*agronomy*

MDPI

Article

# Variation for Concentrations of Various Phytoestrogens and Agronomic Traits Among a Broad Range of Red Clover (*Trifolium pratense*) Cultivars and Accessions

**Valerie Little [1], Kevin F.M. Reed [1,3] and Kevin F. Smith [2,*]**

1 Agriculture Victoria, Private Bag 105 Hamilton, Victoria 3300, Australia; valerie.little@ecodev.vic.gov.au
2 Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Private Bag 105 Hamilton, Victoria 3300, Australia
3 Reed Pasture Science, Brighton East, Victoria, Australia; rps@eftel.net.au
* Correspondence: kfsmith@unimelb.edu.au

Academic Editor: John W. Forster
Received: 15 September 2016; Accepted: 2 May 2017; Published: 5 May 2017

**Abstract:** Agronomic characteristics and phytoestrogen concentrations were measured on 17 cultivars and 47 accessions of red clover (*Trifolium pratense*). These accessions included a range of currently recommended cultivars—from Australia and overseas—and germplasm accessed from genetic resource collections. All lines were grown in the field at Hamilton Vic in 2000 and 2001. Significant genetic variation was detected for key agronomic parameters such as growth habit, leaf shape and markings, leaf area, herbage yield, flowering time, and prolificacy. Significant variation in the concentration of the four main phytoestrogens was found; total isoflavone concentration ranged from 0.14–1.45% DM. Maximum concentrations of daidzein, genistein, formononetin, and biochanin were 0.06, 0.08, 0.86, and 0.91% DM respectively. Multivariate analysis showed that the accessions grouped into 10 distinct clusters that had between 1 and 10 members. Several accessions were superior to existing cultivars—notably Mediterranean accessions with regard to cool season vigour—and valuable for breeding programs to develop high yielding cultivars with either high (for possible medicinal purposes) or low (for grazing) phytoestrogen concentrations.

**Keywords:** red clover; variability; phytoestrogen

## 1. Introduction

Red clover (*Trifolium pratense* L.) is a short-lived perennial legume [1] with a strong tap root. The optimum temperatures for productivity in red clover are between 20 °C and 25 °C [2] and has been widely sown in temperate regions of the former Soviet Union, Argentina, Chile, Columbia, Mexico, USA, Canada, Japan, Australia, and New Zealand [2]. Generally, although the plant is quite frost tolerant, herbage production in winter is poor. However, some cultivars that have been bred by crossing superior New Zealand varieties with Mediterranean genotypes have shown improved winter production [3]. The species is seen as an alternative to the more widely sown white clover and lucerne in Australia and New Zealand but the need exists for further breeding and development of adapted cultivars [4,5].

When consumed by ruminants, oestrogenic isoflavones in red clover can result in the production of equol, an oestrogenic substance that may impair reproductive processes. If exposed to Hamua red clover, ewe weaners rapidly exhibit teat elongation [6] and ewes grazing red clover exhibit irregular oestrus, cystic ovaries, and persistent glandular cysts affecting both the cervix and the endometrium. Prolonged grazing on oestrogenic pastures for several years may result in permanent and progressive infertility in ruminants [7]. To this end, low phytooestrogen cultivars have been developed in Australia to provide safe grazing for sheep—e.g., Redwest and Redquin, selected from Hamua and Quinequeli, respectively [8]. In Australia, the cultivars Genstar and Genstar Null have been selected for high isoflavone concentrations for pharmaceutical use [9].

While the effects of red clover oestrogens on the reproduction of sheep and cattle are largely negative, red clover extracts have been the subject of research for their application in human medicine, especially in the treatment of menopausal symptoms in women [10]. Oestrogen plays an important role in the human body and when oestrogen levels decrease with the onset of menopause, negative physical effects can occur. Phytoestrogens are considered promising for preventing degenerative diseases such as osteoporosis that are associated with oestrogen deficiency [11] although further research is required to validate the role of red clover and red clover-derived phytoestrogens in the treatment of a range of menopausal symptoms [12–14].

There is significant inter-varietal variation for oestrogenic isoflavone content in red clover [15]. Identification of material with a high concentration of phytoestrogens or desirable ratios of specific oestrogens, combined with good agronomic performance, could progress the genetic improvement of red clover for pharmaceuticals.

Various genotypes of red clover have been grouped by the use of cluster analysis for quantitative and qualitative characters. Kouame and Quesenberry [16] evaluated more than 800 accessions of red clover that represented 41 countries of origin. Great diversity was detected among accessions from Eastern and Northern Europe, providing a structure for identifying a limited number of core accessions in order to represent a large collection [16].

A set of red clover cultivars and accessions was grown out to characterise the accessions for agronomic performance and biochemical characters. The material was selected to represent a broad range of geographic originswith the aim of identifying new sources of germplasm for hybridization to existing cultivars for future varietal development.

## 2. Results

### *2.1. Range and Variance of Agronomic Traits*

There were significant genetic effects for all traits, for example visual assessment of winter dry matter yield varied from 2.0 to 4.9 (on a 1–10 scale) and leaf width varied from 13.7 mm to 22.2 mm (Table 1). The proportion of variance expressed by genetic effects was large, ranging from 0.97 for flowering time to 0.45 for stem colour. Variance components for all traits were significant (greater than twice the standard error) except in the case of leaf shape (Table 1). Therefore, a large proportion of variation observed in this experiment was due to genetic effects. The mean and range of characteristics for each trait across all entries are presented in Table 1. There was a range for leaf markings present in the accessions that varied from no markings to a full, intermediate V mark [17] —data not shown.

**Table 1.** Mean and range of characterisation measurements of red clover accessions. ($\sigma^2_g$ = genetic variation, s.e = standard error; R = repeatability).

| Trait | Range | Mean | $\sigma^2_g \pm$ s.e | R |
|---|---|---|---|---|
| Autumn Recovery (Aut rec) | 0.95–2.68 | 1.704 | 0.214 ± 0.044 | 0.694 |
| Flowering Time (FT) | 28.0–83.2 | 50.320 | 136.9 ± 25.60 | 0.877 |
| Growth Habit, Spring/Summer (gh.ss) | 2.19–5.84 | 3.976 | 1.580 ± 0.290 | 0.967 |
| Leaf Shape (leaf sh) | 1.10–2.40 | 2.166 | 0.035 ± 0.018 | 0.467 |
| Flowering Prolificacy (prolificacy) | 3.02–5.92 | 4.789 | 0.520 ± 0.104 | 0.820 |
| Stem Colour (stem) | 1.10–2.53 | 1.780 | 0.089 ± 0.033 | 0.447 |
| Stipule Pigmentation (stipule) | 1.50–3.73 | 2.278 | 0.314 ± 0.088 | 0.632 |
| Cool Season Yield (DM, g/plant) (winter) | 1.97–4.94 | 2.938 | 0.411 ± 0.086 | 0.734 |

**Table 1.** *Cont.*

| Trait | Range | Mean | $\sigma^2_g \pm$ s.e | R |
|---|---|---|---|---|
| Leaf Width (mm) (leaf w) | 13.66–22.16 | 17.085 | 4.08 ± 1.11 | 0.658 |
| Leaf Length (mm) (leaf l) | 17.87–28.85 | 22.397 | 9.20 ± 2.66 | 0.638 |
| Leaf Area (cm$^2$) (leaf a) | 1.56–2.44 | 1.944 | 0.063 ± 0.018 | 0.589 |
| Biochanin (g/kg DM) (Bioch) | 0.09–0.91 | 0.480 | 0.021 ± 0.01 | 0.656 |
| Daidzein (g/kg DM) (Diadz) | 0.003–0.060 | 0.010 | 0.0001 ± 0.0 | 0.791 |
| Formononetin (g/kg DM) (Form) | 0.06–0.86 | 0.354 | 0.011 ± 0.01 | 0.588 |
| Genistein (g/kg DM) (Genist) | 0.001–0.076 | 0.017 | 0.0001 ± 0.001 | 0.374 |
| Total Isoflavones (g/kg DM) | 0.14–1.45 | 0.865 | 0.051 ± 0.028 | 0.670 |

The BLUP estimates for agronomic and biochemical traits across red clover lines are presented in Tables 2 and 3. The data demonstrate considerable variation for most traits. For example, the BLUP estimates for flowering time ranged from 28 days from the commencement of data collection (SA 19676) to 83.2 days after the commencement (M 98) ($p < 0.05$). Variation in leaf width was also evident with the smallest (SA 20017) being 38 % smaller at 13.7 mm than the largest (A 9086) at 22.2 mm ($p < 0.05$). Recovery of plants in autumn was closely correlated with cool season vigour which varied markedly; 17 accessions ranked ahead of the best cultivar (PAC 19, Chile). Accessions from Morocco, Tunisia, Sardinia, and Turkey were superior for cool season vigour with yield increases up to 55% above the best cultivar. Variation was also present for biochemical traits with BLUP estimates for biochanin content ranging from 0.09% (SA 32.374) to 0.91% DM (S 36) and total isoflavone content ranging from 0.14% to 1.45% DM.

**Table 2.** Mean effects of agronomic traits across red clover accessions.

| Line No. | Cultivar/ Accession | Origin | Autumn Recovery | Flowering Time (days) | Growth Habit Spring/ Summer | Leaf Shape | Prolificacy of Flowering | Stem Colour | Winter DM Yield (kg) | Leaf Width (mm) | Leaf Length (mm) | Total Leaf Area ($log_e$ $cm^2$) | Stipule Pigmentation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | PAC 19 | Chile | 1.94 | 71.9 | 2.47 | 2.12 | 4.12 | 1.53 | 6.58 | 19.6 | 24.3 | 2.19 | 2.04 |
| 2 | A9807 | Unknown | 1.71 | 49.1 | 2.54 | 1.89 | 4.86 | 1.68 | 5.23 | 17.8 | 24.6 | 2.19 | 1.76 |
| 3 | Rajah | Denmark | 1.51 | 71.7 | 4.73 | 2.24 | 4.79 | 1.59 | 3.90 | 14.6 | 17.9 | 1.61 | 1.62 |
| 4 | SA 26.449 | Turkey | 2.13 | 45.3 | 4.95 | 2.24 | 4.73 | 1.59 | 7.41 | 15.8 | 19.6 | 1.78 | 2.09 |
| 5 | PI 4379 | Unknown | 1.60 | 43.3 | 3.09 | 2.16 | 5.41 | 1.87 | 5.78 | 17.3 | 23.7 | 2.11 | 2.33 |
| 6 | SA 20.017 | Turkey | 1.86 | 30.4 | 5.45 | 2.16 | 4.65 | 1.72 | 6.10 | 13.7 | 18.0 | 1.58 | 2.92 |
| 7 | PI 4377 | Unknown | 1.56 | 56.6 | 3.03 | 2.28 | 5.10 | 1.66 | 5.08 | 16.5 | 21.6 | 1.88 | 1.87 |
| 8 | S48/9732 | Turkey | 1.75 | 45.4 | 5.61 | 2.15 | 4.65 | 1.75 | 6.07 | 16.1 | 21.3 | 1.89 | 2.67 |
| 9 | El Sureno | Argentina | 0.95 | 43.2 | 2.76 | 2.15 | 5.31 | 1.66 | 4.36 | 19.5 | 25.8 | 2.23 | 2.41 |
| 10 | SA 758 | Morocco | 2.68 | 69.7 | 5.34 | 2.12 | 3.24 | 1.72 | 7.95 | 17.6 | 22.8 | 2.14 | 3.37 |
| 11 | Renegade | USA | 1.67 | 43.7 | 2.53 | 2.11 | 4.93 | 1.93 | 5.37 | 17.6 | 24.9 | 2.05 | 2.04 |
| 12 | H: 4949 | Unknown | 1.67 | 51.2 | 3.06 | 2.24 | 5.76 | 1.55 | 6.33 | 17.6 | 22.5 | 2.09 | 1.76 |
| 13 | Krano | Denmark | 1.82 | 46.8 | 2.81 | 2.06 | 5.27 | 1.62 | 5.67 | 17.5 | 22.8 | 1.99 | 1.82 |
| 14 | PI 5290 | Unknown | 1.47 | 52.7 | 2.95 | 2.14 | 5.35 | 2.02 | 5.92 | 18.3 | 23.6 | 1.91 | 2.39 |
| 15 | T 106 | Tunisia | 2.54 | 46.4 | 4.91 | 2.06 | 4.71 | 2.53 | 9.88 | 17.6 | 23.8 | 2.15 | 3.08 |
| 16 | SA 15.891 | Turkey | 1.74 | 71.5 | 4.45 | 2.14 | 3.43 | 1.89 | 5.29 | 14.2 | 19.1 | 1.56 | 2.43 |
| 17 | H: 7565 | Unknown | 1.25 | 56.5 | 3.46 | 2.35 | 5.11 | 1.73 | 5.41 | 17.4 | 21.2 | 1.97 | 2.13 |
| 18 | SA 22.825 | UK | 1.25 | 42.2 | 4.22 | 2.17 | 5.53 | 1.63 | 5.11 | 15.5 | 21.7 | 1.85 | 1.78 |
| 19 | Cherokee | USA | 1.42 | 38.3 | 2.90 | 2.11 | 5.14 | 1.62 | 5.89 | 17.5 | 24.1 | 2.05 | 1.84 |
| 20 | SA 32.382 | Turkey | 1.92 | 34.7 | 4.99 | 2.13 | 4.45 | 1.80 | 6.42 | 15.7 | 20.2 | 1.73 | 2.61 |
| 21 | S42/9635 | Turkey | 2.27 | 66.2 | 5.64 | 2.02 | 4.37 | 2.34 | 6.91 | 16.1 | 22.0 | 1.94 | 3.19 |
| 22 | M 98 | Morocco | 2.75 | 83.2 | 5.65 | 2.11 | 3.39 | 1.73 | 8.64 | 17.2 | 22.1 | 2.05 | 2.93 |
| 23 | SA 32.377 | Turkey | 2.02 | 48.3 | 5.23 | 2.28 | 4.17 | 1.85 | 6.87 | 17.4 | 22.5 | 2.05 | 2.02 |
| 24 | SA 21.963 | Uruguay | 1.40 | 36.9 | 2.63 | 2.04 | 5.35 | 1.76 | 5.75 | 17.5 | 25.7 | 2.16 | 2.20 |
| 25 | S36 | Sardinia | 2.48 | 55.2 | 5.71 | 2.10 | 3.79 | 1.97 | 8.53 | 15.9 | 20.5 | 1.82 | 2.48 |
| 26 | SA 901 | UK | 1.61 | 49.3 | 3.36 | 2.34 | 5.19 | 1.71 | 5.54 | 17.0 | 21.1 | 1.84 | 2.20 |
| 27 | SA 8.440 | Russia | 1.48 | 44.3 | 3.17 | 2.33 | 4.60 | 1.57 | 5.39 | 15.5 | 19.7 | 1.72 | 2.20 |
| 28 | Colenso | New Zealand | 1.34 | 58.0 | 2.89 | 2.28 | 5.31 | 1.59 | 5.08 | 17.5 | 22.2 | 2.08 | 1.91 |
| 29 | SA 32.376 | Turkey | 2.16 | 53.5 | 5.05 | 2.22 | 4.77 | 1.76 | 6.93 | 16.8 | 22.0 | 1.96 | 2.05 |
| 30 | SA 26.500 | Turkey | 2.05 | 46.0 | 4.93 | 2.15 | 4.66 | 2.08 | 6.72 | 16.3 | 24.3 | 2.05 | 2.66 |
| 31 | Hamidori | Japan | 1.50 | 60.4 | 2.78 | 2.26 | 4.54 | 1.71 | 4.60 | 15.7 | 18.9 | 1.67 | 1.50 |
| 32 | M 80 | Morocco | 2.64 | 66.9 | 5.48 | 2.10 | 3.75 | 1.70 | 9.17 | 16.7 | 21.8 | 2.12 | 3.73 |
| 33 | S44/9664 | Turkey | 1.93 | 40.9 | 5.54 | 2.08 | 5.31 | 2.29 | 6.68 | 16.1 | 22.2 | 1.88 | 2.89 |
| 34 | Quinqueli | Chile | 1.16 | 56.9 | 3.47 | 2.18 | 4.87 | 2.00 | 5.61 | 19.0 | 24.7 | 2.15 | 2.14 |
| 35 | SA 18.686 | Australia | 1.47 | 43.5 | 3.29 | 2.22 | 5.56 | 1.67 | 6.15 | 18.0 | 23.2 | 2.04 | 2.12 |
| 36 | SwisSelectn | Switzerland | 1.63 | 49.4 | 2.19 | 2.22 | 4.74 | 1.45 | 5.51 | 19.4 | 24.8 | 2.23 | 1.78 |
| 37 | S46/9679 | Turkey | 1.93 | 53.2 | 5.79 | 2.12 | 4.61 | 1.76 | 5.88 | 16.5 | 20.9 | 1.86 | 2.35 |
| 38 | M 154 | Morocco | 2.54 | 69.8 | 5.74 | 2.46 | 3.16 | 1.75 | 8.10 | 16.8 | 21.2 | 1.98 | 2.32 |
| 39 | A9806 | Turkey | 1.26 | 52.8 | 2.80 | 2.19 | 5.36 | 1.67 | 5.11 | 22.2 | 28.9 | 2.44 | 1.80 |
| 40 | Redquin | Australia | 1.45 | 45.1 | 2.82 | 2.22 | 5.42 | 1.71 | 4.91 | 19.8 | 24.4 | 2.14 | 1.79 |

**Table 2.** *Cont.*

| Line No. | Cultivar/Accession | Origin | Autumn Recovery | Flowering Time (days) | Growth Habit Spring/Summer | Leaf Shape | Prolificacy of Flowering | Stem Colour | Winter DM Yield (kg) | Leaf Width (mm) | Leaf Length (mm) | Total Leaf Area ($log_e$ $cm^2$) | Stipule Pigmentation |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 41 | Makimidori | Japan | 1.51 | 57.2 | 2.76 | 2.18 | 4.75 | 1.96 | 5.17 | 15.4 | 18.6 | 1.70 | 2.24 |
| 42 | SA 32.381 | Turkey | 2.10 | 49.8 | 5.18 | 2.18 | 4.05 | 1.55 | 7.32 | 17.7 | 24.2 | 2.20 | 2.46 |
| 43 | S47/9706 | Turkey | 1.87 | 57.3 | 5.54 | 2.16 | 4.69 | 2.31 | 6.20 | 14.1 | 18.3 | 1.59 | 2.64 |
| 44 | Concorde | Unknown | 1.52 | 53.6 | 2.99 | 2.21 | 5.23 | 1.82 | 4.56 | 15.5 | 20.0 | 1.80 | 1.78 |
| 45 | Maneta | France | 1.69 | 68.3 | 2.57 | 2.24 | 3.92 | 1.71 | 5.65 | 17.8 | 22.2 | 1.92 | 2.13 |
| 46 | SA 19676 | Afghanistan | 1.12 | 28.0 | 2.90 | 2.13 | 4.89 | 1.71 | 4.07 | 16.1 | 21.8 | 1.89 | 1.91 |
| 47 | Hamua | New Zealand | 1.21 | 53.0 | 2.92 | 2.07 | 5.92 | 1.59 | 4.95 | 16.7 | 22.7 | 1.95 | 2.36 |
| 48 | PI 4383 | Unknown | 2.10 | 64.6 | 3.53 | 2.00 | 3.02 | 1.86 | 6.38 | 17.4 | 23.5 | 2.07 | 2.38 |
| 49 | PI 4378 | Unknown | 1.14 | 51.6 | 2.85 | 2.32 | 5.28 | 1.83 | 5.39 | 16.9 | 22.2 | 1.99 | 1.94 |
| 50 | SA 22.000 | USA | 1.14 | 43.6 | 2.57 | 2.24 | 5.51 | 1.66 | 4.59 | 18.7 | 23.8 | 2.00 | 2.36 |
| 51 | SA 32.380 | Turkey | 1.19 | 41.6 | 3.83 | 2.07 | 5.19 | 1.59 | 4.93 | 17.2 | 24.3 | 2.09 | 2.03 |
| 52 | SA 32.374 | Turkey | 2.09 | 29.5 | 5.29 | 1.88 | 4.36 | 1.84 | 6.91 | 16.8 | 22.2 | 1.90 | 2.11 |
| 53 | S59/9895 | Turkey | 1.56 | 34.2 | 5.73 | 2.12 | 4.35 | 1.70 | 5.97 | 15.5 | 19.8 | 1.71 | 2.40 |
| 54 | SA 32.061 | Russia | 2.16 | 51.0 | 5.85 | 2.22 | 4.28 | 1.91 | 6.83 | 16.5 | 21.0 | 1.88 | 2.23 |
| 55 | FLMR7 | USA | 1.53 | 48.3 | 4.02 | 2.20 | 5.38 | 1.74 | 4.62 | 15.6 | 20.1 | 1.66 | 2.64 |
| 56 | LE 116 | Uruguay | 1.63 | 33.2 | 2.49 | 2.15 | 5.75 | 1.56 | 6.61 | 21.0 | 27.6 | 2.36 | 1.95 |
| 57 | PI 6435 | Unknown | 1.32 | 41.7 | 2.77 | 2.06 | 5.30 | 1.72 | 5.12 | 20.2 | 27.5 | 2.32 | 2.73 |
| 58 | Kenland | USA | 2.04 | 51.4 | 5.64 | 2.30 | 4.07 | 1.77 | 6.72 | 16.3 | 21.2 | 1.94 | 2.70 |
| 59 | Astred | Portugal | 1.28 | 43.2 | 2.70 | 2.40 | 5.30 | 1.74 | 5.12 | 18.0 | 22.7 | 2.04 | 1.78 |
| 60 | S49/9742 | Turkey | 0.99 | 40.9 | 2.88 | 2.01 | 5.46 | 2.05 | 4.89 | 17.4 | 24.9 | 2.16 | 2.33 |
| 61 | SA 12.274 | Turkey | 1.19 | 50.6 | 3.91 | 2.34 | 5.74 | 1.62 | 5.03 | 15.9 | 20.5 | 1.84 | 2.10 |
| 62 | P42 | Unknown | 1.90 | 55.2 | 5.51 | 2.08 | 5.00 | 2.03 | 5.95 | 15.4 | 20.1 | 1.84 | 3.13 |
| 63 | T 98 | Tunisia | 1.50 | 40.0 | 5.63 | 2.15 | 4.79 | 1.60 | 5.65 | 16.3 | 20.6 | 1.91 | 2.52 |
| 64 | 37796 | Unknown | 1.81 | 42.2 | 4.04 | 2.06 | 4.84 | 1.83 | 7.08 | 20.5 | 27.1 | 2.33 | 1.73 |
| S.e.d | | | 0.229 | 3.47 | 0.287 | 0.216 | 0.460 | 0.319 | 0.871 | 1.77 | 2.78 | 0.233 | 0.504 |

**Table 3.** Mean effects of isoflavone concentrations (%DM) across red clover accessions.

| Cultivar/ Line No. | Cultivar/Accession | Origin | Daidzein | Genistein | Formononetin | Biochanin | Total Isoflavone |
|---|---|---|---|---|---|---|---|
| 1 | PAC 19 | Chile | 0.011 | 0.04 | 0.59 | 0.62 | 1.26 |
| 3 | Rajah | Denmark | 0.009 | 0.01 | 0.67 | 0.53 | 1.25 |
| 4 | SA 26.449 | Turkey | 0.006 | 0.00 | 0.28 | 0.59 | 0.88 |
| 5 | PI 4379 | Unknown | 0.019 | 0.04 | 0.47 | 0.57 | 1.10 |
| 6 | SA 20.017 | Turkey | 0.007 | 0.03 | 0.31 | 0.62 | 0.97 |
| 7 | PI 4377 | Unknown | 0.014 | 0.02 | 0.68 | 0.63 | 1.34 |
| 8 | S48/9732 | Turkey | 0.008 | 0.05 | 0.33 | 0.33 | 0.71 |
| 9 | El Sureno | Argentina | 0.005 | 0.08 | 0.59 | 0.78 | 1.45 |
| 10 | SA 758 | Morocco | 0.008 | 0.03 | 0.13 | 0.10 | 0.27 |
| 11 | Renegade | USA | 0.014 | 0.02 | 0.48 | 0.50 | 1.01 |
| 13 | Krano | Denmark | 0.007 | 0.01 | 0.27 | 0.78 | 1.06 |
| 14 | PI 5290 | Unknown | 0.008 | 0.01 | 0.51 | 0.67 | 1.20 |
| 15 | T 106 | Tunisia | 0.060 | 0.01 | 0.29 | 0.17 | 0.53 |
| 16 | SA 15.891 | Turkey | 0.005 | 0.03 | 0.41 | 0.56 | 0.99 |
| 18 | SA 22.825 | UK | 0.010 | 0.01 | 0.32 | 0.82 | 1.16 |
| 19 | Cherokee | USA | 0.010 | 0.01 | 0.37 | 0.74 | 1.13 |
| 20 | SA 32.382 | Turkey | 0.012 | 0.00 | 0.34 | 0.47 | 0.82 |
| 21 | S42/9635 | Turkey | 0.007 | 0.01 | 0.44 | 0.54 | 0.99 |
| 22 | M 98 | Morocco | 0.006 | 0.00 | 0.02 | 0.13 | 0.17 |
| 23 | SA 32.377 | Turkey | 0.013 | 0.03 | 0.22 | 0.31 | 0.58 |
| 24 | SA 21.963 | Uruguay | 0.009 | 0.01 | 0.57 | 0.62 | 1.20 |
| 25 | S36 | Sardinia | 0.009 | 0.02 | 0.16 | 0.91 | 1.09 |
| 26 | SA 901 | UK | 0.003 | 0.01 | 0.29 | 0.76 | 1.06 |
| 27 | SA 8.440 | Russia | 0.008 | 0.01 | 0.23 | 0.45 | 0.68 |
| 28 | Colenso | New Zealand | 0.007 | 0.02 | 0.56 | 0.71 | 1.30 |
| 29 | SA 32.376 | Turkey | 0.012 | 0.01 | 0.39 | 0.39 | 0.80 |
| 30 | SA 26.500 | Turkey | 0.014 | 0.01 | 0.28 | 0.27 | 0.57 |
| 32 | M 80 | Morocco | 0.006 | 0.00 | 0.58 | 0.07 | 0.14 |
| 33 | S44/9664 | Turkey | 0.008 | 0.05 | 0.19 | 0.37 | 0.62 |
| 34 | Quinqueli | Chile | 0.005 | 0.01 | 0.62 | 0.66 | 1.23 |
| 35 | SA 18.686 | Australia | 0.004 | 0.01 | 0.25 | 0.78 | 1.05 |
| 37 | S46/9679 | Turkey | 0.006 | 0.01 | 0.35 | 0.44 | 0.80 |
| 38 | M 154 | Morocco | 0.008 | 0.00 | 0.07 | 0.18 | 0.27 |
| 42 | SA 32.381 | Turkey | 0.009 | 0.01 | 0.31 | 0.35 | 0.67 |
| 43 | S47/9706 | Turkey | 0.018 | 0.03 | 0.37 | 0.23 | 0.65 |
| 46 | SA 19676 | Afghanistan | 0.005 | 0.00 | 0.08 | 0.23 | 0.31 |
| 47 | Hamua | New Zealand | 0.005 | 0.01 | 0.52 | 0.70 | 1.23 |
| 49 | PI 4378 | Unknown | 0.026 | 0.03 | 0.55 | 0.57 | 1.17 |
| 51 | SA 32.380 | Turkey | 0.007 | 0.02 | 0.17 | 0.34 | 0.53 |
| 52 | SA 32.374 | Turkey | 0.013 | 0.05 | 0.30 | 0.21 | 0.57 |
| 53 | S59/9895 | Turkey | 0.004 | 0.01 | 0.09 | 0.09 | 0.19 |
| 54 | SA 32.061 | Russia | 0.010 | 0.01 | 0.27 | 0.49 | 0.78 |
| 55 | FLMR7 | USA | 0.006 | 0.00 | 0.28 | 0.50 | 0.79 |
| 56 | LE 116 | Uruguay | 0.009 | 0.01 | 0.86 | 0.51 | 1.39 |
| 58 | Kenland | USA | 0.007 | 0.04 | 0.12 | 0.19 | 0.36 |
| 59 | Astred | Portugal | 0.018 | 0.02 | 0.55 | 0.68 | 1.26 |
| 61 | SA 12.274 | Turkey | 0.004 | 0.01 | 0.56 | 0.72 | 1.29 |
| 62 | P42 | Unknown | 0.010 | 0.01 | 0.38 | 0.66 | 1.06 |
| 63 | T 98 | Tunisia | 0.013 | 0.00 | 0.25 | 0.16 | 0.43 |
| S.e.d | | | 0.011 | 0.340 | 0.231 | 0.271 | 0.408 |

### 2.2. *Principal Component Analysis of Phenotypic and Biochemical Variance among Red Clover Accessions*

The principal component plot (Figure 1) shows correlations between the various measured traits. Leaf length and width measurements tended to be positively correlated with leaf area. Lines such as number 44 (cv Concorde), which had long and wide leaves, had high prolificacy of flowering scores and higher total isoflavone concentrations. The biplot shows that there is the potential to identify lines with high concentrations of individual isoflavones and also with contrasting agronomic attributes.

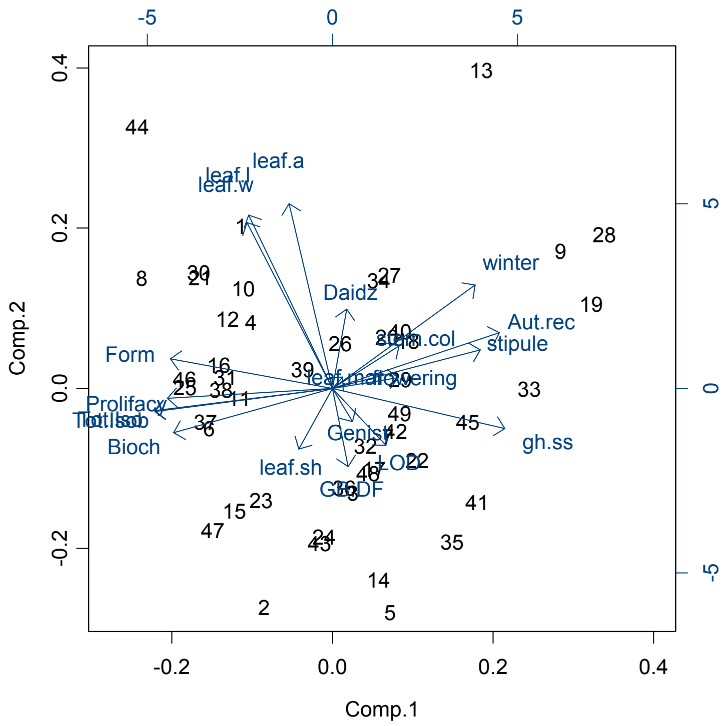


**Figure 1.** Principal component plot of biochemical and agronomic traits measured in 49 red clover cultivars/accessions. A description of the trait abbreviations used in this figure is given in Table 1.

### *2.3. Cluster Analysis of Red Clover Accessions*

Clustering of the data grouped the 64 accessions into ten distinct clusters that had between one and twenty accessions (Figure 2). There were three accessions (M 98, A 9806, FLMR 7) that did not satisfactorily cluster with any other accessions and therefore needed to be classed as separate clusters.

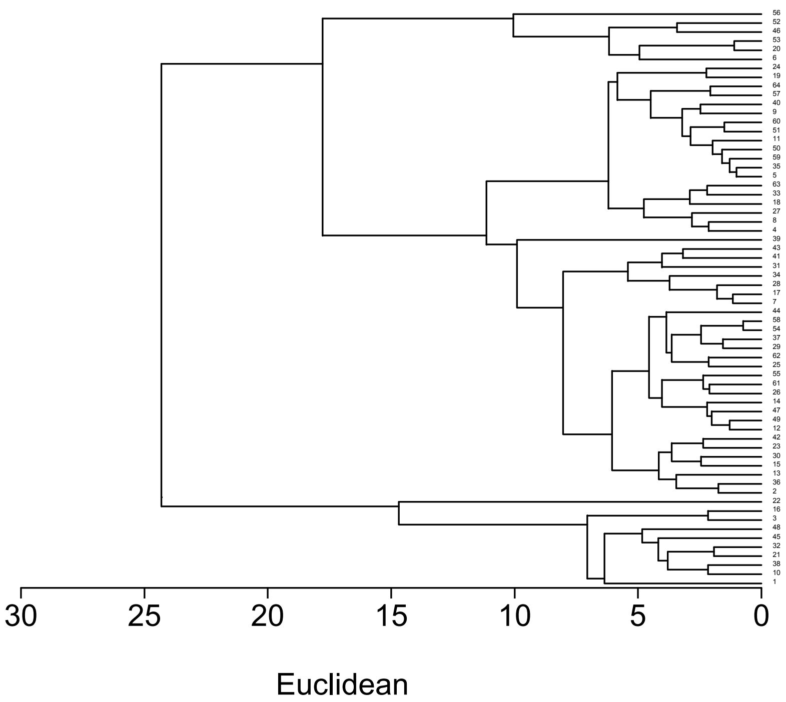


**Figure 2.** Clustering of 64 red clover accessions based on agronomic and phytoestrogen data.

## 3. Discussion

The traits measured in this experiment were similar to those descriptors used by Kouame and Quesenberry [12]. However, the addition of phytoestrogens provides valuable new insight into genetic variation for this important trait in the growth of red clover for pharmaceutical purposes. Many of the traits were highly correlated (Figure 1). For example, those accessions with long leaves such as A 9806, FLMR 7, LE 116, and SA 16658 tended also to have wide leaves and consequently had increased leaf area. However, there were some accessions such as SA 21963 and Renegade that had long but narrow leaves. Mediterranean accessions which displayed good autumn recovery in this experiment (e.g., S 42 and M 98) tended to have decreased flowering prolificacy. Plant breeders need to be mindful of this information when selecting material based on its autumn yield performance in isolation from other characteristics such as prolificacy of flowering.

There were several instances where accessions were found to have herbage yields that were superior to Astred, a popular cultivar commonly used in Australia and New Zealand. Astred is a stoloniferous cultivar selected from a Portuguese accession. It can reproduce through seed or vegetatively through stolons and daughter plants. In the absence of root diseases, Astred can be a most persistent variety; 55% ground cover has been measured after 16 years in Tasmanian pasture trials [18].

There are several different clustering approaches that have been successfully used to characterise plant accessions into similarity groups. Kouame and Quesenberry [16] used germplasm evaluation data from 800 accessions of red clover, which represented 41 countries of origin, and discovered large variations for most characters over all origins. They clustered the accessions using standardised values of 15 morphological and physiological descriptors and were able to identify three distinct groups that corresponded to early, medium, and late maturity groups. Different similarity groups were then identified within each of these three groups. This analysis revealed a large range of diversity among the red clover accessions over all origins, with the most diversity occurring in accessions collected from eastern and northern Europe, perhaps reflecting the history of breeding the species in those regions.

In this experiment, the 64 accessions evaluated fit into 10 distinct clusters. The accessions M 98, A9806, and FLMR 7 were not clustered to any other accessions and therefore were regarded as single clusters. There were several clusters however, where accessions were tightly linked to the commercial cultivars Astred, Colenso, and PAC 19. Accessions SA 23061 and SA 901 were tightly clustered together with Astred, while P1 4377 and P 42 were closely linked to Colenso, illustrating that these lines should be good sources of genetic variation similar to these commercially successful cultivars. The accessions linked closely with PAC 19 formed a cluster that was quite distinct from all other clusters. The mid-winter yield assessment confirmed the importance of Mediterranean material as a source of outstanding cool season vigour as has previously been emphasised in studies on white clover [19] and perennial grasses [20].

The fact that accessions clustered closely with the main cultivars, Astred and Colenso, shows that there are plants with similar characteristics that may serve as potential sources of resistance to diseases such as the root rot diseases that have limited the commercial use of red clover in Australia [21]. The study reported in this paper reflects a major characterization of red clover genetic resources under Australian conditions and may serve as a source of novel germplasm for future breeding and evaluation programs for either grazing or phytoestrogen production.

## 4. Materials and Methods

### 4.1. Germplasm

Over two years, 17 cultivars and 47 accessions were planted and characterized in the field. The cultivars and accessions and their country of origin are listed in Table 2.

### 4.2. Establishment

Seed of each of the 64 lines was sown in seedling trays filled with seed raising media. Seedlings were cultivated in unheated glasshouse with natural lighting for eight weeks and then removed from the glasshouse to harden outside prior to transplanting.

Seedlings were transplanted into a prepared seedbed in the field at Hamilton, Victoria (37°44′ S, 142°01′ E, alt. 200 m) in autumn 1999. Hamilton has a mean average annual rainfall of 703 mm. The site was finely cultivated with power harrows and fertilized with 200 kg/ha of a compound (NPK) fertilizer. The basalt-derived, duplex soil type at the experimental site was a clay-loam overlying heavy clay. Weed mat was laid over the prepared site and holes made at appropriate intervals for the plants.

### 4.3. Trial Design

The seedlings were transplanted into the field experiment as a row column design, latinised within columns. There were eight replicate blocks within which each entry was represented by a cell of four plants, randomised within the block design.

### 4.4. Plant Measurements

Plant measurements were taken over a two-year period for the following characters: growth habit (rating score 1 = erect, score 6 = prostrate), leaf markings and shape [22], leaf length, width and area, flowering time (days after November 1st 1999 when the plant had three or more fully opened flower heads, prolificacy of flowering (number of flowers per plant, rating score 1 = few, 9 = many), stem colour (score 1 = green, score 5 = red) and stipule pigmentation [22]. Recovery after summer drought was rated in autumn 2000 (1 = poor, 3 = good). Cool season vigour was assessed in July 2000; all plants were rated (1 = poor, 10 = good) and 10 plants (1 for each rating) were randomly selected from each block. These plants were cut at 50 mm above ground level and the harvested material dried and weighed in order to calibrate the visual score and so provide an estimate of the yield of dry matter for all plants. Samples for biochemical analysis were collected in October 2000 at hay cutting time. Two young, fully developed leaves were harvested from each plant and were bulked into three replicates.

### 4.5. Biochemical Analysis

The concentration of the phytoestrogens, daidzein, genistein, formononetin, and biochanin in leaves were determined for 49 of the cultivars/accessions in the analytical laboratories of Novogen Ltd., North Ryde, NSW. Isoflavones were analyzed using a modification of previously published methods [23,24]. Aliquots (10 mL) of alcohol extract from leaf material were mixed with 100 mL of glucuronidase. The mixture was incubated for 24 h at 37 °C after which it was extracted on a C-18 solid phase extraction column (Waters Pty. Ltd., Sydney, Australia). Isoflavones were eluted with 3 mL of methanol and 10 mL of the extract was injected into the high-performance liquid chromatography system. The high-performance liquid chromatography system consisted of a 25-cm, 5 nM, C-18 stationary phase column (Symmetry, Waters Pty. Ltd.) and a gradient acetonitrile/water mobile phase. The limit of detection of the assay for each of the isoflavones measured was 5 ng/mL. The interassay coefficient of variation (CV) was <15%.

### 4.6. Statistical Analysis

Data from the experiment were analysed using the method of residual maximum-likelihood (REML) [25] to derive best linear unbiased predictor (BLUP) estimates for each of the characteristics measured for each entry. To display the relationship among accessions and traits in the data (using BLUP estimates), a biplot graphical representation was used. For this a principal component analysis (PCA) was performed using S-Plus 2000 (MathSoft, Inc., Cambridge, MA, USA). The relationship between the accessions and the traits were displayed using a point-vector plot, with points representing accessions, and directional vectors representing traits. The angles between the vectors reflected the correlation structure among traits. By drawing a perpendicular line from the treatment points to the trait vectors, the trait measurements for the accessions can be compared with the average, which is represented by the origin. Equal scaling of the component axes was needed for accurate projection of the points onto the trait vectors. Finally, an agglomerative, hierarchical grouping technique was used with squared Euclidean distance as the dissimilarity measure, based on the BLUP estimates, was carried out using S-Plus 2000 (MathSoft, Inc. Cambridge, MA, USA).

## 5. Conclusions

Significant variation for agronomic and biochemical traits was found amongst accessions. In several instances, their yield attributes were superior to the current major cultivar used in Australia and New Zealand, Astred. Clustering helped to identify several red clover accessions that were tightly linked to commercial cultivars (viz. Astred, Colenso, and PAC 19) and a valuable source of variation for a genetic improvement program.

**Supplementary Materials:** The following are available online at www.mdpi.com/2073-4395/7/2/34/s1.

**Acknowledgments:** The authors wish to thank L. Huang and A.J. Husband from Novogen Ltd. for assistance with biochemical analyses.

**Author Contributions:** K.R. and K.S. conceived and designed the experiments; V.L. performed the experiments; K.S. and V.L. analyzed the data; K.S. wrote the paper.

**Conflicts of Interest:** The authors declare no conflict of interest.

## References

1. Smith, R.S. Red Clover (*Trifolium pratense*). In *Alternative Pasture Legumes 1993, Proceedings of the 2nd National Alternative Pasture Legumes Workshop, Coonawarra, Australia, 25–28 July 1993*; Michalk, D.L., Craig, A.D., Collins, W.J., Eds.; South Australian Research and Development Institute: Urrbrae, Australia, 1994.
2. Fergus, E.N.; Hollowell, E.A. Red Clover. *Adv. Agron.* **1960**, *12*, 365–436.
3. Clark, S.G.; Reed, K.F.M. Performance of Grassland Hamua × Moroccan red clover crosses in a marginal environment. In Proceedings of the 4th Australian Society of Agronomy Conference, Melbourne, Australia, August 1987; Australian Society of Agrononoy: Melbourne, Australia, 1987.
4. Moot, D.J. An overview of dryland legume research in New Zealand. *Crop Pasture Sci.* **2012**, *63*, 726–733. [CrossRef]
5. Nichols, P.G.H.; Revell, C.K.; Humphries, A.W.; Howie, J.H.; Hall, E.J.; Sandral, G.A.; Ghamkhar, K.; Harris, C.A. Temperate pasture legumes in Australia—Their history, current use, and future prospects. *Crop Pasture Sci.* **2012**, *63*, 691–725. [CrossRef]
6. Kenny, P.T.; Reed, K.F.M. Effects of pasture type on the growth and wool production of weaner sheep during summer and autumn. *Aust. J. Exp. Agric. Anim. Husb.* **1984**, *24*, 322–331. [CrossRef]
7. McDonald, M.F. Effects of plant oestrogens in ruminants. *Proc. Nutr. Soc.* **1995**, *NZ 20*, 43–51. [PubMed]
8. Oram, R.N. *Register of Australian Herbage Plant Cultivars*; CSIRO: Melbourne, Australia, 1990.
9. IP Australia. Plant Breeders Rights Database. IP Australia: Australian Government: Canberra. Available online: www.ipaustralia.gov.au/get-the-right-ip/plant-breeders-rights (accessed on 1 May 2017).
10. Dornstauder, E.; Jisa, E.; Unterrieder, I.; Krenn, L.; Kubelka, W.; Jungbauer, A. Estrogenic activity of two standardized red clover extracts (Menoflavon®) intended for large scale use in hormone replacement therapy. *J. Steroid Biochem. Mol. Biol.* **2001**, *78*, 67–75. [CrossRef]
11. Alimoradi, A.; Mahdizad, H.F. Effects of phytoestrogens on bone mineral density during the menopause transition: A systematic review of randomized controlled trials. *Climacteric* **2016**, *19*, 535–545.
12. Ghazanfarpour, M.; Sadeghi, R.; Roudsari, R.L.; Khorsand, I.; KhadivZedah, T.; Muoio, B. Red clover for treatment of hot flashes and menopausal symptoms: A systematic review and meta-analysis. *J. Obstet. Gynaecol.* **2016**, *36*, 301–311. [CrossRef] [PubMed]
13. Chen, M.N.; Lin, C.C.; Liu, C.F. Efficacy of phytoestrogens for menopausal symptoms: A meta-analysis and systematic review. *Climacteric* **2016**, *18*, 260–269. [CrossRef] [PubMed]

14. Clifton-Bligh, P.B.; Nery, M.L.; Clifton-Bligh, R.J.; Visvalingam, S.; Fulcher, G.R.; Byth, K.; Baber, R. Red clover isoflavones enriched with formonetin lower serum LDL cholesterol—A randomized, double-blind, placebo-controlled study. *Eur. J. Clin. Nutr.* **2015**, *69*, 134–142. [CrossRef] [PubMed]
15. Francis, C.M.; Quinlivan, B.J. Selection for formononetin content in red clover (Trifolium pratense). In *Plant Introduction, Breeding and Seed Production, Proceedings of the XII International Grassland Congress, Moscow, Soviet Union, 11–20 June 1974*; Organizing Committee of the International Grassland Congress: Moscow, Soviet Union, 1974; pp. 84–88.
16. Kouame, C.N.; Quesenberry, K.H. Cluster analysis of a collection of red clover germplasm. *Genet. Resour. Crop Evol.* **1993**, *40*, 39–47. [CrossRef]
17. Williams, W.M. Genetics and Breeding. In *White Clover*; Barker, M.J., Williams, W.M., Eds.; CAB International: Wallingford, UK, 1987.
18. Smith, R.S.; Bishop, D.J. Astred—A stoloniferous red clover. In Proceedings of the 17th International Grassland Congress, Palmerston North, New Zealand, 8–21 February 1993; Organising Committee International Grassland Congress: Palmerston North, New Zealand, 1993; pp. 421–423.
19. Reed, K.F.M.; Cade, J.W.; Williams, A.E. The significance of Mediterranean plant introductions for increasing the winter growth of pasture in the high rainfall areas of Victoria. In Proceedings of the 1st Australian Agronomy Conference, Lawes, Australia, April 1980; Wood, I.M., Ed.; Australian Society of Agronomy: Sydney, Australia, 1980; p. 238.
20. Reed, K.F.M.; Clement, S.L.; Feely, W.F.; Clark, B. Improving tall fescue for cool season vigour. *Aust. J. Exp. Agric.* **2004**, *44*, 873–881. [CrossRef]
21. Dear, B.S.; Moore, G.A.; Hughes, S.J. Adaptation and potential contribution of temperate perennial legumes to the southern Australian wheatebelt: A review. *Aust. J. Exp. Agric.* **2003**, *52*, 973–983. [CrossRef]
22. Cade, J.; Stapleton, P. *How to Identify Sub Clover Varieties. Agricultural Notes—Agdex 137/33*; Natural Resources and Environment: Victoria, Australia, 1984; pp. 1–4.
23. Setchell, K.D.; Welsh, M.B.; Lim, C.K. High-performance liquid chromatographic analysis of phytoestrogens in soy protein preparations with ultraviolet, electrochemical and thermospray mass spectrometric detection. *J. Chromatogr.* **1987**, *386*, 315–323. [CrossRef]
24. Franke, A.A.; Custer, L.J.; Cerna, C.M.; Narala, K. Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. *Proc. Soc. Exp. Biol. Med.* **1995**, *208*, 18–26. [CrossRef]
25. GenStat Committee. *GenStat®Release 7.1*; VSN International Ltd.: Oxford, UK, 2003.