NIR–Prediction of water–soluble carbohydrate in white clover and its genetic relationship with cold tolerance

Luis Inostroza[1*], Iris Lobos[2], Hernán Acuña[3], Catalina Vásquez[3], Gerardo Tapia[1], and Gerson Monzón[4]

ABSTRACT

In temperate climates, cold stress constrains productivity of white clover (*Trifolium repens* L.), the most important perennial forage legume in intensive grazing systems for ruminants. Metabolism of water sugar carbohydrate (WSC) has been proposed as an important trait conferring cold tolerance to white clover. Conventional methodologies for WSC determination are considered high-cost and time-consuming. Near-infrared (NIR) spectroscopy is a robust, reliable, and high-throughput methodology to estimate chemical composition of forage species. The objectives of this work were to determine the accuracy of NIR spectroscopy for predicting WSC in stolon samples of white clover, and to evaluate the genetic relationship between WSC and cold tolerance. A white clover association mapping (WCAM) population was stablished in three location that represent a winter low temperature gradient associated with altitude. Dry matter production and some morphological traits were evaluated during three growing seasons. Samples for WSC determination were collected three time during a winter period. Samples were scanned with a NIR system, and a prediction model for WSC was fitted using partial least squares (PLS) regression. The adjusted prediction model achieved suitable predictive ability ($R^2 > 0.85$). The WSC *per se* did not show significant genetic relationship with morphological and agronomically important traits. However, the WSC degradation rate (WSCdr) across the winter period showed significant genetic correlation with DM production during spring ($r_g = 0.64$), which is the result of genetic/physiological mechanism expressed during the cold period. The NIR spectroscopy is a reliable and high-throughput methodology to predict WSC in stolon samples of white clover. The metabolism of WSC, evaluated as WSCdr, is involved in the cold tolerance of the WCAM population. The methodology implemented in this work is suitable to be applied in a plant breeding program routine.

Key words: Broad sense heritability, genetic correlation, high-throughput phenotyping, PLS regression *Trifolium repens*.

[1]Instituto de Investigaciones Agropecuarias, INIA Quilamapu, Avda. Vicente Méndez 515, Chillán, Chile.
[*]Corresponding author (linostroza@inia.cl).
[2]Instituto de Investigaciones Agropecuarias, INIA Remehue, Ruta 5 Sur, km 8, Osorno, Chile.
[3]Universidad de Concepción, Facultad de Agronomía, Av. Vicente Méndez 595, Chillán, Chile.
[4]Universidad de Tarapacá, Facultad de Ciencias Agronómicas, Campus Azapa, km 12 Valle de Azapa, Casilla 6-D Arica, Chile.

INTRODUCTION

The intensive grazing systems for ruminants in temperate climates are mainly based on the perennial grasses/white clover mixed sward (Acuña and Inostroza, 2013; Barrett et al., 2015). The economic and environmental sustainability of mixed sward depend on the annual white clover (*Trifolium repens* L.) contribution. Under proper agronomical management practices, there can be large annual fluctuations in white clover yield; these are commonly attributed to the inferior cold tolerance and growth potential at low temperature (5-10 °C) of white clover compared with companion grasses (Wachendorf et al., 2001; Collins et al., 2002).

Cold tolerance has been the most studied abiotic stress in white clover (Annicchiarico et al., 2015; Barrett et al., 2015). Several physiological and morphological traits conferring cold tolerance have been described (Dalmannsdóttir et al., 2001; Frankow-Lindberg, 2001; Wachendorf et al., 2001; Collins et al., 2002; Goulas et al., 2003). Cold tolerance related traits can be cluster in two categories: those conferring plant survival during winter and those conferring early vigor at the beginning of the growing season (spring). Early vigor traits are related with photosynthetic and growth capacity under sub-optimal temperature. Whereas, plant survival traits are mainly related with the synthesis and catabolism of compatible osmolytes during winter (Annicchiarico et al., 2015). The most common compatible osmolytes found in white clover comprise soluble sugar and protein and amino acids including proline (Frankow-Lindberg, 2001; Goulas et al., 2003; Annicchiarico et al., 2015).

In white clover a physiological relationship between stolon-WSC and cold tolerance has been described (Dalmannsdóttir et al., 2001). At the beginning of the cold season, white clover induces an acclimation process, which includes starch accumulation in stolons and roots. During the cold period, starch provides energy for basic metabolic activity, and its hydrolysis releases WSC. A higher concentration of WSC in stolon increases osmotic potential and lower the freezing point of the cell (Dalmannsdóttir et al., 2001; Castonguay et al., 2006). Studies in controlled conditions have demonstrated that a higher stolon WSC increase plant survival under cold condition (Dalmannsdóttir et al., 2001; Frankow-Lindberg, 2001). Based in this relationship, stolon WSC has been proposed as a promising selection criterion for breeding programs that aims to develop cold tolerant cultivars. However, the performance of this criterion has not been robust enough in practice. For instance, Annicchiarico et al. (2001) reported nonsignificant variation in the stolon contents of

either water-soluble or total non-structural carbohydrates in a sample of 11 white clover populations that differed widely in their levels of freezing tolerance. Similar results were found by Collins et al. (2002) in a set of white clover populations collected across Europe. Several factors could affect the performance of WSC as selection criteria (Collins et al., 2002). One of the most important is the moment when the measurement is made. In both works cited before (Annicchiarico et al., 2001; Collins et al., 2002), the stolon WSC was measured in a unique and arbitrary moment during a cold period. In alfalfa, a strong relationship between root/crown-WSC and cold tolerance has been reported (Castonguay et al., 2006), which was found after periodical measurement of WSC across a winter period.

From a plant breeding point of view, conventional determination of WSC is considered high time-consuming and high-cost (Deaville and Flinn, 2000). Reason why, most studies have evaluated a few number of genotypes/ populations and just once time across an experimental period. A high throughput methodology to determiner WSC is required to a successful implementation of this trait as selection criterion in a plant breeding program. Near-infrared spectroscopy is considered a robust, reliable, and high-throughput methodology to estimate chemical composition of forage species (Alomar et al., 2009; Nie et al., 2009; Krahmer et al., 2013; Piaskowski et al., 2016).

The NIR spectroscopy has been successfully used to estimate forage-quality traits in diverse species including white clover (Berardo, 1997; Lister and Dhanoa, 1998; Alomar et al., 2009; Krahmer et al., 2013). There are several works where NIR-spectroscopy has already been used to predict WSC (Nie et al., 2009; Widdup et al., 2010; Lobos et al., 2013; Piaskowski et al., 2016).

A white clover association mapping (WCAM) population was developed to study the cold tolerance of white clover in temperate environments (Acuña et al., 2014). The population includes genotypes collected in cold and marginal areas of the Patagonia Region of South America (from 39 to 52° S lat). This population represents a valuable genetic resource to identify physiological traits and genomic regions controlling cold tolerance in white clover, the most important abiotic stress that constrains productivity of temperate mixed swards. The objectives of this work were to determine the accuracy of NIR spectroscopy for predicting WSC in stolon samples of white clover and to evaluate the genetic relationship between WSC and cold tolerance in the WCAM population.

MATERIALS AND METHODS

Plant material
The white clover association mapping (WCAM) population included 192 cold-tolerance divergent genotypes (96 sensitive and 96 tolerant). The 192 individuals were selected from six populations (three cold-sensitive and three cold-tolerant) naturalized in the Argentinean and Chilean Patagonia region (Acuña et al., 2014). From each foundational population, 32 individuals were selected. The WCAM population includes small- and medium-leaved white clover types with prostrate and erect growing patterns. For phenotyping, plants were clonally propagated under greenhouse conditions by rooting stolon sections (Inostroza et al., 2016).

Plant establishment and agricultural management
The WCAM population was established in three locations that represent a winter low temperature gradient associated with altitude. The locations were Santa Rosa (36°32' S, 71°55' W; SR_{140}), Atacalco (36°53' S, 71°37' W; AT_{650}), and Puente Marchant (36°54' S, 71°32' W; PM_{1050}), located at 140, 650 and 1050 m a.s.l., respectively (Table 1). The soil was ploughed and rolled, and glyphosate (3 L ai ha^{-1}) was applied 20 d before planting. Fertilizer was applied at planting in an area of 0.01 m^2 (10 × 10 cm) for each plant at a rate of 400 kg ha^{-1} triple superphosphate (46% P_2O_5 and 21.7% CaO), 200 kg ha^{-1} potassium muriate (62% K_2O), and 100 kg ha^{-1} urea (46% N). Experiments were established in spring 2013 (October-November) (Table 1) using a plant spacing of 1 × 1 m. The genotypes were arranged in an alpha lattice experimental design with 24 incomplete blocks (IB), each with eight genotypes, and with two resolvable replicates.

In all locations, plants were irrigated through a pressurized irrigation system with 2 L h^{-1} drip emitters. During the growing season (October-April), plants were irrigated three times per day for 1 h each time. Periodically, broadleaf weeds were controlled manually and grasses with

Table 1. Planting date, geographic, edaphic, and climatic variables at three experimental locations. The climatic descriptors correspond to the second growing season (2014-2015). Values between brackets correspond to the minimum and maximum absolute temperature.

Location	Planting date	Altitude	Georeference	Soil texture	Annual rain	Growing season	Air temperature	Soil temperature
		m a.s.l.			mm		°C	
Santa Rosa (SR_{140})	28 Oct 2013	140	36°32'11.84" S 71°55'10.13" W	Clay-loam	1030	2014-2015	13.2 (-3.6-34.7)	13.3 (5.5-20.9)
Atacalco (AT_{650})	8 Nov 2013	650	36°53'35.25" S 71°37'55.68" W	Clay-loam	2300	2014-2015	12.2 (-4.4-34.5)	14.8 (0.9-34.4)
Puente Marchant (PM_{1050})	12 Nov 2013	1050	36°54'24.76" S 71°32'20.37" W	Sandy-clay-loam	1760	2014-2015	10.0 (-7.4-29.2)	10.5 (-0.8-24.4)

clethodim 1 L ha^{-1}. The air temperature, relative humidity, global radiation, wind speed, and soil temperature (5 cm depth) were recorded at 1-h intervals with an automatic meteorological station (WatchDog 2900ET, Spectrum Technologies, Aurora, Illinois, USA) installed in each experimental site (Table 1).

Water-soluble carbohydrate (WSC) determination
In all locations, stolon WSC were measured three times during winter 2014 using the anthrone reactive method (Yemm and Willis, 1954). In PM$_{1050}$, WSC was determined only two times, because when the first sampling was planned, plants were covered with 50 cm of snow. The dates of sampling were scheduled to cover the entire winter period. Samples were taken at the beginning (10 June), middle (6 August), and the end (9 September) 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 by 1 h and then 40 °C by 16 h (Frankow-Lindberg, 2001). In total 3072 samples were collected (192 genotypes × 2 replicates × 3 locations × 3 or 2 times). A sub-sample of 360 stolons were selected (10% of total samples) for conventional WSC determination. The sub-sample included 10 cold-tolerant and 10 cold-sensitive genotypes. These individuals were selected based on their agronomic performance during the first growing season. For cold-tolerant and cold-sensitive genotypes the higher and lower yielding genotypes into each group were selected, respectively.

The 360 stolon samples were ground in a mortar. The WSC were extracted from 10 mg sample with 3 mL extraction buffer containing 80% ethanol, 10 mM Hepes-KOH (pH = 7.5), and incubated overnight at 60 °C. Then, samples were centrifuged at 60 rpm for 30 min. The anthrone reagent was added to each supernatant and placed over a hotplate at 80 °C for 20 min. Finally, the absorbance of the sample was measured at 620 nm in an EPOCH microplate UV-Vis Spectrophotometer (BioTek, Winooski, Vermont, USA) using COSTAR 3596 96 well-plates (Corning Incorporated, Corning, New York, USA). The stolon-WSC was expressed as milligram WSC per unit of stolon dry weight (mg g^{-1}). Then the WSC degradation rate (WSCdr) was calculated as the slope of the relationship between WSC content and the time of every measurement.

NIR Spectroscopy and chemometric analyses
Dried and grounded stolon samples (3072 samples, 0.1 g sample^{-1}) were scanned over a spectral wavelength range of 12000-4000 cm^{-1} using an MPA-FT NIR analyzer (Bruker Optik GmbH, Ettlingen, Germany). Each spectral measurement was obtained from 32 scans performed at a wavenumber resolution of 16 cm^{-1} (Figure 1).

Partial least-squares regression (PLSR) with leave-one-out (LOO) cross validation was performed to fit predictive models using chemometrical software OPUS version 6.0 (Bruker Optik GmbH, Ettlingen, Germany). Predictive models were fitted using the 360 stolon samples chemically
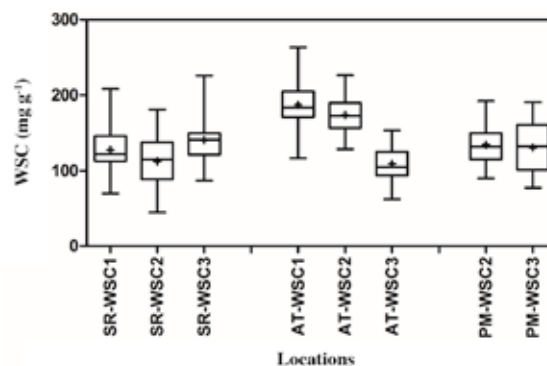

Figure 1. Frequency distribution (box plot) for stolon water-soluble carbohydrates (WSC) measured at the beginning (WSC1), middle (WSC2) and the end (WSC2) of a winter season in the white clover association mapping population. The WSC were measured in three locations: Santa Rosa (SR), Atacalco (AT) and Puente Marchant (PM).

analyzed for WSC. Spectral signatures were subjected to five pre-processing transformations using the optimization tool in OPUS 6.0. Several PLS regression models were fitted based on the pre-processing transformations. Three criteria were used to select the best model: i) low root mean square error of cross validation (RMSECV), ii) high coefficients of determination in cross-validation, and iii) a ratio of prediction to deviation (RPD) value higher than 2.4 (Nie et al., 2009).

Dry matter production and stolon growth pattern
Dry matter (DM) production was evaluated during three growing season by harvesting the aboveground biomass at a height of 2 cm with an electric shearing machine (ShowMaster, Oster, McMinnville, Tennessee, USA). During the first growing season, DM production was evaluated in only one cut at 3-mo after planting (summer DM accumulation). During the second growing season DM production was evaluated in three cuts in SR$_{140}$ and AT$_{650}$ (7 October 2014, 24 November 2014, and 2 February 2015) and two cuts in PM$_{1050}$ (25 November 2014 and 3 February 2015). During the third growing season DM production was evaluated in two cuts in the three locations (7 December 2015 and 15 March 2015). For all DM determinations, the fresh samples were dried in a forced air oven at 65 °C until constant weight.

Stolon growth and morphology were measured across a growing season. Two stolon per plant were randomly selected and marked with a colored wire. Marks were put in the internode section between the second and third plenty expanded leaves. Periodically stolon length (StL, distance between mark and growing point), stolon diameter (StD, second internode), and internode length (StInodL, distance between second and first plenty expanded leaves) were recorded with a digital caliper. Measurements were taken on summer (9 and 23 January, 7 and 21 February 2014), fall (5 and 25 April 2014) and spring (12 November 2014, 1 December 2014, and 10 January 2015). Stolon elongation rate (StER, cm d^{-1}) was calculated for every period as the slope of the linear regression between StL and time.

Data analyses

A phenotypic linear mixed model was implemented to estimate the variance components using the Restricted Maximum Likelihood (REML) method within the ASReml-R package (Gilmour et al., 2009) in R software (https://www.r-project.org/) using the following equation:

$$Y_{ijlm} = \mu + l_j + + r_i + IB_m + g_i + g \times l_{ij} + \varepsilon_{ijlm}$$

where Y_{ijlm} is the phenotypic value of ith genotype (g) in the jth location (l), l[th] replicate (r), and m[th] incomplete block (IB), μ is the overall population mean, l is the fixed effect of location, IB is the random incomplete block effect $\sim IDD(0, \sigma_i^2)$, g is the random effect of the genotype $\sim IDD(0, \sigma_g^2)$, $g \times l$ is the random interaction effect of location by genotype $\sim IDD(0, \sigma_{g\times l}^2)$, and ε the random experimental error $\sim IDD(0, \sigma_\varepsilon^2)$.

The variance components were used to estimate the broad sense heritability (H[2]) on a clone mean basis (Nyquist and Baker, 1991), which was calculated as follows:

$$H^2 = \frac{\sigma_g^2}{\sigma_g^2 + \dfrac{\sigma_{g\times l}^2}{l} + \dfrac{\sigma_\varepsilon^2}{lr}}$$

Bivariate analyses, extending the model above, were performed to estimate the genetic correlation (r_g) between stolon-WSC and some cold tolerance related traits using ASReml-R. The genotypic random effect was modeled as an unstructured matrix $G = \begin{bmatrix} \sigma_{g1}^2 & COV_{g1g2} \\ COV_{g1g2} & \sigma_{g2}^2 \end{bmatrix}$ to obtain the the covariance between the pair of traits (1 and 2), the error random effect was also modeled as unstructured matrix, $R = \begin{bmatrix} \sigma_{\varepsilon1}^2 & COV_{\varepsilon1\varepsilon2} \\ COV_{\varepsilon1\varepsilon2} & \sigma_{\varepsilon2}^2 \end{bmatrix}$ while all other components were modeled as a diagonal matrix. The genetic correlation (r_g) was then calculated as $r_g = \dfrac{COV_{g1g2}}{\sqrt{\sigma_{g1}^2 \sigma_{g2}^2}}$.

RESULTS

WSC-NIR prediction model

Chemically-determined stolon WSC samples showed broad range of variability (Figure 1), which was suitable for fitting the prediction model. In overall, WSC varied between 40.1 and 282.5 mg g^{-1}; broad range of variation was also observed within every location and date of sampling (Figure 1). Stolon WSC was significantly affected by location and date of sampling (P < 0.05). At the begging of the winter period the highest stolon WSC was observed in all locations. A reduction in the stolon WSC was observed across the winter period only in AT_{650}.

The best prediction model was obtained with Subtraction of Straight-Line pre-processing method. Spectral regions including second and first overtone (7506-6094 cm^{-1}) and combination vibrations (5454-4242 cm^{-1}) allowed to fit the best prediction model (Figures 2 and 3; Table 2). The selected model showed a high calibration coefficient of determination (R[2]$_c$ = 0.85). Furthermore, it accounted for a low RMSECV value (15.3), high coefficients of determination in the cross-validation procedure (R[2]$_{CV}$ = 0.83) and a RPD value of 2.5 (Table 2).
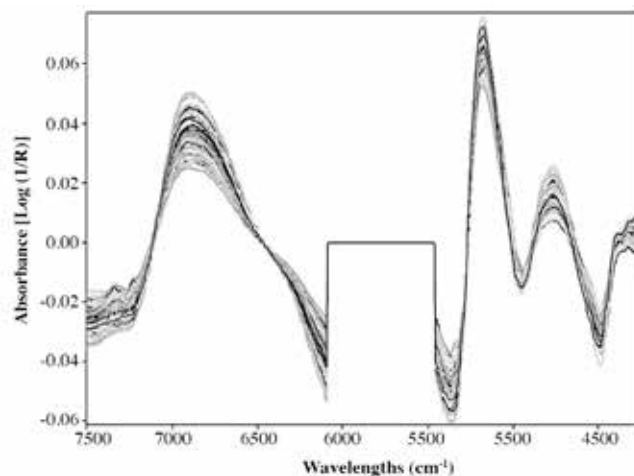

Figure 2. Effect of straight-line subtraction spectral preprocessing on original spectra of white clover stolon samples.
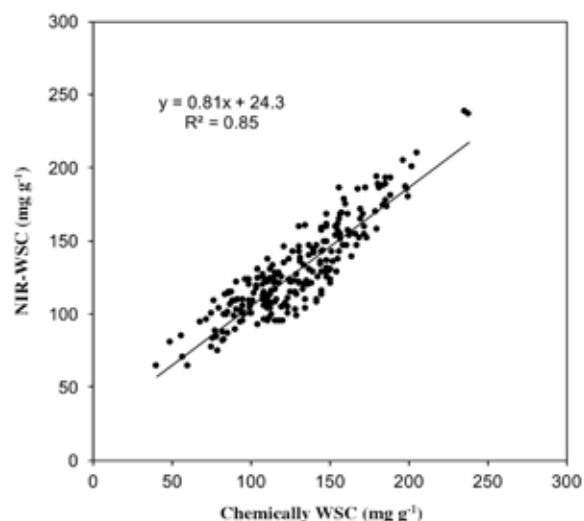

Figure 3. Relationship between water-soluble carbohydrates (WSC) estimated chemically and by NIR-spectroscopy.

Table 2. Calibration and validation statistics of partial least squares (PLS) models for determination of water-soluble carbohydrate (WSC) in white clover stolons.

Trait	Set calibration			Set cross-validation		
	R^2_c	RMSEC	RPD	R^2_{CV}	RMSEP	RPD
Stolon WSC	0.85	15.3	2.6	0.83	14.8	2.5

R^2_c: Coefficient of determination in calibration; RMSEC: root mean square error of calibration; RPD: residual prediction deviation; R^2_{CV}: coefficient of determination in cross-validation; RMSEP: root mean square error of prediction.

Genetic relationship between WSC and cold-tolerance related traits

Spring DM production was significantly affected by the cold condition. It was reduced in 34% and 39% in AT_{650} and PM_{1050} relative to the warmer environment (SR_{140}; Table 3). Spring DM production showed broad genotypic variability within each location. Furthermore, a medium/high proportion of the total variance was accounted by the σ^2_g component ($H^2 = 0.60$; Table 3).

NIR-predicted stolon water-soluble carbohydrate (NIR-WSC) showed same pattern observed in chemically evaluated white clover stolon samples. The higher stolon NIR-WSC was observed at the beginning of the winter period in all locations. Then, high WSC degradation rate was only observed in AT_{650} (Table 3). Across the winter period stolon NIR-WSC showed significant genotypic (G) and G×E interaction effects. The σ^2_g and $\sigma^2_{g\times l}$ components showed similar contribution to total variance with a H^2 value of 0.38 in average (Table 3). The WSCdr also showed G and G×E effects, however, the contribution to the total variance of $\sigma^2_{g\times l}$ component was almost tree fold higher than σ^2_g component. The WSCdr reached a low value of H^2 (0.13; Table 3).

Stolon traits were measured three times during growing season (summer, fall and spring). All stolon traits changed across the growing season, but StD was the most stable (Figure 4b). Stolon internode length and StER were drastically reduced in fall and spring relative to summer in all locations (Figure 4a and 4c). In summer and fall, the higher StInodL was observed in SR_{140}. In spring, no significant differences (P < 0.05) were observed in StInodL between locations (Figure 4). During the colder periods of the growing season (fall and spring), the higher StER was observed in SR_{140}, except for spring-StER in PM_{1050} (Figure 4). Stolon traits broad sense heritability varied across the growing season. In general, the lowest H^2 values were observed in spring. In average, H^2 values
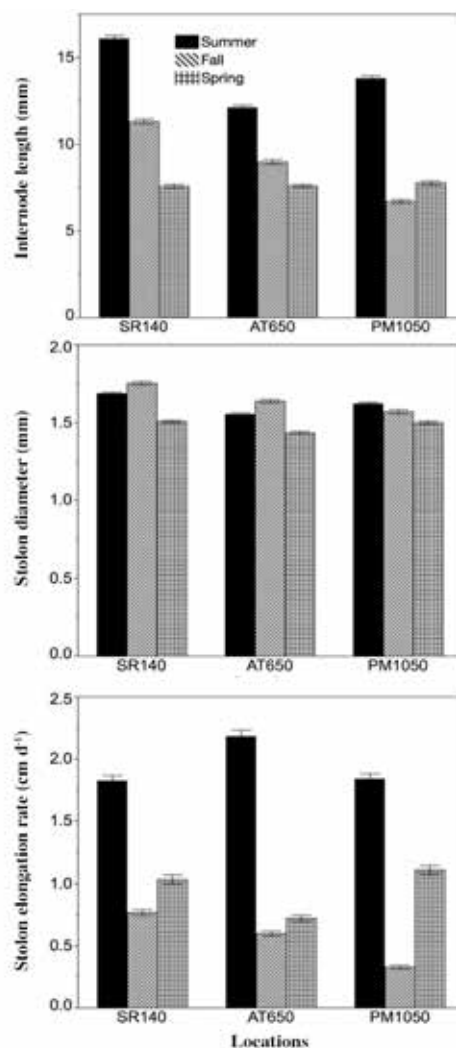

Figure 4. Internode length, stolon diameter and stolon elongation rate evaluated in the white clover association mapping population in three locations (SR140, AT650 and PM1050) and three dates across a growing season (summer, fall and spring). Bars indicate standard errors of the mean.

Table 3. Range, mean, genotypic variation (σ^2_g), genotype by location interaction ($\sigma^2_{g\times l}$), and pooled error (σ^2_ε) variance components and their associated standard errors (± SE), clone mean broad sense heritability (H^2) and genotypic correlation with spring dry matter production (r_g SpringDM) estimated for the stolon water-soluble carbohydrate (WSC), and WSC degradation rate (WSCdr). Traits evaluated in the white clover association mapping population grown in three locations.

Traits	Locations	Mean ± SE	Range	σ^2_g	$\sigma^2_{g\times l}$	σ^2_ε	H^2	r_g Spring DM
WSC1, mg g^{-1}	SR_{140}	127.7 ± 1.5	83.0-193.6	204.2 ± 61.8	243.3 ± 67.4	707.7 ± 52.8	0.37 ± 0.08	0.15 ± 0.28
	AT_{650}	153.1 ± 1.6	102.9-212.0					
	PM_{1050}							
WSC2, mg g^{-1}	SR_{140}	124.3 ± 1.4	79.4-180.5	257.4 ± 71.5	183.9 ± 78.9	965.6 ± 73.2	0.40 ± 0.08	0.18 ± 0.14
	AT_{650}	141.0 ± 2.1	77.5-233.4					
	PM_{1050}	120.1 ± 2.0	64.1-173.1					
WSC3, mg g^{-1}	SR_{140}	128.8 ± 1.8	75.1-186.5	216.6 ± 62.9	134.7 ± 70.7	911.5 ± 68.8	0.37 ± 0.08	0.30 ± 0.13
	AT_{650}	109.9 ± 1.5	67.0-173.2					
	PM_{1050}	130.0 ± 1.5	73.6-235.9					
WSCdr, mg d^{-1}	SR_{140}	-0.20 ± 0.04	-2.7-1.2	60.8 ± 56.3	204.8 ± 80.1	964.0 ± 72.5	0.13 ± 0.08	0.64 ± 0.10
	AT_{650}	-1.15 ± 0.03	-4.2-0.9					
	PM_{1050}	-0.26 ± 0.06	-4.0-0.8					
Spring DM, g plant^{-1}	SR_{140}	61.9 ± 3.5	2.0-260.6	754.8 ± 108.4	107.3 ± 72.0	1340.9 ± 83.6	0.60 ± 0.05	
	AT_{650}	46.1 ± 2.4	2.0-168.0					
	PM_{1050}	42.2 ± 2.0	1.4-258.7					

WSC1, WSC2 and WSC2, stolon water-soluble carbohydrates evaluated at the beginning, middle and the end of a winter season, respectively.

of 0.46, 0.70 and 0.50 were observed for StER, StD and StInodL, respectively (Table 4).

None genetic relationship was found between stolon NIR-WSC and Spring DM production. However, significant genetic correlation between WSCdr and spring DM was found (r_g = 0.64, Table 3). All stolon traits showed significant genetic relationship with Spring DM (Table 4), with rg values ranging from 0.44 (StD) and 0.80 (StER). The genetic relationship between WSCdr and stolon traits also varied across the growing season; higher values of rg was found with StER (0.70) and InodL (0.60) during spring (Table 4).

DISCUSSION

The stolon-WSC prediction model fitted with our dataset reached suitable statistical parameters in term of its predictive ability (R^2 = 0.85 and RPD = 2.5, Table 1, Figure 3). This work represents the first time where a prediction model is fitted using stolon samples in white clover. White clover is one of the most important perennial forage legumes worldwide (Annicchiarico et al., 2015; Barrett et al., 2015). Thus, several NIR spectroscopy studies has been performed to predict forage quality (Berardo, 1997; Lister and Dhanoa, 1998; Alomar et al., 2009; Krahmer et al., 2013). However, all these works have used samples of aerial biomass (leaves + petiole). Berardo (1997) predicted the chemical composition of white clover, including crude protein, crude fiber, crude lipid, among others, with levels of accuracy like obtained in the present work. Lister and Dhanoa (1998), predicted leaves-WSC in white clover. They obtained predictions models with R^2 values ranging between 0.85-0.93, which is into the range of our results. In this sense, the model obtained represents a reliable and high-throughput tool to estimate stolon-WSC in white clover.

Chemically determined WSC showed a broad range of variation, which was the expression of the genotypic, location and temporal effects during the winter season (Figure 1). The variance of this dataset showed a suitable amplitude and homogeneity, which are desirable conditions to obtain a good calibration (Nie et al., 2009). The WSC values obtained in this study were into the range of

values reported for white clover (Dalmannsdóttir et al., 2001; Frankow-Lindberg, 2001; Collins et al., 2002). An interesting result to highlight was that the chemically estimated WSC and NIR-estimated WSC showed the same pattern across location and date of sampling (Table 3 and Figure 1); higher WSCdr was observed only in AT_{650}. Independent of the error associated to the predictions, the fitted model allows to observe how plants respond to the environment. In this sense, whether this trait is really conferring cold tolerance to white clover, the prediction model described in this work would be highly effective as selection criteria into a white clover breeding program.

Independent of the well-known theoretical/physiological relationship between WSC and cold tolerance (Dalmannsdóttir et al., 2001; Frankow-Lindberg, 2001; Collins et al., 2002), most empirical studies did not find phenotypic relationship between these two traits. For instance, Collins et al. (2002) evaluated the phenotypic relationship between stolon-WSC and some cold-tolerance related traits. Their results suggested that stolon-WSC would not play an important role on white clover cold tolerance. They found low phenotypic relationship between WSC and cold tolerance related traits (r values between -0.28 and -0.22). Annicchiarico et al. (2001) reported nonsignificant differences in stolon WSC evaluated in 11 white clover populations that differed widely in their levels of freezing tolerance. Our results confirm those found in previous works (Annicchiarico et al., 2001; Collins et al., 2002). The genetic relationship between WSC and cold tolerance (evaluated as Spring DM) was nonsignificant (Table 3). Additionally, the WSC evaluated three times across the winter season showed low values of broad sense heritability and significant G×E interaction. These two properties of WSC would limit its performance as selection criterion into a breeding program (Inostroza et al., 2015).

In our study, SprDM was considered a key trait describing cold tolerance in the WCAM population. Mainly because SprDM is the result of the expression of morpho-physiological traits/mechanisms belonging to both components governing cold tolerance (winter survival and early-vigor) in temperate climate. The importance of stolon-WSC on white clover cold tolerance changed when the WSCdr was considered; a genetic correlation (rg) of 0.64

Table 4. Genotypic (σ^2_g), genotype by location interaction ($\sigma^2_{g\times l}$), and pooled error (σ^2_ε) variance components and their associated standard errors (± SE), clone mean broad sense heritability (H^2) and genotypic correlation with spring dry matter production (r_g SpringDM) and water-soluble carbohydrate degradation rate (r_g WSCdr) estimated for some stolon traits evaluated in the white clover association mapping population. Stolon traits were evaluated three times across a growing season: Summer (Sm), fall (Fl) and spring (Sp).

Traits	σ^2_g	$\sigma^2_{g\times l}$	σ^2_ε	H^2	r_g SpringDM	r_g WSCdr
Stolon elongation rate, cm d^{-1} (Sm)	235.2 ± 38.9	57.8 ± 34.0	644.1 ± 39.1	0.54 ± 0.05	0.64 ± 0.07	0.43 ± 0.33
Stolon elongation rate, cm d^{-1} (Fl)	45.4 ± 8.2	13.5 ± 7.8	144.8 ± 8.9	0.50 ± 0.05	0.80 ± 0.06	0.37 ± 0.29
Stolon elongation rate, cm d^{-1} (Sp)	65.7 ± 16.7	37.4 ± 21.5	399.8 ± 24.4	0.35 ± 0.06	0.62 ± 0.10	0.70 ± 0.45
Stolon diameter, mm (Sm)	36.0 ± 4.1	3.1 ± 1.0	18.6 ± 1.1	0.83 ± 0.02	0.49 ± 0.07	0.31 ± 0.20
Stolon diameter, mm (Fl)	26.7 ± 3.7	1.9 ± 2.4	48.3 ± 2.9	0.65 ± 0.04	0.53 ± 0.07	0.36 ± 0.26
Stolon diameter, mm (Sp)	16.2 ± 2.3	0.01 ± 0.001	34.5 ± 1.6	0.63 ± 0.03	0.44 ± 0.08	0.26 ± 0.26
Internode length, mm (Sm)	49.7 ± 7.2	8.8 ± 5.0	94.7 ± 5.7	0.63 ± 0.04	0.76 ± 0.05	0.31 ± 0.28
Internode length, mm (Fl)	26.2 ± 4.1	3.9 ± 3.2	61.9 ± 3.8	0.58 ± 0.04	0.77 ± 0.05	0.10 ± 0.21
Internode length, mm (Sp)	5.1 ± 1.4	4.2 ± 1.9	36.7 ± 2.2	0.30 ± 0.06	0.66 ± 0.10	0.60 ± 0.40

was found between WSCdr and SprDM (Table 3). However, the WSCdr also showed a low H^2 value and significant G×E interaction (Table 3). For northern climates, a low rate of utilization/degradation of WSC has been suggested as a cold tolerance mechanism (Frankow-Lindberg, 2001; Wachendorf et al., 2001). Because in that extreme cold condition breeders favor plant survival. However, a lower WSCdr should be associated to dormant or completely inactive plant during winter, which affects early vigor in spring (Annicchiarico et al., 2001; Helgadottir et al., 2008). Our results showed that a higher WSCdr helps plant survive and increased re-growth vigor in spring.

CONCLUSION

Near-infrared (NIR) spectroscopy allowed to predict water sugar carbohydrate (WSC) in stolon samples of white clover with reliable level of accuracy. The prediction model fitted represents a high-throughput tool to estimate stolon-WSC into a plant breeding routine. In this work, WSC was evaluated three times across a winter period. All these measurements did not show significant genetic relationship with cold tolerance related traits in white clover. However, when the three measurements were bulked into an index (WSC degradation rate, WSCdr), a significant relationship was observed. In this sense, our results allow to conclude that metabolism of WSC during the cold season, and not WSC *per se*, is conferring white clover cold tolerance to white clover.

ACKNOWLEDGEMENTS

REFERENCES

Acuña, H., and Inostroza, L. 2013. Phosphorus efficiency of naturalized Chilean white clover in a grazed field trial. Grass and Forage Science 68:125-137. doi:10.1111/j.1365-2494.2012.00879.x.

Acuña, H., Inostroza, L., and Pino, M.T. 2014. Selection of contrasting cold-tolerant white clover genotypes from twentyeight populations naturalized in southern Chile and Argentina. Grassland Science in Europe 19:858-860.

Alomar, D., Fuchslocher, R., Cuevas, J., Mardones, R., and Cuevas, E. 2009. Prediction of the composition of fresh pastures by near infrared reflectance or interactance-reflectance spectroscopy. Chilean Journal of Agricultural Research 69:198-206.

Annicchiarico, P., Barrett, B., Brummer, E.C., Julier, B., and Marshall, A.H. 2015. Achievements and challenges in improving temperate perennial forage legumes. Critical Reviews in Plant Sciences 34:327-380. doi:10.1080/07352689.2014.898462.

Annicchiarico, P., Collins, R.P., Fornasier, F., and Rhodes, I. 2001. Variation in cold tolerance and spring growth among Italian white clover populations. Euphytica 122:407-416. doi:10.1023/a:1012918100232.

Barrett, B.A., Faville, M.J., Nichols, S.N., Simpson, W.R., Bryan, G.T., and Conner, A.J. 2015. Breaking through the feed barrier: options for improving forage genetics. Animal Production Science 55:883-892. doi:http://dx.doi.org/10.1071/AN14833.

Berardo, N. 1997. Prediction of the chemical composition of white clover by near-infrared reflectance spectroscopy. Grass and Forage Science 52:27-32. doi:10.1046/j.1365-2494.1997.00050.x.

Castonguay, Y., Laberge, S., Brummer, E.C., and Volenec, J.J. 2006. Alfalfa winter hardiness: A research retrospective and integrated perspective. Advances in Agronomy 90:203-265.

Collins, R., Helgadóttir, Á., Fothergill, M., and Rhodes, I. 2002. Variation amongst survivor populations of white clover collected from sites across Europe: Growth attributes and physiological responses to low temperature. Annals of Botany 89:283-292. doi:10.1093/aob/mcf037.

Dalmannsdóttir, S., Helgadóttir, Á., and Gudleifsson, B.E. 2001. Fatty acid and sugar content in white clover in relation to frost tolerance and ice-encasement tolerance. Annals of Botany 88:753-759.

Deaville, E.R., and Flinn, P.C. 2000. Near-infrared (NIR) spectroscopy: An alternative approach for the estimation of forage quality and voluntary intake. p. 301-320. In Givens, D., Owen, E., Axford, R., and Omed, H. (eds.) Forage evaluation in ruminant nutrition. CABI Publishing, Wallingford, UK.

Frankow-Lindberg, B.E. 2001. Adaptation to winter stress in nine white clover populations: Changes in non-structural carbohydrates during exposure to simulated winter conditions and 'spring' regrowth potential. Annals of Botany 88:745-751.

Gilmour, A.R., Gogel, B.J., Cullis, B.R., and Thomson, R. 2009. ASReml-R Reference manual release 3.0. VSN International Ltd., Hemel Hempstead, UK.

Goulas, E., Le Dily, F., Ozouf, J., and Ourry, A. 2003. Effects of a cold treatment of the root system on white clover (*Trifolium repens* L.) morphogenesis and nitrogen reserve accumulation. Journal of Plant Physiology 160:893-902. doi:10.1078/0176-1617-00937.

Helgadottir, A., Marum, P., Dalmannsdottir, S., Daugstad, K., Kristjansdottir, T.A., and Lunnan, T. 2008. Combining winter hardiness and forage yield in white clover (*Trifolium repens*) Cultivated in northern environments. Annals of Botany 102:825-834. doi:10.1093/aob/mcn159.

Inostroza, L., Acuña, H., and Méndez, J. 2015. Multi-physiological-trait selection indices to identify *Lotus tenuis* genotypes with high dry matter production under drought conditions. Crop and Pasture Science 66:90-99. doi:http://dx.doi.org/10.1071/CP14193.

Inostroza, L., Acuña, H., Muñoz, P., Vasquez, C., Ibañez, J., Tapia, G., et al. 2016. Using aerial images and canopy spectral reflectance for high-throughput phenotyping of white clover. Crop Science 56:2629-2637. doi:10.2135/cropsci2016.03.0156.

Krahmer, A., Gudi, G., Weiher, N., Gierus, M., Schutze, W., and Schulz, H. 2013. Characterization and quantification of secondary metabolite profiles in leaves of red and white clover species by NIR and ATR-IR spectroscopy. Vibrational Spectroscopy 68:96-103. doi:10.1016/j.vibspec.2013.05.012.

Lister, S.J., and Dhanoa, M.S. 1998. Comparison of calibration models for the prediction of forage quality traits using near infrared spectroscopy. In France, J. (ed.) Thirtieth Meeting of the Agricultural Research Modellers' Group, London. Journal of Agricultural Science 131(2):241-242.

Lobos, I., Gou, P., Hube, S., Saldaña, R., and Alfaro, M. 2013. Evaluation of potential NIRs to predict pastures nutritive value. Journal of Soil Science and Plant Nutrition 13:463-468.

Nie, Z., Tremblay, G.F., Bélanger, G., Berthiaume, R., Castonguay, Y., Bertrand, A., et al. 2009. Near-infrared reflectance spectroscopy prediction of neutral detergent-soluble carbohydrates in timothy and alfalfa. Journal of Dairy Science 92:1702-1711. doi:http://dx.doi.org/10.3168/jds.2008-1599.

Nyquist, W.E., and Baker, R.J. 1991. Estimation of heritability and prediction of selection response in plant populations. Critical Reviews in Plant Sciences 10:235-322. doi:10.1080/07352689109382313.

Piaskowski, J.L., Brown, D., and Campbell, K.G. 2016. Near-infrared calibration of soluble stem carbohydrates for predicting drought tolerance in spring wheat. Agronomy Journal 108:285-293. doi:10.2134/agronj2015.0173.

Wachendorf, M., Collins, R.P., Connolly, J., Elgersma, A., Fothergill, M., Frankow-Lindberg, B.E., et al. 2001. Overwintering of *Trifolium repens* L. and succeeding growth: Results from a common protocol carried out at twelve European sites. Annals of Botany 88:669-682.

Widdup, K.H., Ford, J.L., Barret, B.A., and Woodfield, D.R. 2010. Development of white clover populations with higher concentrations of water soluble carbohydrate. p. 277-282. Proceedings of the New Zealand Grassland Association. New Zealand Grassland Association (NZGA), Lincoln, New Zealand.

Yemm, E.W., and Willis, A.J. 1954. The estimation of carbohydrates in plant extracts by anthrone. Biochemical Journal 57:508-514. doi:10.1042/bj0570508.