Coumestrol content of lucerne under drought stress

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Abstract
The response of coumestrol to water stress was assessed in two lucerne cultivars 'Wairau' and 'Kaituna'. Coumestrol content of lucerne is of importance because its ingestion by ewes prior to and during the mating period can decrease ovulation rate. Some farmers take the conservative approach of removing animals from wilted lucerne during this period in case of elevated coumestrol, but the evidence for or against this practice is lacking. This experiment found that coumestrol content was 2.2 mg/kg DM in well-watered lucerne and was not elevated in wilted lucerne (1.3 mg/kg DM). After two weeks coumestrol in well-watered lucerne decreased to 0.95 mg/kg DM, while a slight increase to 3.0 mg/kg DM occurred in senesced plants but remained below the levels expected to affect ewe reproduction. This result indicates that farmers can safely graze ewes on wilted lucerne for mating.

Keywords
Medicago sativa, alfalfa, phyto-oestrogen, water stress, dryland.

Introduction
Lucerne (*Medicago sativa* L.) is an important crop in dryland pasture systems due to its long taproot and high water use efficiency (Tonmukayakul et al. 2009). This means that it is often exposed to conditions of moderate to severe water deficit in summer/autumn dry periods. These dry periods are typified by a lack of growth in grass pastures, leading to a reliance on lucerne, which due to a long tap root can reach water unavailable to many other pasture species. However, in prolonged dry periods, shallow soils, or situations where the soil has not fully recharged between droughts, lucerne can also become water stressed. These dry periods can continue into the mating period of sheep and wilting lucerne may be the only feed on offer to ewes.

Coumestrol is a phyto-oestrogen that can cause decreased ovulation rate, and thus decreased lambing rate, in ewes ingesting it during and prior to the mating period. Coumestrol levels above 25 mg/kg DM have been reported to affect ewe fecundity (Smith et al. 1979). Roots of soybean plants grown in hydroponic systems were found to have increased coumestrol in response to water stress (Tripathi et al. 2015). While fungal damage and aphid herbivory are known to cause elevated coumestrol (Loper and Hanson 1964; Loper 1968; Sherwood et al. 1970), whether or not lucerne produces coumestrol as a stress response to water deficit has not been determined. Despite this, some dryland sheep farmers avoid grazing lucerne for the mating period, however this neglects a valuable source of feed at a time when live weight gains are desired for increasing ovulation rate.

The aim of this experiment was to determine whether lucerne produced coumestrol in response to water stress. This experiment also tested whether recovery from water stress caused increased coumestrol levels, as unpublished data has shown a tendency for this to occur in field experiments.

Methods
Experimental design
This experiment took place in an 'Aluminex Glasshouse' at Lincoln University, Canterbury, New Zealand. The mean temperature in the glasshouse during the experiment was 18.5°C (range 15.1 – 24.9°C). There were three water stress treatments: well-watered, water stressed and re-watered to field capacity following wilting, and two harvest dates (21 September and 6 October 2016). Two cultivars of lucerne were used, a recent cultivar 'Grasslands Kaituna' and a cultivar from the 1980s 'Wairau'. Seeds from these cultivars were planted in two litre pots of inoculated potting mix nine months prior to the experiment and plants cut just above the crown every six weeks. Pots were arranged in a randomised block design with three blocks.

At the beginning of the experiment (26 August 2016), plants were cut and the soil was brought to field capacity by saturating for two hours followed by four hours of drainage. Every 48 hours, for two weeks, each pot was watered back to field capacity. After two weeks of re-growth, watering was ceased for the water-stressed and re-watered treatments, and maintained for the well-watered plants. When the plants were wilted without recovery to turgidity overnight randomly selected pots (n = 3) of the well-watered and wilted treatments were harvested from each block.

Following the first harvest (21 September 2016) the re-watered treatment was watered to and maintained at field capacity. Fifteen days later the well-watered, water stressed, and re-watered treatments were harvested (6 October 2016).

Data collection
At each harvest the middle leaflet from the first fully expanded leaf was taken from two stems per pot. The leaflets were weighed together to give fresh weight (FW) and submerged in water for 24 hours in the dark. The surfaces of the leaflets were dried and the turgid weight (TW) of the leaf was then measured. The leaflets were oven dried and the dry weight (DW) recorded. The relative water content (RWC) of the leaflets was then calculated: RWC (%) = (FW-DW) / (TW-DW) × 100. Disease symptoms were assessed using the criteria from James (1971) and plants were harvested to 30 mm height. Material was oven dried at 60°C, ground through a 1 mm sieve and 0.5 g extracted overnight in 5 mL methanol. Coumestrol content was measured by HPLC with the methodology described in Fields et al. (2016).

Statistical analysis
Statistical analyses were performed in Genstat 16.1 with a balanced analysis of variance (ANOVA). Fisher's protected least significant difference (LSD) *post hoc* test was used to separate means when the ANOVA was significant ($\alpha = 0.05$). The standard error of the mean (SEM) is presented where mean data are reported in the format 'mean ± SEM'.

Results
Water stressed vs. well-watered lucerne
Wilting occurred in all plants in the water stressed treatment on 21 September 2016, twelve days after watering had ceased. Two weeks later, on 6 October 2016, the above-ground material of the water stressed plants had senesced.

RWC was affected by an interaction ($P < 0.001$) between treatment and harvest date. RWC did not change ($P = 0.449$) in well-watered plants between the two harvest dates (mean of 86 ± 1.2%) and was higher ($P < 0.001$) than the RWC of water stressed plants. The RWC of the water stressed plants decreased ($P < 0.001$) from 49 ± 5.0% on 21 September to 25 ± 1.1% on 6 October 2016.

Coumestrol content was affected by an interaction ($P = 0.001$) between treatment and harvest date (Figure 1). At the first harvest there was no difference ($P = 0.105$) in coumestrol content between well-watered and water stressed plants. Between the first and second harvests, coumestrol increased ($P = 0.005$) in water stressed plants from 1.3 ± 0.43 mg/kg DM at the first harvest date, where plants were wilted but green, to 3.0 ± 0.57 mg/kg DM at the second harvest date when foliage had senesced. On the other hand, coumestrol content of well-watered plants decreased ($P = 0.034$) from 2.2 ± 0.59 mg/kg DM to 0.95 ± 0.107 mg/kg DM between harvests. At the second harvest, senesced plants had a higher ($P = 0.002$) coumestrol content than well-watered plants.

Coumestrol content was also affected by an interaction ($P = 0.010$) between treatment and cultivar. Water stressed 'Wairau' had higher ($P = 0.006$) coumestrol (2.8 ± 0.71 mg/kg DM) than well-watered 'Wairau' (1.1 ± 0.15 mg/kg DM) and was higher ($P = 0.038$) than stressed 'Kaituna' (1.6 ± 0.44 mg/kg DM) but was not different ($P = 0.220$) to well-watered 'Kaituna' (2.1 ± 0.62 mg/kg DM). There was no difference ($P = 0.324$) between well-watered and stressed 'Kaituna' (2.1 vs. 1.6 mg/kg DM). Light fungal symptoms (<1% of total leaf area affected) were observed in the well-watered lucerne at the second harvest, whereas no fungal symptoms were observed at the first harvest or in the water-stressed plants.

Re-watered treatment
After water-stressed plants were re-watered at the first harvest, RWC increased ($P < 0.001$) from 50 ± 4.3% to 79 ± 1.1% two weeks later. This increased RWC was lower than well-watered treatment plants (84 ± 2.1%) but was higher ($P < 0.001$) than the water stressed plants (25 ± 1.1%). Dry weight at the second harvest was 6.8 ± 2.25 g and was lower ($P < 0.001$) than in the well-watered plants with a dry weight of 9.1 ± 0.39 g. Dry weight of the water stressed plants was lowest ($P < 0.001$) at 4.4 ± 0.18 g.

The re-watered plants had an average coumestrol content of 1.1 ± 0.13 mg/kg DM which was not different ($P = 0.712$) from well-watered plants (0.95 ± 0.107 mg/kg DM), but was lower ($P = 0.002$) than water stressed plants (2.9 ± 0.63 mg/kg DM). There was no difference ($P = 0.513$) between re-watered ‘Kaituna’ and re-watered ‘Wairau’. Coumestrol of re-watered plants had not increased ($P = 0.691$) relative to the content of the water stressed plants two weeks prior (1.3 ± 0.43 mg/kg DM).

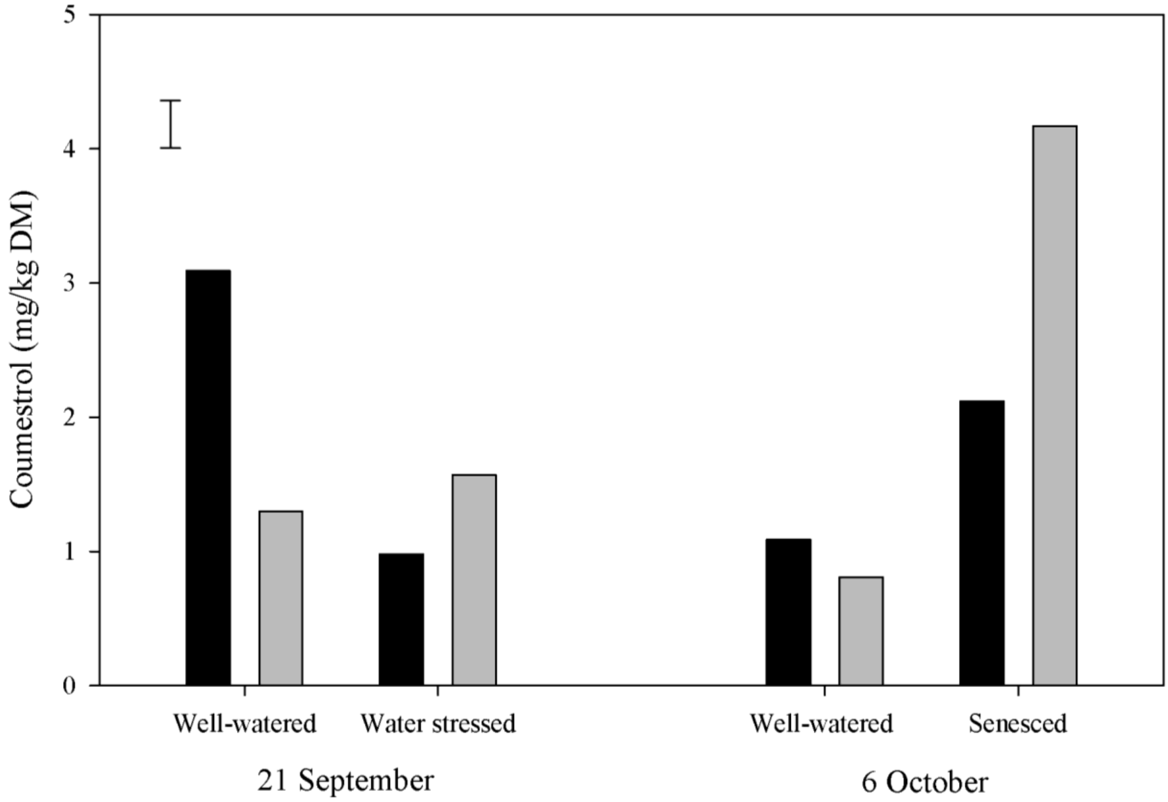

Figure 1. Coumestrol content (mg/kg DM) of glasshouse grown ‘Grasslands Kaituna’ (■) and ‘Wairau’ (■) lucerne under well-watered and water stressed treatments on 21 September 2016 and 6 October 2016. The error bar is the standard error of the mean for the interactions between cultivar × treatment and date × treatment.

Discussion
Coumestrol content was low across all plants, below the levels (ca. 25 mg/kg DM) reported (Smith et al. 1979) to affect ewe reproductive performance. This suggests drought stress alone should not be a cause for concern when mating sheep that are grazing on lucerne. When the lucerne was wilted but green, coumestrol content was not elevated relative to that of the well-watered plants. Severe drought conditions that resulted in foliage death did result in slight elevation of coumestrol content, from 1.3 to 3.0 mg/kg DM. The increase in coumestrol content of senesced lucerne may have been due to non-symptomatic fungal infection or a late stage stress response of the senesced plant cells. The older cultivar ‘Wairau’ accumulated more coumestrol than ‘Kaituna’ at senescence and overall the coumestrol content of water stressed ‘Kaituna’ was no different to the well-watered ‘Kaituna’.

Lucerne that was wilted and then re-watered recovered, albeit with less dry weight than the well-watered plants. The re-watered lucerne did not have increased coumestrol content relative to the well-watered plants. This confirms that the recovery of drought stressed plants after a simulated rainfall event did not cause increased coumestrol.

Conclusion

The results of this experiment indicate that water stress alone is not an important cause of increased coumestrol levels in lucerne crops. Even in senesced 'Wairau' plants, coumestrol levels were below those which would be a risk to reproductive performance of ewes. We contend that wilted lucerne which is only suffering from water stress, but otherwise disease and aphid free, can safely be used in sheep farming systems during mating.

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