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Agronomic characteristics of annual *Trifolium* legumes and nutritive values as predicted by near-infrared reflectance (NIR) spectroscopy

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Abstract. A range of annual legume genotypes comprising one line of *Trifolium subterraneum*, four lines of *T. michelianum*, 11 of *T. resupinatum* var. *resupinatum*, and one line of *T. resupinatum* var. *majus* were grown in glasshouses under temperature regimes of 10−15°C and 16−21°C. Dry matter (DM) weights of stem, leaf, and flower tissues were measured when plants had six nodes, at first flower appearance, and at senescence. All samples were scanned by near-infrared reflectance spectroscopy (NIRS). One-third of the samples, covering the range of spectral characteristics, were analysed for *in vitro* digestible organic matter (DOMD), organic matter, crude protein (CP), neutral detergent fibre (NDF), lignin, cellulose, and the hemicellulosic polysaccharide monomers arabinose, xylose, mannose, galactose, and rhamnose. These data were used to develop calibration equations from which the composition of the remaining samples was predicted by NIRS. The higher temperature resulted in plants reaching respective phenological stages earlier, but did not affect either DM yields of total plant, stem, leaf, and petiole tissues or the proportions of each fraction. *In vitro* DOMD and arabinose and galactose levels decreased, while lignin, cellulose, NDF, xylose, mannose, and rhamnose levels increased with advancing maturity. *In vitro* DOMD was positively associated with contents of CP, arabinose, galactose, and the arabinose/xylose ratio and was negatively associated with contents of lignin, cellulose, NDF, xylose, mannose, and rhamnose. Lignin contents were highly correlated with levels of both xylose and mannose. Stems were more digestible than leaves in subterranean clover and *T. resupinatum* var. *majus*. The study also demonstrated that NIRS can be used routinely as a quick, inexpensive, and reliable laboratory technique to predict feed components of annual *Trifolium* legumes.

Additional keywords: annual legume, cell wall polysaccharides, morphology, NIRS, yield.

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Introduction

Annual legumes are major feed sources for ruminants in southern Australia. The success of the most widespread annual legume, subterranean clover (*Trifolium subterraneum* L.), is largely due to its ability to establish and persist under heavy grazing and also to the wide range of cultivars available for different climatic and edaphic conditions (Nichols *et al*. 2007). Persian clover (*Trifolium resupinatum* L.) and balansa clover (*T. michelianum* Savi) are alternative legumes to subterranean clover. There are two botanical varieties of *T. resupinatum* that are used in agriculture, var. *majus* and var. *resupinatum*, with var. *majus* having longer, thicker, and more hollow stems and larger leaves than var. *resupinatum* (Zohary and Heller 1984). They are adapted to withstand waterlogged conditions better than subterranean clover and can grow in moderately saline areas (Nichols *et al*. 2006).

Subterranean clover has an exceptionally high nutritive value as a feed for sheep when green (Stockdale 1992*a*, 1992*b*). However, the nutritive value of senesced plant material is lower during the summer months, when ewe and weaner nutrition is critical (Kenny and Reed 1984; Ridley *et al*. 1986). In contrast, the Persian clover var. *majus* type cv. Maral gave satisfactory animal production results in Victoria (Kenny and Reed 1984). It is not clear whether these differences could be accounted for by the relatively higher nutritive value reported for the Persian clover at senescence or differences in pasture availability.

Available evidence indicates that environmental factors such as temperature have significant impacts on agronomic and nutritive characteristics of annual legumes (Evans *et al*. 1992). There is considerable variation within Persian clover in nutritive value, particularly when senesced (Nichols *et al*. 2006).

Apparent nitrogen digestibility was lower for sheep fed on Persian clover cv. Kyambro (var. *resupinatum*) than on subterranean clover cv. Junee (Li *et al*. 1992). Differences between the types of Persian clovers are apparent, with Kyambro being considerably lower in digestibility than Maral (Craig 1989). It is not known whether there is any variation within balansa clovers, and the relationships between agronomic and morphological traits and measures of nutritive value have not been examined.

Thomas *et al*. (2010) found that sheep preferred plants with higher nutritive value when the quality of vegetation was poor. Therefore, selection of senesced annual legumes with better nutritive characteristics should benefit sheep production. The chemical composition of a feed is routinely used as a rapid and economic method for predicting digestibility and other measures of nutritive value. Lignification is the major factor limiting digestibility of plant cell wall polysaccharides by ruminants (Grabber 2005; Hatfield *et al*. 2009). The composition of plant cell wall polysaccharides may influence their rate and extent of digestion by rumen microorganisms, independent of lignin effects (Buxton *et al*. 1987). Separation of cell wall polysaccharides into their constituent monosaccharides in part explains differences in digestibility among forages and plant fractions (Albrecht *et al*. 1987*a*; Wedig *et al*. 1987). Ben-Ghedalia and Miron (1984) found that xylans from lucerne (*Medicago sativa*) cell walls were the least digestible cell-wall carbohydrates. Burritt *et al*. (1984) found a correlation of –0.85 ($P < 0.01$) between proportion (%) of xylose and *in vitro* dry matter digestibility of grasses. Nordkvist and Aman (1986) found a very strong relationship between xylose content and *in vitro* dry matter degradability ($r = –0.98$, $P < 0.01$) of lucerne and suggested that the nutritive value of lucerne could be predicted from an analysis of the xylose content. Thus, selection of annual legumes with low lignin and xylose contents is likely to increase their nutritive value for ruminants.

An understanding of the morphological characteristics that influence the nutritive value of plants would aid in selection and in developing management systems for pasture plants. Stem tissue usually plays a dominate role in determination of herbage digestibility (Buxton and Hornstein 1986; Albrecht *et al*. 1987*b*). Research with lucerne and red clover (*Trifolium pratense*) showed that decreases in digestibility with progressive growth were almost exclusively associated with the stem (Kuhbauch 1983). With increasing stem length, more structural support for the plant is required, which results in increases in stem cell wall thickness, fibre content, and/or lignification.

Analysis of lignin and xylose by wet chemical methods, as for analyses of many other constituents, is time-consuming, requires chemical reagents, and produces waste products. Near-infrared reflectance spectroscopy (NIRS) has been used to quantify a wide range of characteristics in various different feed ingredients, including cereal grains and oilseed meals (Albrecht *et al*. 1987*a*; Stimson *et al*. 1991; Kovalenko *et al*. 2006; Li *et al*. 2007; Deaville *et al*. 2009). It is a relatively fast and inexpensive, non-destructive technique.

This study examined two hypotheses: (*i*) the nutritive value of annual legumes is correlated with their agronomic characteristics and chemical composition, and selection of superior lines can be achieved by selecting desirable agronomic characteristics and chemical composition; and (*ii*) NIRS can be used across different phenological stages and temperature regimes as a cost-effective tool to analyse organic matter (OM), *in vitro* digestible OM (DOMD), crude protein (CP), neutral detergent fibre (NDF), lignin, cellulose, and the hemicellulosic polysaccharide monomers of annual legumes.

Materials and methods

Growing and harvesting plants

Twelve lines of Persian clover (11 *resupinatum* and one *majus*) and four of balansa clover with different flower times were chosen from material identified by R. Snowball (curator of the Australian Trifolium Genetic Resource Centre) as worthy of further evaluation on the basis of their agronomic characteristics. Subterranean clover cv. Junee was included as a control. Seeds were sown on 5 July in 2.5-L pots containing a mix of three parts sand to two parts peat plus lime and a basal fertiliser mix in temperature-controlled glasshouses with natural lighting. There were two temperature treatments of 15/10°C and 21/16°C (day/night). Plants were watered daily and fertilised weekly with Aquasol (Yates Australia, Padstow, NSW), a complete nutrient fertiliser. Each pot was thinned to three plants at 25 days after sowing.

Eighteen pots per line were randomly allocated into three harvesting dates under each temperature regime for three pots per line per harvest per temperature regime. Harvests were made on the basis of phenological stages: when plants had six nodes on average (H1), at first flower appearance (H2), and when plants were fully senesced (H3). For the first two harvests, each plant was separated into stem, leaf, and petiole tissue samples. Morphological measurements included stem length and number of nodes with fully emerged leaves on the main stem, stem diameter immediately below the cotyledonary node and below the eighth node on the main stem, and total numbers of branches and leaves per plant. At the final harvest, whole plants of each pot were harvested and the weights of individual fractions were determined. All samples were frozen in liquid nitrogen and freeze-dried (Christ Freeze Dryers, Osterode am Harz, Germany). Tissue fractions from each line and temperature were pooled and milled in a mill (Christy and Morris, UK) through a 1-mm screen for use in NIRS and wet chemistry.

NIR analysis

Samples placed in a 30-mm-diameter quartz window and pressure pad sample holder were scanned by a monochromating NIRS (Pacific Scientific Model 6250, Pacific Scientific, USA) and absorption spectra recorded in the range 1100–2500 nm. The instrument had logarithmic response amplification of signals from the detectors. Signals were digitised and recorded as log 1/R by computer. The software for scanning, mathematical processing, and statistical analysis was by Infrasoft International (J. S. Shenk and Associates, State College, PA, USA). A subset of samples (30%) which covered the range of variation present in spectral characteristics was selected. The subset was analysed by wet chemical methods to provide data for establishing calibration equations. This served as the ‘closed’

population calibration set used to predict unknowns from the 'open' population.

The various terms used in NIRS analysis have been described by Stimson *et al*. (1991) and Hruskchka (2001). The standard error of the laboratory (SEL) data is defined as the standard deviation between duplicate laboratory measurements. The standard error of calibration (SEC) data is the standard error between laboratory reference and NIRS measured values in the calibration set. The standard error of prediction (SEP) is the standard error between the predicted values of the validation set and the corresponding laboratory analyses. The SEL, SEC, and SEP as proportion (%) of the mean were also calculated. Regression relationships of laboratory-determined and NIRS-predicted values were used to test whether an equation had successfully predicted the values of the validation set.

Calibration equations for each constituent were chosen by the optimum combination of the following statistics: small SEC, large r^2, and F-test >10 on each selected wavelength. For equation validation, the criteria were: small SEP, large r^2, and small bias (Marten *et al*. 1984; Stimson *et al*. 1991). Ideally, there should be no bias (intercept) and the slope should be 1. Equations selected as the best fit for a particular chemical fraction were then used to predict the composition of the remaining samples.

Chemical analyses

Dry matter was determined by drying the samples in a forced-draught oven at 90°C for 48 h. Organic matter was determined by ashing the samples in a muffle furnace at 500°C for 5 h. Crude protein (N × 6.25) was determined by the Kjeldahl method using a Kjel-Foss™ nitrogen analyser (Kjel-Foss Automatic 16210, A/S N, Foss Electric, Denmark) (AOAC 1984). Neutral and acid detergent fibres and lignin were determined by the method of Goering and Van Soest (1970). Cellulose content was calculated by subtracting lignin-plus-ash from acid detergent fibre (Van Soest and Robertson 1980; Möller 2009). Arabinose, xylose, mannose, galactose, and rhamnose were analysed by the method of Choct and Annison (1990). *In vitro* DOMD in the samples was measured using pepsin-cellulase according to the method of McLeod and Minson (1978).

Statistical analyses

The StatView program version 4.57 (Abacus Concepts, Inc., Berkeley, CA, 1996) was used to analyse all data. Total dry matter (DM) yield and DM distribution of stem, leaf, and petiole were analysed by one-way analyses of variance (ANOVA) on separate harvests and temperature regimes with the individual plant as a replicate unit for H1 and H2 and the pot as the unit for H3. Junee subterranean clover and Persian clover var. *majus* type had incomplete flowering at the second harvest. Therefore, these lines were excluded when correlation coefficients were calculated between morphologic measures (i.e. stem length, number of nodes, stem diameter, number of branches, and number and % of leaves), *in vitro* DOMD, and chemical composition. One-way ANOVA was also used to analyse the differences among species groups, Junee subterranean clover, balansa clovers, Persian clover var. *majus* type and var. *resupinatum* type for H1 and H3 under each temperature regime. Stage H2 was excluded due to incomplete flowering of Junee subterranean clover and Persian clover var. *majus* type. Standard errors of means (s.e.m.) and least significant differences (l.s.d. at $P = 0.05$) or P values are given in the respective tables or text.

Results

Temperature effects

Higher temperature resulted in plants reaching the respective phenological stages earlier (Table 1). Most lines, except Junee subterranean clover and var. *resupinatum* type SA19690 at the higher temperature regime, reached senescence 4–6 weeks earlier than those at the lower temperature. There was no consistent effect of temperature on DM yield of total plant (Table 1), stem, leaf, and petiole tissues, or the proportional distribution of each fraction (data not shown).

Total DM yield and distribution of dry weight in fractions

Dry matter yield of individual lines for three harvests and two temperature regimes are shown in Table 1. In general, balansa clovers produced more DM under both temperature regimes than did Persian clovers for H1 and H2. The DM yield of Persian clover 26202-3 for H1 was the highest among the var. *resupinatum* lines at both temperature regimes. Balansa clovers 45856-1 and 45855-1 produced the highest total DM among the balansa clovers for all harvests at lower and higher temperatures, respectively. There were no differences in DM yield among Junee subterranean clover, Persian clovers, and balansa clovers for H3 under both temperature regimes ($P > 0.05$).

For H1, the leaves constituted 41–55% of total plant DM weight, whereas stems were only 12–25% and petioles were 28–37%. However, for H2, the stem proportion was 51–65% of the total DM weight, with the proportions of leaf and petiole being 16–28% and 7–14%, respectively. There was a significant correlation between stem length and DM yield ($r = 0.80$, $P < 0.01$). The DM distribution of stem, leaf, and petiole for H3 was similar to H2 (data not presented).

Correlation between morphological characters, chemical composition, and digestibility

Stem length, node number, stem diameter, and branch number were positively associated with contents of OM, NDF, lignin, cellulose, xylose, and mannose ($P < 0.01$) and were negatively associated with *in vitro* DOMD, CP, arabinose, and galactose ($P < 0.01$) across harvests H1 and H2 and the two temperature regimes (Table 2). Leaf percentage was positively associated with *in vitro* DOMD ($r = 0.633$, $P < 0.01$) and CP ($r = 0.879$, $P < 0.01$) (Table 2).

In vitro DOMD was positively associated with CP, arabinose, galactose, and arabinose : xylose ratio and was negatively associated with lignin, cellulose, NDF, xylose, mannose, and rhamnose of stem, leaf, and petiole across H1 and H2 and the two temperature regimes (Table 3). The relationships were similar for the senesced stems (Table 3). Lignin was positively related to xylose ($r = 0.91$, $P < 0.001$) and mannose ($r = 0.81$, $P < 0.001$). Cellulose was positively correlated with xylose ($r = 0.86$, $P < 0.001$) and mannose ($r = 0.83$, $P < 0.001$) and was negatively associated with CP ($r = –0.84$, $P < 0.001$). The correlation coefficient between xylose and mannose was 0.76 ($P < 0.001$) (data not shown).

Table 1. Dry matter yield and days after sowing (in parentheses) at 15/10°C and 21/16°C (day/night) when harvested at three stages of growth (H1, H2, and H3) for *Trifolium michelianum*, *T. resupinatum*, and *T. subterraneum*

H1, Plants with six nodes; H2, first flower appearance; H3, fully senesced. Across rows within harvests, values followed by different letters are significantly different ($P < 0.05$)

Line	H1		H2		H3	
	15/10°C	21/16°C	15/10°C	21/16°C	15/10°C	21/16°C
	g/plant (days)				g/pot (days)	
Trifolium subterraneum L.						
Junee	1.00a (74)	0.42b (56)	4.20 (154)	6.01 (153)[A]	18.1 (203)	16.6 (190)
T. resupinatum L. var. *resupinatum*						
cv. Persian Prolific	0.76a (74)	0.39b (56)	4.23 (115)	3.26 (83)	15.2 (203)	20.9 (160)
26202-3	0.97 (74)	0.72 (56)	4.28 (118)	4.28 (95)	19.3 (193)	16.4 (151)
SA18904	0.73a (74)	0.46b (57)	8.79a (134)	4.29b (102)	14.2 (191)	18.5 (160)
SA19704	0.74 (74)	0.65 (57)	9.34 (147)	5.70 (130)	38.7 (193)	19.0 (155)
LEGW30040	0.62 (74)	0.60 (56)	10.5 (139)	6.76 (112)	15.4 (193)	21.4 (151)
PI120159	0.41 (74)	0.43 (56)	6.12 (155)	8.91 (130)	15.7 (191)	6.55 (160)
P1204932	0.53 (74)	0.64 (56)	5.67 (155)	8.73 (147)	11.8 (193)	25.1 (161)
WCS11RES	0.33 (75)	0.29 (57)	7.07 (155)	7.68 (146)	14.07 (210)	15.5 (161)
CIZIRES-B	0.89a (74)	0.48b (56)	7.26 (154)	7.07 (131)	22.2a (203)	17.1b (155)
Kyambro	0.39 (74)	0.26 (56)	7.78 (148)	7.88 (139)	25.8 (193)	17.3 (161)
SA12240	0.68 (74)	0.61 (57)	4.32 (154)	7.03 (130)	–	20.2 (158)
T. resupinatum L. var. *majus*						
SA19690	0.57 (74)	0.37 (57)	6.57 (155)[A]	4.37 (153)[A]	21.6 (210)	15.5 (190)
T. michelianum Savi						
86522	0.69a (75)	0.40b (57)	8.73 (139)	8.44 (120)	15.5 (203)	11.5 (159)
45856-1	1.26a (75)	0.67b (57)	12.2a (149)	5.49b (118)	25.0 (191)	14.9 (160)
45855-1	1.01 (75)	0.81 (57)	8.13 (134)	9.24 (131)	14.1 (193)	29.7 (155)
86817-1	0.97a (74)	0.43b (56)	9.01 (147)	6.73 (103)	25.1 (203)	20.4 (155)
s.e.m.	0.106	0.0858	1.532	1.346	3.620	2.376
l.s.d. ($P = 0.05$), *P* value	0.294, <0.01	0.238, <0.01	4.246, 0.011	3.730, 0.046	10.550, 0.013	6.861, <0.01

[A]Incomplete flowering.

Table 2. Correlations between morphological measurements and chemical composition of stems for four balansa clovers and 11 Persian clover var. *resupinatum* lines across both temperatures for the first two harvests

$^{**}P < 0.01$

Variable	Stem length	No. of nodes	Stem diameter	No. of branches	% Leaf
OM	0.72**	0.65**	0.62**	0.55**	–0.76**
In vitro DOMD	–0.64**	–0.63**	–0.18	–0.60**	0.50**
CP	–0.81**	–0.81**	–0.74**	–0.61**	0.89**
NDF	0.87**	0.87**	0.48**	0.73**	–0.78**
Lignin	0.82**	0.79**	0.38**	0.69**	–0.68**
Cellulose	0.87**	0.86**	0.51**	0.74**	–0.81**
Arabinose	–0.88**	–0.87**	–0.65**	–0.72**	0.91**
Xylose	0.90**	0.88**	0.54**	0.75**	–0.82**
Mannose	0.72**	0.71**	0.34**	0.68**	–0.62**
Galactose	–0.89**	–0.88**	–0.74**	–0.73**	0.96**
Rhamnose	–0.06	–0.04	–0.35**	0.12	0.16
Arabinose/xylose	–0.87**	–0.88**	–0.62**	–0.73**	0.89**

Table 3. Pearson correlation coefficients (*r*) between *in vitro* DOMD and chemical composition of stem, leaf, and petiole across H1 and H2, and of stem at H3

H1, Plants with six nodes; H2, first flower appearance; H3, fully senesced.
$^{*}P < 0.05$; $^{**}P < 0.01$

Variable	Across H1 and H2			H3
	Stem	Leaf	Petiole	Stem
CP	0.41**	0.41**	0.49**	0.64**
NDF	–0.85**	–0.73**	–0.67**	–0.79**
Lignin	–0.89**	–0.83**	–0.82**	–0.80**
Cellulose	–0.82**	–0.67**	–0.85**	–0.83**
Arabinose	0.54**	0.17	0.43**	0.52**
Xylose	–0.78**	–0.71**	–0.89**	–0.84**
Mannose	–0.75**	–0.64**	–0.77**	–0.54**
Galactose	0.38**	0.29*	0.59**	0.21
Rhamnose	–0.52**	–0.78**	–0.80**	–0.56**
Arabinose/xylose	0.63**	0.56**	0.77**	0.71**

NIRS prediction

The SELs as a proportion of the mean were generally <5%, except for lignin and rhamnose, which were 16.4% and 13.5%, respectively (Table 4). The SECs of *in vitro* DOMD, CP, NDF, and OM were <5% of the mean, except lignin at 18.9%, which was associated with a high SEL of 16.4%. The SECs for cell wall polysaccharide constituents were 9.3–16% of the mean (Table 4).

The r^2 values for the calibration equations were ≥0.95 for *in vitro* DOMD, CP, cellulose, NDF, and xylose. Lignin, arabinose, mannose, galactose, and rhamnose had lower r^2 values (Table 4).

Table 4. Statistical parameters of calibration and validation equations for NIRS analyses of samples
Values in parentheses are percentage of the mean

Variable	Mean (g/kg DM)	Laboratory SEL	Calibration SEC	r^2*	Prediction (validation) SEP	r^2**	Bias	Slope
OM	868	4.55 (0.525)	10.7 (1.2)	0.86	15.8 (1.8)	0.69	0.65 (0.07)	1.02
In vitro DOMD	596	12.1 (2.04)	16.3 (2.7)	0.95	20.3 (3.4)	0.92	2.59 (0.43)	1.03
CP	182	2.73 (1.50)	7.19 (3.95)	0.99	10.8 (5.9)	0.98	0.65 (6.0)	0.99
NDF	304	4.95 (1.62)	11.5 (3.8)	0.99	22.1 (7.3)	0.97	3.22 (1.10)	0.99
Lignin	39.4	6.47 (16.4)	7.45 (18.9)	0.79	8.30 (21.0)	0.75	0.51 (1.30)	1.0
Cellulose	208	6.28 (3.03)	10.2 (4.9)	0.99	19.7 (9.5)	0.96	1.52 (0.70)	0.99
Arabinose	25.3	1.08 (4.27)	3.79 (15.0)	0.86	5.29 (20.9)	0.73	0.20 (0.79)	1.08
Xylose	30.6	0.441 (1.44)	2.85 (9.3)	0.96	3.85 (12.6)	0.92	0.21 (0.69)	0.96
Mannose	5.98	0.222 (3.71)	0.69 (11.5)	0.77	0.88 (14.7)	0.63	0.07 (1.2)	0.99
Galactose	18.2	0.691 (3.79)	1.75 (9.6)	0.68	2.24 (12.3)	0.49	0.07 (0.38)	1.01
Rhamnose	8.10	1.08 (13.5)	1.30 (16.0)	0.58	1.38 (17.0)	0.55	0.06 (0.74)	0.93

The components with higher concentrations tended to have lower SEPs as a proportion of the mean. The SEPs of *in vitro* DOMD, CP, cellulose, NDF, and OM were <10% of the means, whereas SEPs for lignin and arabinose were high, at up to 21% of the mean.

The slopes of the components measured were close to 1.00 and the biases for NIRS analysis were ≤1.3% of the mean, except for CP. The squared simple correlation coefficients (r^2) between NIRS and laboratory-analysed data for validation equations ranged from 0.98 for CP to 0.49 for galactose (Table 4).

Effects of temperature and maturity on chemical composition

There were large differences in each chemical component between the two temperature regimes (Table 5). *In vitro* DOMD and arabinose and galactose levels were higher under the lower temperature regime, whereas CP, lignin, cellulose, NDF, OM, xylose, mannose, and rhamnose levels were higher under the higher temperature regime. *In vitro* DOMD, CP, arabinose, galactose, and arabinose : xylose ratio decreased as maturity advanced, whereas NDF, cellulose, lignin, rhamnose, xylose, and mannose increased as maturity advanced (Table 6). However, CP contents were higher at senescence than at flowering in Persian clover var. *resupinatum* type

Table 5. Effect of temperature on chemical composition (g/kg DM) for four balansa clovers and 11 Persian clover var. *resupinatum* lines
Across rows, means followed by different letters are significantly different ($P < 0.05$)

Variable	Temperature 10/15°C	16/21°C
OM	857b	872a
In vitro DOMD	626a	608b
CP	176b	205a
NDF	290b	317a
Cellulose	197b	234a
Lignin	36.6b	44.5a
Arabinose	27.5a	22.6b
Xylose	28.4b	33.7a
Mannose	5.32b	6.58a
Galactose	19.1a	18.3b
Rhamnose	7.62b	8.48a

Table 6. Chemical composition (g/kg DM) of balansa clovers, Junee subterranean clover, Persian clover var. *majus*, and Persian clover var. *resupinatum* lines for H1 and H3
H1, Plants with six nodes; H3, fully senesced. Across rows and within harvests, means followed by different letters are significantly different ($P < 0.05$)

Variable	H1 Balansa	H1 Junee	H1 var. *majus*	H1 var. *resupinatum*	H3 Balansa	H3 Junee	H3 var. *majus*	H3 var. *resupinatum*
OM	863.0	850.1	861.8	850.1	872.3a	860.3b	869.5ab	861.8ab
DOMD	682.8a	634.3b	674.9a	674.2a	566.0b	523.9c	596.7a	545.3c
CP	247.1	254.2	248.0	272.5	147.7b	164.9ab	212.5a	153.1b
NDF	239.8	263.4	221.4	231.2	370.3	394.9	303.4	366.5
Lignin	28.4b	38.2a	25.5b	27.8b	52.4b	65.1a	39.8c	54.2b
Cellulose	136.9	137.2	133.8	130.7	295.2	283.1	251.5	282.8
Arabinose	30.1ab	39.1a	27.6b	32.7ab	19.6b	28.0a	22.7b	20.7b
Xylose	19.2	19.0	18.3	19.4	38.8ab	38.6ab	31.5b	41.9a
Mannose	5.0	4.9	4.9	4.8	7.3a	7.5a	6.6b	7.0ab
Galactose	21.3	21.7	20.2	21.4	17.0	16.9	16.9	17.4
Rhamnose	7.2	7.1	7.0	7.1	9.4	9.3	9.1	9.2
Arabinose/xylose	1.60b	2.10a	1.56b	1.73ab	0.54b	0.77a	0.77a	0.54b

PII20159, WCS11RES, Kyambro, and SA12240 (data not shown).

Comparisons between species groups

In vitro DOMD content of balansa clovers and Persian clover var. *majus* type was significantly ($P < 0.05$) higher than that of Junee subterranean clover for H1 and H3 (Table 6). Persian clovers var. *resupinatum* type were high in CP when green (H1), although the difference was not statistically significant ($P > 0.05$), whereas var. *majus* type was higher ($P < 0.05$) in CP when senesced (H3) compared with other species groups. Lignin was higher ($P < 0.05$) in Junee than other species groups at all harvests. Persian clover var. *majus* type was superior to other Persian clovers var. *resupinatum* type, balansa clovers, and Junee in terms of higher contents of *in vitro* DOMD and CP and lower contents of NDF, lignin, and xylose than other species groups when senesced (Table 6).

Comparisons between plant parts

Leaf tissue was more digestible than stem and petiole tissues in balansa clovers and Persian clovers var. *resupinatum* type throughout three harvests. The digestibilities of stem and leaf for Junee subterranean clover and Persian clover var. *majus* were similar when the plants were green (Table 7). However, their stems were more digestible than leaves and petiole when the plants had senesced (Table 8). The difference in *in vitro* DOMD between stems and leaves tended to increase with maturity in Junee. Leaves had much higher CP content than did stems and petioles. However, this did not make leaves more digestible in Junee subterranean clover and Persian clover var. *majus*. Lignin content was generally higher in stem tissue than in leaf and petiole tissue. Cellulose content was lower in leaf tissues than in stem and petiole tissues (Tables 7 and 8). The NDF contents were higher in stem than leaf and petiole tissues. Organic matter contents of leaf tissues were higher at H1 (Table 7) and lower at H3 (Table 8) than stem and petiole tissues.

Contents of arabinose and xylose were the highest in stem tissue and the lowest in leaf tissues (Tables 7 and 8). Mannose content was similar in stem and petiole tissues, and both were slightly higher than in leaf tissue. Mannose content of stem was lower in Persian clover var. *majus* than in the other

Table 7. Chemical composition (g/kg DM) of balansa clovers, Junee subterranean clover, Persian clover var. *majus*, and Persian clover var. *resupinatum* lines at H1 (plants with six nodes)

Across rows and within fractions, means followed by different letters are significantly different ($P < 0.05$)

Variable	Leaf				Petiole				Stem			
	Balansa	Junee	var. *majus*	var. *resupinatum*	Balansa	Junee	var. *majus*	var. *resupinatum*	Balansa	Junee	var. *majus*	var. *resupinatum*
OM	887.8	867.9	880.7	873.3	859.1	842.2	851.1	844.6	842.1	830.4	850.8	830.6
DOMD	710.6a	640.8b	685.6a	701.0a	661.7a	622.2b	653.3ab	655.9ab	676.1a	639.8b	689.3a	664.9ab
CP	362.8	356.8	378.5	383.8	175.5	168.3	168.7	196.7	203.0	237.5	179.6	233.6
NDF	126.3b	151.4a	113.8c	115.6c	304.8b	349.2a	291.0b	300.8b	288.2	289.6	272.0	281.8
Lignin	23.7b	36.8a	25.5b	24.2b	31.1b	40.7a	26.5b	30.0b	30.5ab	37.0a	24.1b	29.4b
Cellulose	58.2	55.7	57.4	53.8	196.6	215.9	202.0	188.1	155.8	140.2	144.5	152.0
Arabinose	20.8	23.7	20.5	21.8	29.8bc	43.2a	27.5c	32.6b	39.8	50.4	37.1	45.0
Xylose	15.1	12.5	15.3	15.7	22.6	26.0	21.7	22.1	20.0	18.5	18.0	20.4
Mannose	4.6	4.2	4.6	4.3	5.3	5.4	5.1	5.1	5.1	5.1	5.0	5.0
Galactose	19.2	18.3	18.7	18.9	21.8a	22.3a	20.2b	21.9a	22.9	24.3	22.4	23.7
Rhamnose	6.6	5.8	6.6	6.2	7.6	7.7	7.4	7.6	7.4	7.7	7.1	7.4
Arabinose/ xylose	1.44	1.90	1.40	1.46	1.34	1.67	1.32	1.52	2.02	2.73	2.10	2.26

Table 8. Chemical composition (g/kg DM) of senesced balansa clovers, Junee subterranean clover, Persian clover var. *majus*, and Persian clover var. *resupinatum* lines at H 3 (fully senesced)

Across rows and within fractions, means followed by different letters are significantly different ($P < 0.05$)

Variable	Leaf				Petiole				Stem			
	Balansa	Junee	var. *majus*	var. *resupinatum*	Balansa	Junee	var. *majus*	var. *resupinatum*	Balansa	Junee	var. *majus*	var. *resupinatum*
OM	863.3	851.8	870.7	856.1	865.3a	862.4a	862.8ab	852.6b	888.4	866.7	874.9	876.7
DOMD	595.6a	509.3b	606.1a	570.9a	528.8	512.3	537.2	523.9	573.6b	550.1b	646.9a	541.2b
CP	215.8b	230.5b	284.6a	226.8b	115.7	123.6	107.8	123.2	111.7b	140.6b	245.0a	109.3b
NDF	252.8a	271.9a	190.6b	236.1a	401.9ab	451.9a	398.1ab	385.6b	456.3a	461.0a	321.5b	477.8a
Lignin	44.0b	60.3a	36.3c	43.2b	49.2b	65.9a	44.7b	49.0b	63.9a	69.1a	38.3b	70.5a
Cellulose	195.9	157.4	163.2	177.9	365.5a	366.0a	366.3a	334.6b	324.3a	325.9a	225.0b	336.0a
Rham	9.5	9.3	8.5	9.0	9.8	9.6	9.8	9.7	8.8	9.2	9.1	8.9
Arabinose	16.5	21.1	20.1	18.9	21.3bc	32.4a	17.7c	21.9b	21.1b	30.4a	30.3a	21.4b
Xylose	26.4	23.2	25.2	28.5	38.6	42.3	36.7	39.9	51.5a	50.2a	32.7b	57.4a
Mannose	7.0	7.0	6.1	6.4	7.8	8.2	7.5	7.3	7.0a	7.3a	6.2b	7.2a
Galactose	17.2	16.9	17.2	18.2	17.6a	17.6a	16.3b	17.8a	16.1	16.2	17.1	16.4
Arabinose/ xylose	0.65	0.93	0.80	0.69	0.56b	0.77a	0.49b	0.56b	0.43b	0.61b	1.01a	0.38b

species groups. Galactose contents were similar in all three fractions. Rhamnose content was similar in stem and petiole tissues.

Discussion

Relationships of nutritive value to agronomic and morphological characteristics

In the present study, there were significant positive correlations between stem length and contents of NDF, lignin, and cellulose. Significant inverse relationships were found between stem length and digestibility in most of the legumes, except for Junee subterranean clover and the var. *majus* type. In these exceptions, the stems were more digestible than were the leaves, despite their having high cell wall contents. Kuhbauch (1983) found significant negative relationships between stem length and *in vitro* DM digestibility of lucerne ($r = -0.951$, $P < 0.01$) and red clover ($r = -0.804$, $P < 0.01$), but this relationship did not exist for white clover ($r = -0.035$, $P > 0.05$). Kuhbauch (1983) also found that stems of white clover (*Trifolium repens*) were more digestible than leaves, suggesting that there were qualitative differences within the cell wall as a whole and/or within particular fibre fractions between these legumes. In the present study, there were significant correlations between morphological measures and monomers of non-starch polysaccharides. Stem length was positively associated with contents of xylose ($r = 0.899$, $P < 0.01$) and mannose ($r = 0.722$, $P < 0.01$), which is in agreement with earlier studies in which mannose (Buxton *et al*. 1987) and xylose (Wedig *et al*. 1987) were negatively associated with DM digestibility. The stem : leaf ratio increased during growth for all lines, which was also in general agreement with previous studies of Nordkvist and Aman (1986) and Albrecht *et al*. (1987*b*).

Kalu *et al*. (1988) found highly significant linear relationships between leaf percentage and crude protein ($r = 0.91$) and *in vitro* true digestibility ($r = 0.92$) of lucerne. They suggested that plant leafiness can be used to predict forage quality. In the present study, there were significant, but lower, correlations between leaf percentage and *in vitro* DOMD ($r = 0.633$, $P < 0.01$) and CP (0.879, $P < 0.01$). The weaker relationships were associated with the various different annual legume genotypes used in this study compared with the single genotype in the study of Kalu *et al*. (1988).

Following flowering, nutritive value declines due to translocation of soluble carbohydrates from stems and leaves to inflorescences and also due to increased cell wall lignification (Hacker and Minson 1981). Therefore, a late-flowering cultivar can be expected to have a higher level of digestibility once flowering commences than an earlier flowering cultivar of the same species. Forage species which maintain high digestibility for long periods during the growing season are of higher value for animal production than are those which may flower early and have high digestibility at a young stage. However, early flowering is desirable for cultivars aimed at short growing seasons to ensure seed production. Therefore, there is a trade-off between persistence for the species and nutritive value in spring and summer.

Analytical methods

Near-infrared reflectance spectroscopy successfully predicted *in vitro* DOMD and other chemical composition characteristics of stem, leaf, and petiole fractions. The SECs for *in vitro* DOMD, CP, lignin, NDF, and OM were smaller than those reported by Stimson *et al*. (1991), which were based only on senesced annual legumes. The SECs for xylose and mannose were smaller than those reported by Albrecht *et al*. (1987*a*) for lucerne stem and leaf. The smaller SECs indicate that the calibration equations accurately predict the contents of *in vitro* DOMD, CP, lignin, NDF, OM, xylose, and mannose of the calibration set. In contrast, large SECs for arabinose and galactose indicate that the equations were less accurate in predicting their respective sugar contents in the calibration set. The larger SECs may be due to differences in reference methods used for cell wall polysaccharide analysis (Fairbrother and Brink 1990). Analysis of cell wall polysaccharides presents many difficulties. Acid hydrolysis is a compromise between the hydrolysis of resistant polysaccharides and the degradation of released monosaccharides (Fairbrother and Brink 1990). The relatively large SECs for lignin may be associated with the difficulties in its analysis due to incomplete knowledge of its structure (Rowland and Roberts 1994).

The calibration equation r^2 values for *in vitro* DOMD, CP, NDF, and OM were larger than those reported by Stimson *et al*. (1991), indicating that the contents of these components analysed in laboratory and by NIRS were highly corrected in the calibration set (Pazdernik *et al*. 1997). The lower r^2 values for arabinose, mannose, galactose, and rhamnose in NIRS analysis may be associated with their low concentrations (<35 g/kg) and small ranges in the plant material analysed (Albrecht *et al*. 1987*a*). The low r^2 value for lignin was similar to that reported by Stimson *et al*. (1991).

The precision with which NIRS predicted laboratory analyses compared favourably with other studies. The SEPs for *in vitro* DOMD, CP, lignin, NDF, and OM were comparable to the study of Stimson *et al*. (1991). The SEPs for xylose and mannose were similar to those reported by Albrecht *et al*. (1987*a*), while SEPs for arabinose and galactose were larger than those in the same report. The small SEPs indicate more accurate predictions by NIRS (Pazdernik *et al*. 1997).

The low r^2 values in the prediction equations for arabinose, mannose, galactose, rhamnose, and lignin (0.49–0.75) may again be associated with their low concentrations and small ranges in the plant material analysed (Albrecht *et al*. 1987*a*). The precision of chemical analysis for lignin, arabinose, and rhamnose was low, as indicated by much higher SEL values (% of the mean) than for *in vitro* DOMD. These errors are incorporated into the NIRS errors of calibration (SEC) and prediction (SEP). The accuracy of NIRS determination was high for all chemical fractions, as indicated by a slope close to 1.00 and small bias errors as a percentage of the mean.

In vitro DOMD in this experiment was determined *in vitro* with a pepsin-cellulase technique. An earlier study by Li *et al*. (1992) indicated that the pepsin-cellulase technique was not suitable for prediction of *in vivo* DM digestibility or digestible OM in DM of senesced subterranean clover cv. Junee, Persian clover var. *resupinatum* cv. Kyambro, and Zodiac (*Medicago*

murex Wild.) because of differences between *in vitro* and *in vivo* values. However, estimation of true digestibility is not the primary application of *in vitro* techniques. Rather, these techniques are for predicting relative digestibility differences among a wide range of forage species and types, particularly when forage samples are small (Marten 1981).

Differences between species groups in chemical composition and digestibility

Temperature regime influenced all parameters measured due to its effects on rate of growth and maturation. Thomas *et al*. (2010) showed that generally when plants approach senescence, some carbohydrates are lost during wilting or maturing, which makes the nitrogen content higher at senescence than at flowering. However, some lines of Persian clover var. *resupinatum* type had higher CP contents at senescence than at flowering. One explanation is that some plants deposit more carbohydrates or other non-nitrogen components as they approach flowering, which dilutes nitrogen content.

Lignin content increased with maturity in the present study, which is in agreement with Nordkvist and Aman (1986) and Buxton (1989). Lignin adds rigidity to cell wall structure (Van Soest 1994). It is well documented that lignin is negatively associated with nutrient digestibility (Buxton 1989; Hornstein *et al*. 1989; Grabber 2005; Hatfield *et al*. 2009). Similar results were obtained in this study. The inhibitory effect of lignin is thought to be mediated by covalent bonding between lignin and hemicelluloses (Morrison 1980) and the physical separation of appropriate enzymes or microorganisms from cellulose (Grabber 2005).

Hemicellulosic monosaccharide components play a dominant role in determining the digestibility of lucerne (Nordkvist and Aman 1986; Hornstein *et al*. 1989) and red clover (Hornstein *et al*. 1989). In the present study, arabinose and galactose decreased with plant maturity in all species groups, which agrees with results reported by Buxton *et al*. (1987) and Hornstein *et al*. (1989).

Mannose content increased with maturity, as previously reported by Nordkvist and Aman (1986) and had a negative relationship with *in vitro* DOMD as previously reported by Buxton *et al*. (1987). The increase in rhamnose content with maturity is in contrast to the situation in lucerne stems and leaves reported by Albrecht *et al*. (1987*b*). They found that rhamnose concentration remained fairly constant with maturation. The difference may be due to the differences in the forage species tested, particularly with lucerne being a perennial species.

The increase in xylose content with maturity was similar to that observed in lucerne and red clover (Albrecht *et al*. 1987*a*; Buxton *et al*. 1987). The negative association of xylose content with *in vitro* DOMD was also similar to results obtained for lucerne (Nordkvist and Aman 1986; Buxton 1989; Hornstein *et al*. 1989), red clover (Hornstein *et al*. 1989), and grasses (Burritt *et al*. 1984).

The arabinose : xylose ratio decreased with maturity as arabinose levels declined and xylose levels rose. Similar results were reported by Burritt *et al*. (1984), Albrecht *et al*. (1987*a*), Buxton *et al*. (1987), and Wedig *et al*. (1987). Wedig *et al*. (1987) concluded that a low arabinose : xylose ratio is characteristic of feeds with low digestibility. The positive association of the arabinose : xylose ratio with *in vitro* DOMD supports the reports of Wedig *et al*. (1987) with lucerne and orchard grass. However, it contrasts with results of Brice and Morrison (1982), who found a negative association between arabinose : xylose ratio and hemicellulose digestibility in senesced ryegrass.

Stem tissue was more digestible than was leaf tissue in subterranean clover cv. Junee. Similar results were observed with subterranean clover cvv. Woogenellup and Yarloop (McIvor and Smith 1973) and cvv. Clare and Trikkala (Stockdale 1992*a*). The finding that stem was more digestible than leaf fractions of subterranean clover contrasts with results for lucerne (Wilman and Altimimi 1984; Nordkvist and Aman 1986; Albrecht *et al*. 1987*b*), red clover (Wilman and Altimimi 1984; Nordkvist *et al*. 1987), white clover (Wilman and Altimimi 1984), and grasses (Poppi *et al*. 1980). Furthermore, the differences in digestibility between leaf and stem increased with maturity, which was similar to results reported by Stockdale (1992*a*).

It was also found that stem tissue was more digestible than leaf in Persian clover var. *majus*, which has an erect growth habit. This greater digestibility of stems than leaves in erect plants does not appear to have been documented before. It is not clear why these erect plants have similar characteristics to prostrate plants in term of digestibilities of stem and leaf. However, var. *majus* types tend to have hollow, thicker stems and larger leaves than do var. *resupinatum* types.

Conclusions

A higher temperature regime resulted in plants reaching respective phenological stages earlier and also had some impact on relative chemical composition. Lignin and xylose contents were shown to influence the digestibility of annual legumes. Genotype selection for long stems should increase DM yield and selection for a high proportion of leaves, and low lignin and xylose concentrations should increase the nutritive value of annual legumes. Persian clover var. *majus* type and balansa clovers were evidently superior to Junee subterranean clover in terms of higher in *in vitro* DOMD, CP, and arabinose and lower in lignin, cellulose, NDF, and xylose contents than was Junee subterranean clover when green and when senesced. Nonetheless, it possible that the ranking observed under the controlled environmental conditions of these experiments may differ from that under field conditions. In this context, future field testing of the cultivars is warranted. Finally, NIRS calibration and validation equations for *in vitro* DOMD, OM, CP, NDF, cellulose, and xylose with small standard errors and large r^2 indicate that NIRS can be used as an accurate, cost-effective, and fast non-destructive tool for routine analyses.

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