran (2004), the MTCI is an indirect estimator of chlorophyll content that is highly sensitive to changes at elevated concentrations and more precise than the Red Edge Position Index (REP). Clevers and Kooistra (2012) estimated the chlorophyll content (g m^{-2}) using the PROSAIL Radioactive Transfer Model, which is a combination of the PROSPECT leaf RT model and the SAIL canopy RT model (Kimes, 1984; Jacquemoud et al., 2009) that use known biophysical parameters to simulate leaf reflectance and transmittance. Clevers and Kooistra found that the MTCI was linearly related to the canopy chlorophyll content of a natural grassland ($y = 0.4701x - 0.2209$, $R^2 = 0.83$). If this equation were applied to the highest MTCI recorded in our experiment in November 2014 and December 2015 (spring measurements), the chlorophyll content would be between 1.25 and 1.38 g m^{-2} , respectively. Another function calculated by Hughes et al. (2017) could allow for expressing leaf chlorophyll content as N concentrations. Because there is limited information regarding MTCI measurements in white clover, whether for individual plants or pastures, the results of our experiment can be compared with N concentrations in white clover, where there is considerable information.

Biochemical characters

The stolon WSC concentrations decreased during the winter (June - August) and early spring (September) (Figure 6) because they were used for surviving the winter (Boller and Nösberger, 1983) and for spring regrowth (Baur-Höch et al., 1990; Goulas et al., 2003), respectively. In a study comparing native Norwegian clover to cultivars growing under less cold conditions, Dalmannsdóttir et al. (2001) described the importance of adequate autumn carbohydrate reserves in stolon for plants to survive the winter. In this experiment, mean WSC concentrations in stolons of the WCAM fell from 184 to 119 mg g^{-1} between June and September (Figure 6). The distribution

histogram (Figure 5) shows that there were 140 genotypes in June, around 80 in August, and only 14 in September in the range of 158 to 207 mg g⁻¹. In September, this range included the highest WCAM values, which confirms the genetic variability of this character, and the association between stolon WSC and cold tolerance. However, several studies of relationship between stolon WSC concentrations and cold tolerance (Collins et al., 2002, Dalmannsdóttir et al., 2001, Frankow-Lindberg, 2001) have not found phenotypic associations between these characters. Collins et al. (2002) evaluated the phenotypic relationship between stolon WSC and cold-tolerance related traits and concluded that WSC concentrations do not play a significant role in white clover cold tolerance. Annicchiarico et al. (2001) concurred with this conclusion, and Inostroza et al. (2015) pointed out the limited use of white clover stolon WSC as a selection criteria in breeding programs. The evaluation of SID should be considered in the context of the results of Boller and Nösberger (1983) regarding stolon diameter and weight. SID decreased from autumn 2014 to summer 2015 (Figure 2), which could be related to the low WSC in stolon in September 2014 (Figure 5).

Dry matter accumulation

Despite the high degree of variability in DMA among the genotypes in the evaluations in 2014 and 2015, the genotypes are concentrated in the histogram categories (Figure 7) that include around 95% of the WCAM population. However, accumulation was approximately 50% lower at the last cutting (March 2016), after two winter cold periods, although the distribution was more homogeneous than at previous cuttings, which could reflect genotype adaptation to cold conditions. These results concur with those of Frankow-Lindberg (1999) who studied the effects of low temperatures on white clover cultivars of Swiss origin that had been subjected to four cold winters. He concluded that plants adapted to winter stress have inherently lower potential biomass production than plants from milder environments when they grow in the absence of winter stress.

Although the distribution of DMA among the WCAM genotypes was similar for the three first evaluations, DMA was higher in April 2015 (Figure 7), as a result of favorable autumn growth conditions, in particular in terms of water soil availability and temperature, which promoted stolon and leaf growth and development more than that

of stolons and roots. Plants promote the growth of the latter morphological structure as a survival mechanism against low temperatures (Boller and Nösberger, 1983, Murray et al., 2000), which could explain the low DMA performance of the smaller group of genotypes (< 25, first category of histograms) at all measurement dates (Figure 7).

The results of correlation analyses were reported in an earlier presentation of this experiment (Inostroza et al., 2015) to find relationships among nine spectral reflectance indices (SRIs) calculated from the spectral reflectance of the plant canopies of the experimental sites (including NDVI and MTCI), and the DMA for the second growing season (2014 - 2015). The DMA correlated strongly with all the SRIs ($P < 0.001$). The correlation coefficient varied among the site environments, the highest and lowest r values were observed respectively at PM (0.42 to 0.72) and SR 0.25 a 0.46. Significant ($P < 0.001$) genetic variability among experimental sites for dry matter production (Table 3) was confirmed by this analysis. Dry matter production ranged from 25.8 to 557.3, 18.9 to 517.2 and 8.9 to 344.6 g plant⁻¹ at SR, AT and PM respectively.

CONCLUSIONS

There was a broad degree of genetic variability for all the evaluated phenotypic traits among genotypes of the characterized white clover associated-mapping population, which is sufficient for the next round of association-mapping analyses. There were no effects of the contrasting environments where the population was tested, but winter cold affected morphology and growth traits, reducing stolon and leaf size and dry matter accumulation, and increasing specific leaf area and the stolon elongation rate. Water-soluble carbohydrate concentrations in stolons reached their highest values in autumn and decreased after the winter cold. These conclusions enable us to use this population as a genetic source for cold-tolerant white clover.

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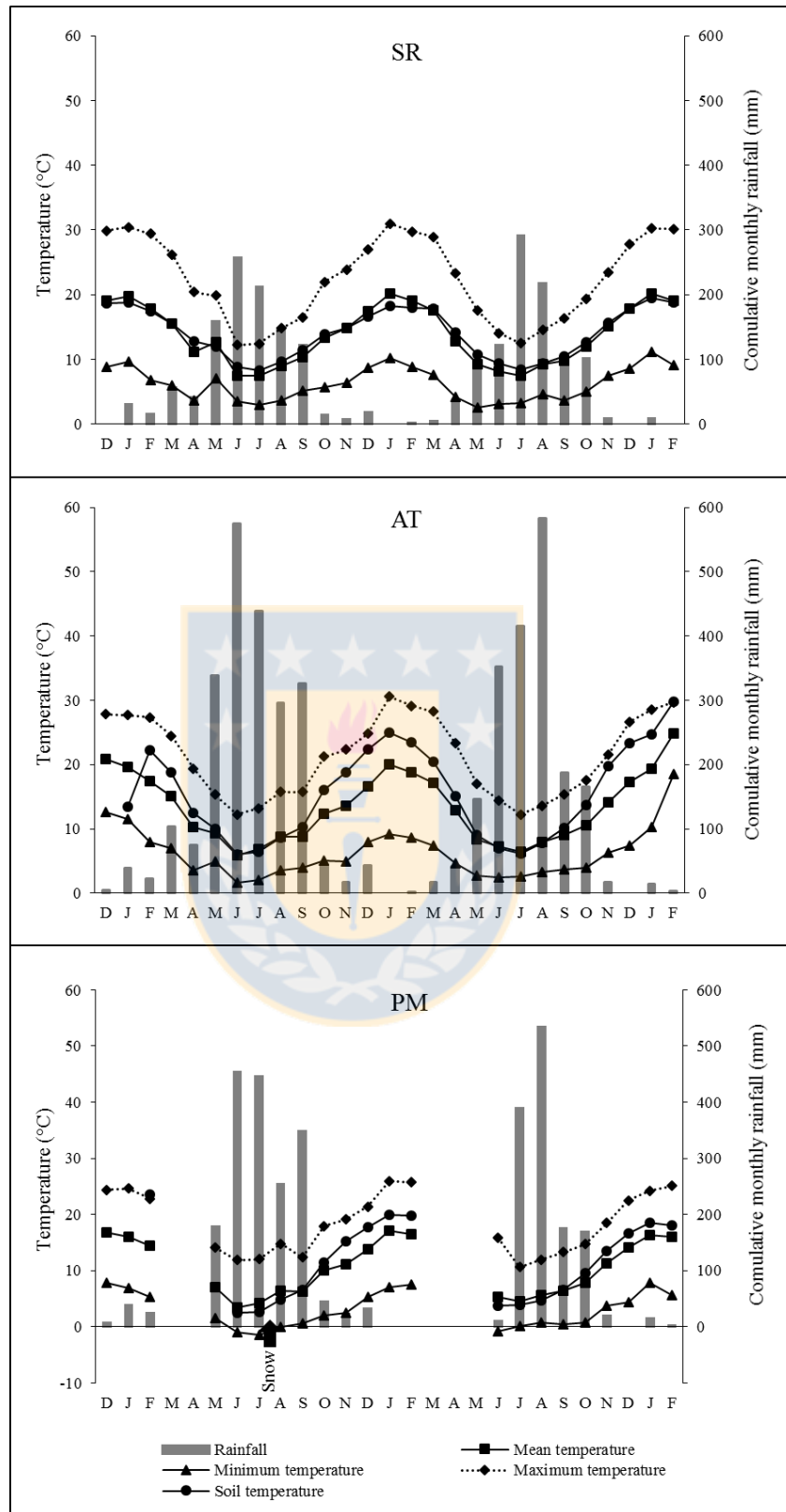


Figure 1. Monthly mean of the daily mean air and soil temperatures, and monthly rainfall during the experiment. December 2013 – February 2016.

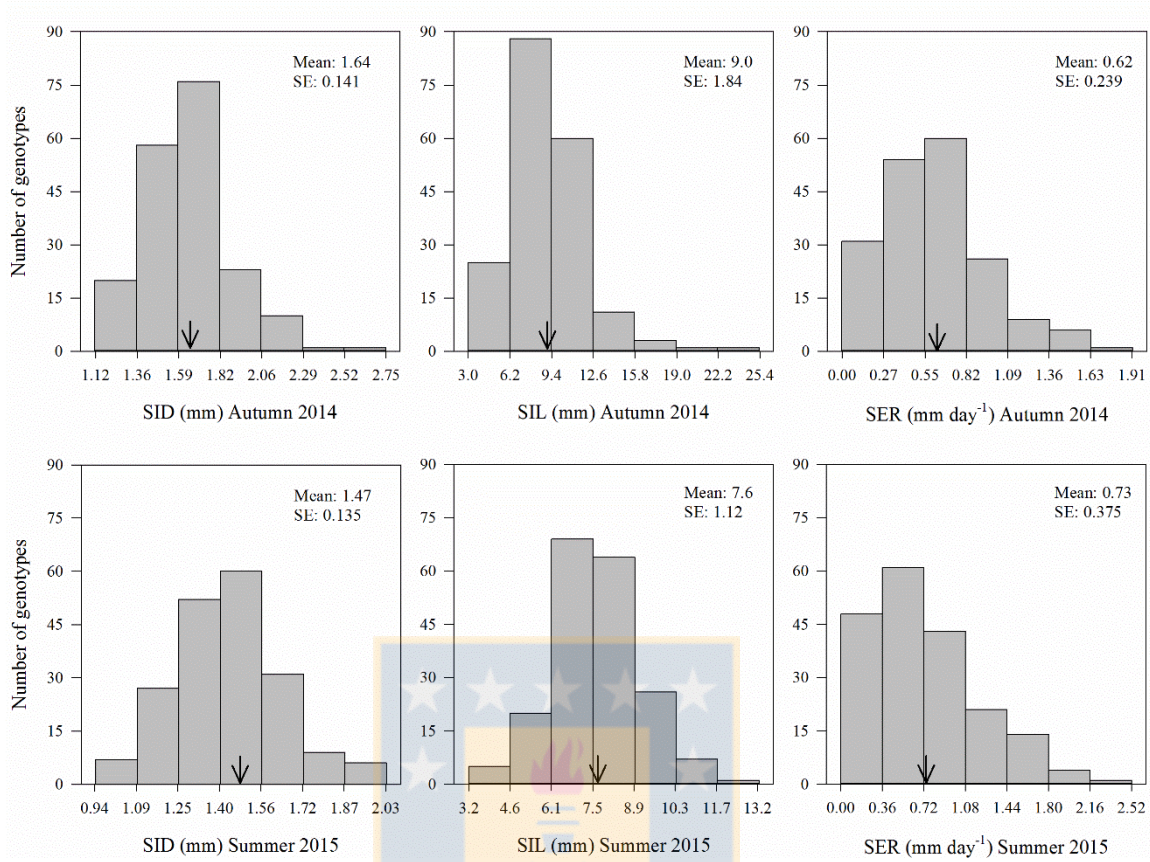


Figure 2. Genotype distribution according to the stolon internode diameter (SID) and length (SIL), and stolon elongation rate (SER) in autumn 2014 and summer 2015, at Atacalco (AT) experimental site.

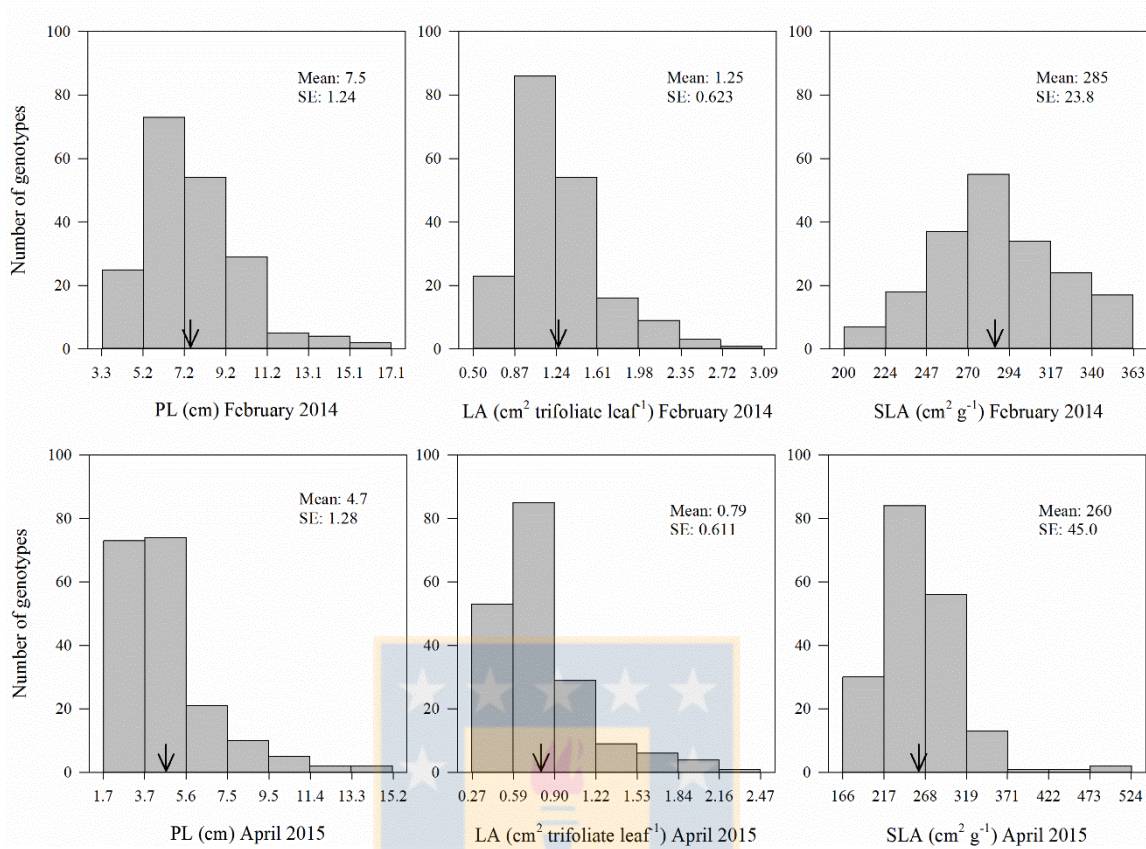


Figure 3. Genotype distribution for petiole length (PL), leaf area (LA) and specific leaf area (SLA) in the summer and autumn of 2014 and 2015, respectively, at the Puente Marchant (PM) experimental site.

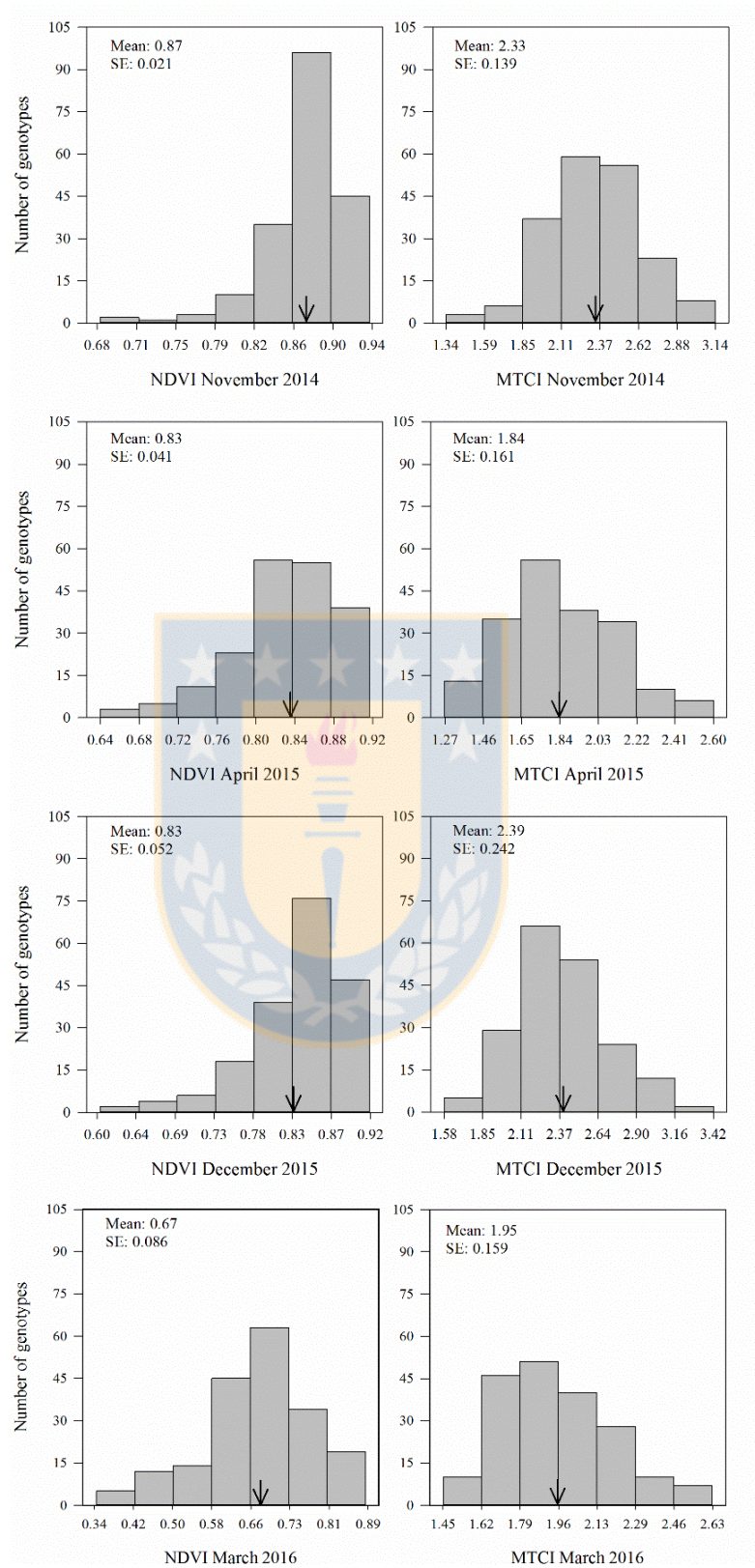


Figure 4. Genotype distribution according to the NDVI and MCTI indexes, average of the three sites in the first (2014-2015) and second (2015-2016) growing seasons.

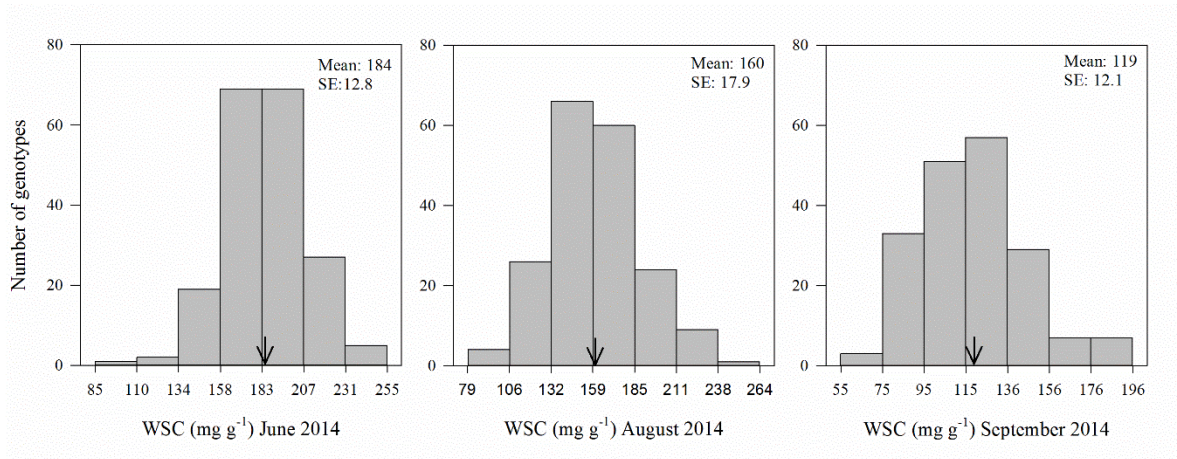


Figure 5. Genotype distribution according to stolon water soluble carbohydrate (WSC) concentrations in late autumn (June), winter (August), and early spring (September) 2014 at the Atacalco (AT) experimental site.



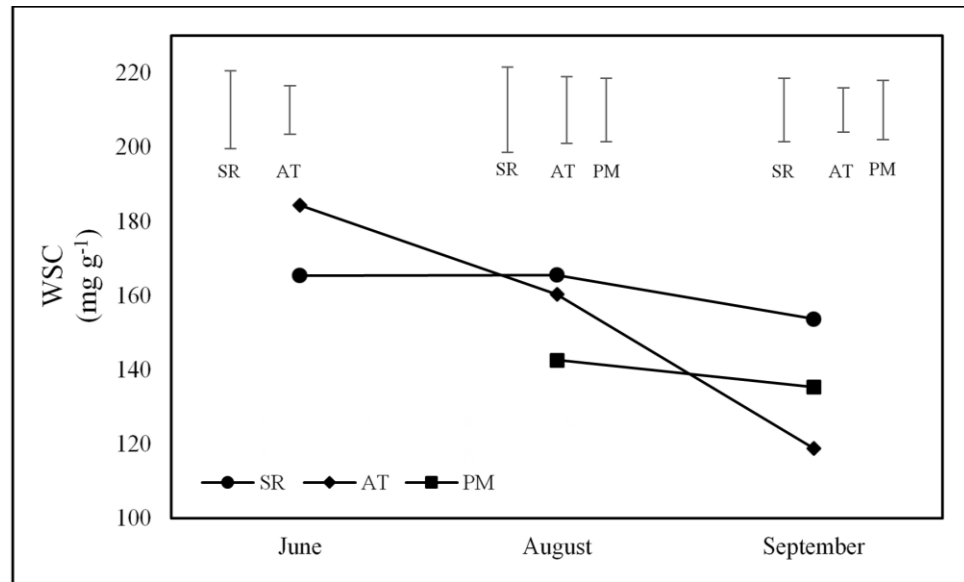


Figure 6. Comparison of genotype stolon water soluble carbohydrate (WSC) concentrations among the experimental sites: Santa Rosa (SR), Atacalco (AT), and Puente Marchant (PM), in late autumn (June), winter (August) and early spring (September).



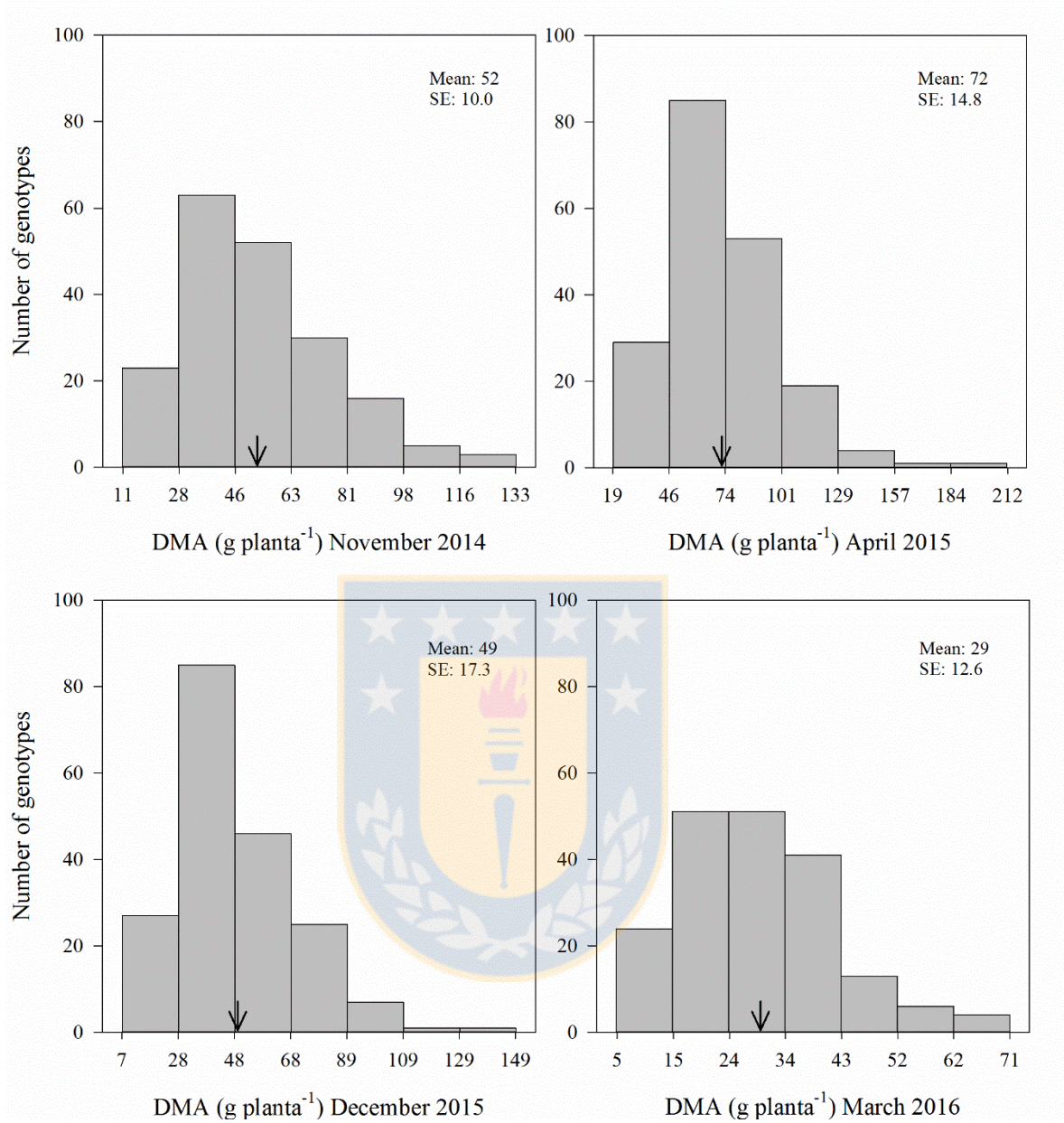


Figure 7. Genotype distribution according to dry matter accumulation (DMA), average of the three experimental sites in the first (2014-2015) and second (2015-2016) growing seasons.

Table 1. Geographic locations of the collection sites of 28 naturalized white clover populations and the two cultivars.

WCN	Georeference	Altitude (m.a.s.l.)	CO	WCN	Georeference	Altitude (m.a.s.l.)	CO	WCN	Georeference	Altitude (m.a.s.l.)	CO
94-14	S39 25 W72 11	ur	CL	94-38	S40 07 W71 39	1050	AR	94-62	S39 10 W71 15	990	AR
94-18	S40 00 W72 33	250	CL	94-41	S39 46 W71 37	820	AR	94-65	S39 33 W70 57	120	AR
94-19	S40 16 W72 39	220	CL	94-45	S39 54 W71 36	810	AR	95-20	S44 15 W71 50	ur	CL
94-22	S40 30 W72 14	ur	CL	94-46	S40 00 W71 30	890	AR	95-36	S45 34 W72 06	ur	CL
94-24	S40 23 W72 47	ur	CL	94-50	S39 37 W71 23	880	AR	95-71	S46 15 W71 52	ur	CL
94-28	S40 48 W71 46	820	AR	94-51	S39 37 W71 23	880	AR	95-77	S47 09 W72 21	ur	CL
94-30	S40 40 W71 53	960	AR	94-52	S39 35 W71 27	880	AR	96-33	S51 34 W72 31	ur	CL
94-33	S40 26 W71 36	970	AR	94-54	S39 33 W71 25	890	AR	96-64	S52 43 W71 03	0	CL
94-35	S39 56 W71 38	850	AR	94-55	S39 04 W71 12	1400	AR	Weka			AU
94-36	S39 57 W71 40	850	AR	94-58	S39 19 W71 03	1290	AR	Haifa			IL

CO: country of origin (CL, AR, AU and IL for Chile, Argentina, Australia and Israel, respectively). WCN: white clover naturalized population (Acuña et al., 2014); ur: unrecorded.

Table 2. Soil nutrient availability, basis of interchange, and microelements content in 0-15 cm soil layer from the three experimental sites: Santa Rosa (SR), Atacalco (AT) and Puente Marchant (PM).

Site	pH H ₂ O	% O.M	Nutrient availability mg kg ⁻¹				Bases interchangeable Cmol (+) kg ⁻¹					CEC	Sat. Al. %	Microelements mg kg ⁻¹				
			N	P	K	S	Ca	Mg	K	Na	Al			Zn	Fe	Cu	Mn	B
SR	6.0	9.5	30.0	5.7	78.6	12.5	3.5	0.8	0.2	0.1	0.0	4.6	0.6	0.4	55.8	1.1	4.2	0.4
AT	6.2	9.4	23.0	6.4	129.9	< 1,0	11.8	2.0	0.3	0.2	0.0	14.3	0.1	0.7	112.7	1.1	28.2	0.3
PM	6.6	5.5	9.0	4.0	89.7	< 1,0	6.7	1.3	0.2	0.1	0.0	8.4	0.3	0.7	45.4	1.2	1.3	0.5



Table 3. Analyses of variance, F values and significance. Stolon morphology and growth: stolon internode diameter (SID), stolon internode length (SIL), and stolon elongation rate (SER); Leaf morphology and growth: petiole length (PL), leaf area (LA), and specific leaf area (SLA); Physiology and herbage production: Normalized Difference Vegetation Index (NDVI), MERIS Terrestrial Chlorophyll Index (MTCI), and dry matter accumulation (DMA); Biochemical characters: Stolon water soluble carbohydrates (WSC). Sites: Santa Rosa (SR), Atacalco (AT), and Puente Marchant (PM).

MORPHOLOGY AND GROWTH								
Characters	Stolon				Characters	Leaf		
	07/04/2014 (Autumn)		12/12/2014 (Summer)			02/02/2014 (Summer)		21/04/2015 (Autumn)
SID					PL			
SR	2.17 ***		1.30 ns		SR	3.79 ***		1.35 *
AT	2.74 ***		2.01 ***		AT	1.96 ***		1.64 **
PM	1.75 ***		2.25 ***		PM	3.19 ***		2.76 ***
SIL					LA			
SR	1.75 ***		1.25 ns		SR	5.17 ***		1.58 **
AT	2.26 ***		1.54 **		AT	5.56 ***		1.71 ***
PM	2.14 ***		1.88 ***		PM	3.65 ***		2.75 ***
SER					SLA			
SR	1.91 ***		1.34 ns		SR	2.37 ***		1.50 **
AT	1.79 ***		1.44 *		AT	2.75 ***		0.94 ns
PM	1.57 **		1.96 ***		PM	1.78 ***		1.24 ns
PHYSIOLOGY AND DRY MATTER ACCUMULATION								
Characters	24/11/2014 (Spring)		21/04/2015 (Autumn)		03/12/2015 (Spring)		16/03/2016 (Summer)	
NDVI								
SR	1.65 **		1.29 ns		1.16 ns		1.31 *	
AT	2.28 ***		1.20 ns		1.3 *		1.14 ns	
PM	1.43 *		1.14 ns		1.28 ns		1.54 **	
MTCI								
SR	1.91 ***		1.28 ns		1.28 ns		1.38 *	
AT	2.15 ***		2.08 ***		1.63 **		1.01 ns	
PM	2.66 ***		1.78 ***		1.33 ns		1.99 ***	
DMA								
SR	2.11 ***		1.92 ***		1.11 ns		0.95 ns	
AT	2.61 ***		1.80 ***		1.58 **		0.74 ns	
PM	2.09 ***		2.45 ***		1.50 *		1.31 ns	
BIOCHEMICAL CHARACTERS								
Characters	10/06/2014 (Autumn)		06/08/2014 (Winter)		09/09/2014 (Winter)			
WSC								
SR	2.30 ***		2.04 ***		1.76 ***			
AT	2.61 ***		1.86 ***		3.45 ***			
PM			1.76 ***		1.83 ***			

*P<0.05; **P<0.01; ***P<0.001; ns: not significant.

CONCLUSIONES GENERALES

Se detectó una alta variabilidad genética en todos los rasgos fenotípicos evaluados entre los 192 genotipos de trébol blanco pertenecientes a una población para mapeo asociativo.

- El efecto de las bajas temperaturas sobre la morfología de los genotipos se observó a través de la disminución del largo y diámetro de los estolones, largo de pecíolos y materia seca acumulada, mientras la tasa de elongación de los estolones y el área foliar específica aumentaron.
- Los índices de vegetación NDVI y MTCI presentaron alta variabilidad genotípica principalmente luego del primer invierno.
- Las concentraciones de carbohidratos solubles en agua en estolones alcanzaron los valores más altos en otoño, disminuyendo de forma drástica luego del invierno a consecuencia del aumento de las temperaturas.
- Solo la evaluación de la concentración de carbohidratos solubles en agua en estolones presentó efecto del genotipo x ambiente.
- La alta variabilidad fenotípica de la población variabilidad fenotípica de la población permitiría utilizarla como una fuente de variabilidad genética en estudios de mapeo asociativos para trébol blanco con tolerancia a frío.

Adicionalmente, los datos obtenidos han permitido el desarrollo de investigaciones derivadas de estos, como la evaluación fenotípica de los genotipos mediante la utilización de imágenes multiespectrales y reflectancia de vegetación, con el objetivo de estimar la relación genotípica entre los resultados de la caracterización fenotípica de alto rendimiento y la producción de materia seca (Anexo1).

Por último, los datos obtenidos durante cada temporada fueron difundidos como resultados de la temporada mediante resúmenes extendidos en simposios y congresos internacionales (Anexo 2-6).

ANEXO 1

Inostroza, L., H. Acuña, P. Muñoz, C. Vásquez, J. Ibáñez, G. Tapia, M. T. Pino and H. Aguilera. 2016. Using aerial images and canopy spectral reflectance for high-throughput phenotyping of white clover. *Crop Sci.* 56(5):2629-2637. doi:10.2135/cropsci2016.03.0156

ABSTRACT

Plant breeders are demanding high-throughput phenotyping methodologies to complement the abundant genomic information currently available. Remote-sensing technologies offer new tools for high-throughput phenotyping in field conditions, and many remote sensors have shown high capacity for describing plant physiological behavior. The objective of this study was to evaluate the genotypic relationship between high-throughput phenotyping based on image analysis and canopy reflectance estimated traits and dry matter (DM) production, the most important trait in forage species. An experiment of a white clover (*Trifolium repens* L.) association-mapping population was established in three locations. Plant DM production was evaluated during two growing seasons. The plant area (PA), normalized difference vegetation index (NDVI), and plant growth were estimated from multispectral aerial images collected with an unmanned aerial vehicle. Additionally, canopy reflectance was evaluated with a spectroradiometer (350–1075 nm) and 10 spectral reflectance indices (SRIs) were calculated, including NDVI. The image-derived PA trait showed the highest genetic correlation with DM production ($r_g = 0.88$, < 0.001) with a broad-sense heritability (H^2) value of 0.56. All the SRIs showed highly significant genetic correlation with DM production with r_g absolute values between 0.54 and 0.72 (< 0.001). However, the popular NDVI index showed one of the lowest DM correlations using both systems. The results indicate that aerial-image-derived traits and SRIs could be used together as a high-throughput proxy to estimate genotypic variation of white clover DM production. Use of these variables could contribute to alleviating phenotypic bottleneck in discovering genes or predicting yield using genomic data.

ANEXO 2

Acuña, H., Inostroza, L., Muñoz, P. y Vásquez, C. 2014. Caracterización fenotípica de una población de trébol blanco de mapeo asociativo para tolerancia a frío: primera temporada de crecimiento. 37º Congreso AAPA–2nd Joint Meeting ASAS-AAPA–XXXIX Congreso SOCHIPA, 20-22 octubre 2014, Buenos Aires, Argentina. Revista Argentina de Producción Animal 34 Supl.1:551-576. Sección Praderas y Forrajes, p 564. <http://ppct.caicyt.gov.ar/index.php/rapa/article/view/5383/pdf>

ANEXO 3

Inostroza, L., Acuña, H., Muñoz, P., Vásquez, C., Ibañez, J. y Aguilera, H. 2014. Caracterización fenotípica de alto rendimiento de una población de trébol blanco mediante imágenes multiespectrales aéreas. 37º Congreso AAPA–2nd Joint Meeting ASAS-AAPA–XXXIX Congreso SOCHIPA, 20-22 octubre 2014, Buenos Aires, Argentina. Revista Argentina de Producción Animal 34 Supl.1:551-576. Sección Praderas y Forrajes, p 555.

<http://ppct.caicyt.gov.ar/index.php/rapa/article/view/5383/pdf>

ANEXO 4

Vásquez, C., Inostroza, L., Muñoz, P., y Acuña, H. 2015. Phenotypic characterization of white clover association-mapping population for cold tolerance: first winter and second growing season. Session 3: Overcoming Abiotic Stresses. Summary. 5th International Symposium of Forage Breeding – Buenos Aires, Argentina, October 19, 20 and 21.

PHENOTYPIC CHARACTERIZATION OF WHITE CLOVER ASSOCIATION-MAPPING POPULATION FOR COLD TOLERANCE: FIRST WINTER AND SECOND GROWING SEASON

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White clover (*Trifolium repens* L.) is the main forage legume species in grazing pastures of the central, central-south and southern zones of Chile. The selection of cold tolerant genotypes is an important step for obtaining cultivars with the ability to improve the clover growth curve and to extend the grazing season in early spring and autumn. The objective of this work was the phenotypic characterization of a 192 white clover genotypes association-mapping population for cold tolerance, after the first winter and during the following growing season, which was developed from 28 naturalized populations originated in the Argentinian-Chilean Patagonia. A field experiment was established in three localities with contrasting winter temperatures in the Biobío region, under an Alpha Lattice, with 24 incomplete blocks, and two replicates, experimental design. Before growth initiation, to evaluate the winter (2014) cold effects, the petiole length (mm), leaflet area (cm²) and specific leaf area (SLA, cm² g⁻¹) were measured in the colder locality. The water soluble carbohydrates (WSC) of stolon were determined three times during winter. The measurements carried out during the second growing season (2014-2015) were related with the stolon morphology and growth (length per unit area [cm 0,1 m²], elongation rate [mm day⁻¹], internode length and diameter [mm], dry weight [g 0.1 m²], and number of growing points [nº 0.1 m²]), and with dry matter production [g plant⁻¹]. Analyses of variance, combining the three localities, to determine genotype, environment and their interaction effects were carried out. Significant differences (P<0.001) for morphological and growth characters were found among genotypes, but they were not affected significantly (P>0.05) by the environment. The genotype x environment interaction was significant for internode diameter, stolon dry weight and length during winter, and stolon elongation rate during spring. The WSC concentration decreased during the winter cold period (from June to August). A high phenotypic variation for morphological characters related with cold tolerance were found within the studied population, particularly after winter cold period, which would allow the identification of cold tolerant genotypes. Acknowledgements: this work is part of the project N°1130340 funded by FONDECYT Chile.

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ANEXO 5

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ANEXO 6

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