Acta vet. scand. 1987, *28,* 255–262.

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Variations in the Content of Plant Oestrogens in Red Clover-Timothy-Grass during the Growing Season

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Kallela, K., I. Saastamoinen and E. Huokuna: Variations in the content of plant oestrogens in red clover-timothy-grass during the growing season. Acta vet. scand. 1987, 28, 255–262. – The investigation concerned variations in the plant oestrogen content of red clover-timothy swards receiving the same basic fertilization, but with different plant compositions and levels of nitrogen fertilizing.

The oestrogen content of pure red clover was high in the early spring and declining by midsummer. The oestrogen content in aftermath remained high and compared to the age of the growth was greater than that of the spring crop. The age of the aftermath from the preceeding harvest, as well as the time of harvesting affected the content.

The plant oestrogen content of red clover grown in the mixed swards was on average somewhat higher that from a pure red clover sward. The plant oestrogen content of a pure timothy sward was usually negligible.

Nitrogen fertilization diminished the share of red clover in the mixed swards and thus the plant oestrogen contents of those swards as well. The effect was very notable in the spring crop. The average content of aftermath was consistently higher than that of the spring crop.

Nitrogen fertilizer had a loowering effect on the estrogen content of pure red clover as well as on the percentage of crude protein in clover. The study found a strong correlation between the plant oestrogen and crude protein contents of red clover. Apparently both are due much to the same factors despite their divergent chemical compositions.

Changes occurring in the total plant oestrogen content are determined predominantly by alternations in the contents of formononetin and biochanin-A, which were usually parallel. Compared to these changes, the quantities and respective variations of daidzein and genistein were insignificant.

plant composition; nitrogen fertilization.

Introduction

In plants the quantity of oestrogens chiefly depends upon the plant species and variety but is also variable in the same plant during the growing season. It is generally high in the spring during the luxuriant growth period, however ample amounts of plant oestrogens have also been found in autumn aftermath (*Kallela* 1964). The formation of oestrogens is probably influenced by many factors such as for example climate, fertilization and soil. In Finland, the most commonly cultivated fodder plants are timothy and red clover. Oestrogenic activity in red clover is comparatively great, whereas timothy contains negligible amounts of plant oestrogens (*Kallela* 1974). Exactly how the quantities of plant oestrogens vary in plants during the course of the growing season has not yet been systematically shown. Therefore, it was considered well founded to investigate the variations during one growing season for the content of plant oestrogen in red clover-timothy swards with varying plant compositions and different levels of nitrogen fertilizing.

Materials and methods

The red clover-timothy swards studied were cultivated during the summer of 1984 at the South Savo Agricultural Research Station at Mikkeli (61°, 40′). The variety of red clover used was the Finnish tetraploid Tepa and the timothy variety the Finnish Tammisto.

The plant compositions to the swards were as follows:

1. pure red clover
2. clover-rich red clover-timothy; 14 kg red clover seed and 6 kg timothy seed per hectare were used for seeding the sward
3. clover-poor red clover-timothy; 6 kg clover and 14 kg timothy per hectare were used
4. pure timothy.

The basic fertilization was identical for all swards; for both harvests 400 kg/ha trace element enriched PK-fertilizer was applied (2-8-12). The timothy and mixed swards in addition received treatments of 0, 50 and 100 kg/ha nitrogen fertilizer for both the spring and aftermath harvests. Plant growth commenced on May 1. Yields were gathered weekly. The first moving of spring growths took place on May 29 when the plants were 4-weeks old and the last mowing was on July 10 when their corresponding age was 10 weeks.

Aftermath yields were gathered on July 31, and on August 7, 14 and 21, respectively. At the time of the first mowing, 8 and 9 weeks had elapsed from the spring mowings (previous mowing on May 29 and June 5), at the second, 7 and 8 weeks (June 12 and 19), at the third, 6 and 7 weeks (June 26 and July 3); and the last moving on August 21 took place 6 weeks from the previous mowing on July 10, 1984.

Immediately after mowing, representative samples for plant oestrogen determinations were collected from the swards. The samples were finely ground in a meat grinder, allowed to stand for 1/2 h at + 37°C in order for the plant oestrogens to hydrolyze (*Francis & Millington* 1965), then mixed into an ample amount of absolute alcohol and stored until analysis.

For the high performance liquid chromatographic determination the samples were filtered. From the filtrates the oestrogenic isoflavones formononetin, biochanin-A, genistein and daidzein were determined by an earlier described method (*Kallela & Saastamoinen* 1978) which was modified so that the conditions were as follows:

Equipment:	Perkin-Elmer 1220 liquid chromatograph UV/detector LC-55 Perkin-Elmer at 254 nm Sigma 10 lab data system
Pre column:	3 µm 3 cm C-18 258-0160
Column:	5 µm 10 cm HS-5 HCODS 258-0152 P-E
Eluent:	40 % acetonitrile in water
Flow rate:	1 ml/min
Temperature:	55°C
Chart speed:	5 mm/min
Pressure:	1500 PSI
Sample size:	2 µl

The fluorescent isoflavones (daidzein and formononetin) were also determined by a

LS-4 fluorometric detector. Commercial plant oestrogen preparations (K & K Laboratories, Inc.) served as standards.

Due to practical difficulties, coumestrol, the content of which is known to be negligible in the plants studied, if present at all, was not determined (*Kallela* 1964).

Results

The results are presented graphically in Figs. 1–5. The average plant oestrogen contents of pure red clover from all swards containing red clover is presented in Fig. 1. Thus the figure includes the pure red clover swards, the selected clover from the clover-rich red clover-timothy swards (fertilizations of 0, 50 and 100 kg N/ha), and the selected red clover from the clover-poor red clover-timothy swards (fertilizations of 0, 50 and 100 kg N/ha).

Fig. 2 illustrates the contents of the variously fertilized (0, 50 and 100 kg N/ha) clover-rich, Fig. 3 those of the clover-poor and Fig. 4 shows the average plant oestrogen content of the pure timothy swards. The average crude protein and plant oestrogen

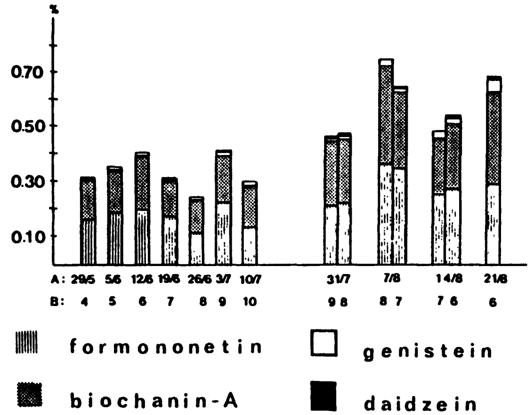

Figure 2. Average plant oestrogen content (% of DM) of cloverrich swards.
A = mowing date. B = crop age (in weeks).

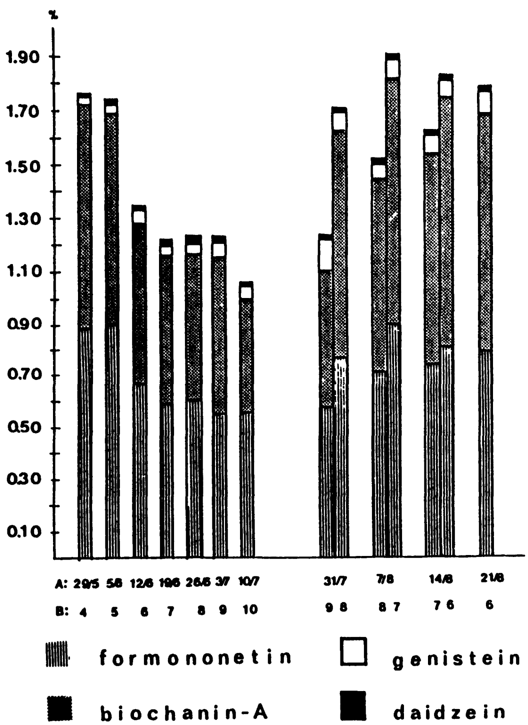

Figure 1. Average plant oestrogen content (% of DM) of all red clover samples.
A = mowing date. B = crop age (in weeks).

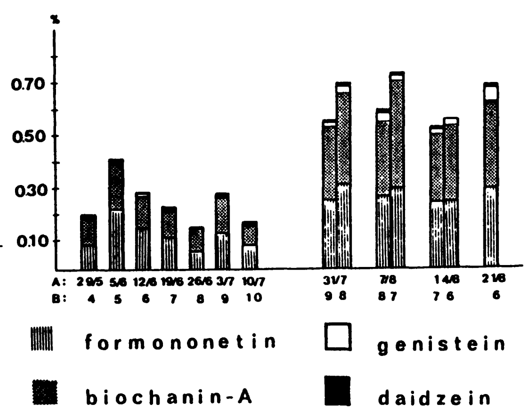

Figure 3. Average plant oestrogen content (% of DM) of cloverpoor swards.
A = mowing date. B = crop age (in weeks).

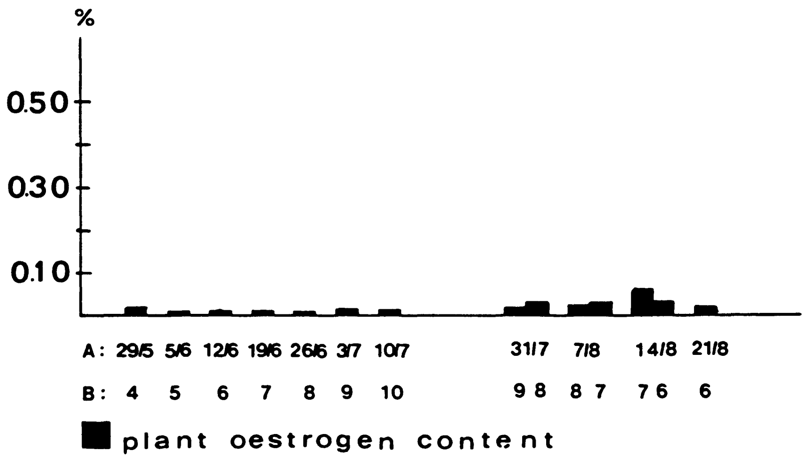

Figure 4. Average plant oestrogen content (% of DM) of pure timothy swards. A = mowing date. B = crop age (in weeks).

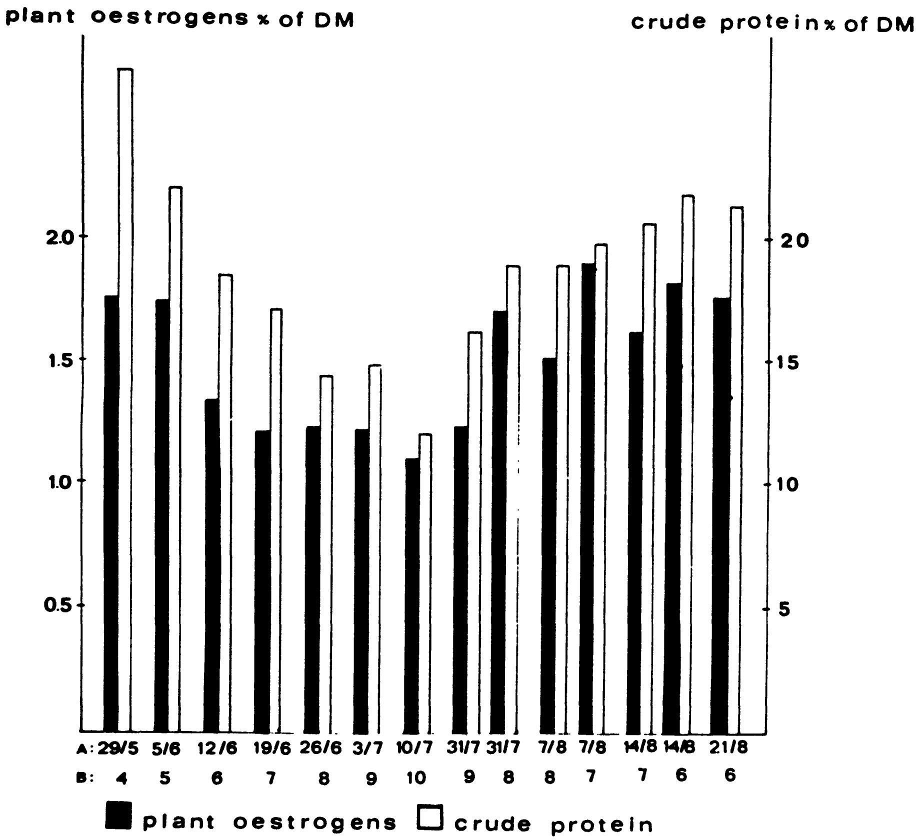

Figure 5. Average plant oestrogen and crude protein contents of all red clover samples. A = mowing date. B = crop age (in weeks).

contents of red clover for all of the swards are presented in Fig. 5.

Detailed information concerning the plant oestrogen contents of all samples investigated can be obtained from the authors. From these data the plant oestrogen contents of red clover at different times of harvesting in pure red clover and in differently fertilized clover-rich and clover-poor swards and, correspondingly, in ungraded clover-rich and clover-poor and pure timothy swards are evident. Detailed information can also be obtained on the crude protein and dry matter contents in the red clover samples, and the harvest yields of the red clover and timothy swards.

A statistical evaluation of the results is presented in Table 1.

Discussion

The purpose of the investigation was to clarify the variations during one growing season in the plant oestrogen contents of Finnish red clover-timothy-grass whose N-fertilization rate and plant composition varied. The plant oestrogen content was determined in both the swards as such, and in the selected red clover of the mixed swards.

Average plant oestrogen content variation in test swards

The plant oestrogen content of the red clover swards as well as that of the selected red clover from the mixed swards of the spring yield was highest in the youngest crops, aged 4–5 weeks and declined greatly by midsummer. The plant oestrogen content rose again in aftermath and compared to the age of the crop, it was regularly higher than that of the early summer yield.

It seems apparent that especially in aftermath the plant oestrogen content is determined not only by the crop's age but also by the time period when mowing is carried out,

Table 1. Statistical relationship (regression coefficients) of plant oestrogen and crude protein content in red clover and their relationship to red clover and total harvest yields of the swards.

	Plant oestrogen content of red clover: (% of DM)						Crude protein content of red clover: (% of DM)			
	crude protein content of red clover (% of DM)		red clover yield (kg DM/ha)		total crop yield (kg DM/ha)		red clover yield (kg DM/ha)		total crop yield (kg DM/ha)	
	spring yield	after-math	spring yield	after-math	spring yield	after-math	spring yield	after-math	spring yield	after-math
Red clover sward	+0.936	+0.865	−0.931	−0.929	−0.931	−0.929	−0.967	−0.776	−0.967	−0.776
Clover-rich sward	+0.720	+0.737	−0.735	−0.791	−0.729	−0.748	−0.854	−0.699	−0.993	−0.882
Clover-poor sward	+0.824	+0.389	−0.889	−0.816	−0.729	−0.698	−0.819	−0.648	−0.905	−0.861

as aftermath of the same age mown earlier in autumn contains a greater abundance of plant oestrogens than does that harvested at a later date.

In addition to the physiological age of the plant and harvest time, the plant oestrogen content of red clover may be influenced by other growing conditions such as rainfall, temperature, etc. One Swedish study found markedly high plant oestrogen contents in plants after very cold nights in autumn (*Pettersson et al.* 1984). Likewise, it has been found that plant diseases tend to increase plant oestrogen contents (*Shutt* 1976).

Determinations performed on both the clover-rich and clover-poor swards show the plant oestrogen contents of the clover-rich swards to be greater in the spring than those of the clover-poor swards. In the spring (May 1, 1984) clover sprouts were also more abundant in the clover-rich swards (mean 53 plants/m^2) than in the clover-poor swards (30 plants/m^2). In both cases the quantity of oestrogens in aftermath clearly rose; relatively more so in the clover-poor swards. Corresponding variations could also be found in the clover yields.

In the samples from the red clover and mixed swards investigated, the total plant oestrogen content was determined mainly by the dominating quantities of formononetin and biochanin-A present which, in addition, as a rule varied in the same direction. By comparison, the contents of genistein and daidzein were rather low. They remained at the same levels throughout the entire growing season and particularly with regard to genistein, appeared to rise somewhat toward midsummer and late autumn.

The plant oestrogen content of timothy was very low, as expected. In the spring yield it was caused mainly by formononetin, and was in aftermath especially due to genistein which increased toward late autumn in the swards not fertilized with nitrogen. Because of exceptionally high genistein contents, additional thin-layer chromatographic determinations on genistein as well as on other isoflavones were performed. These investigations proved that with respect to genistein the results were apparently too high, as genistein is not completely separated from impurities by the HPLC-determination applied. However, the possible drawback of the analytical method employed on the clover and mixed sward samples is rather slight.

The plant oestrogen content of pure red clover as compared to red clover grown in mixed swards.

The growth circumstances of the test swards (soil, basic fertilization, climate) were very much similar except for the N-fertilization. By comparing the plant oestrogen content of the red clover grown in a red clover sward to that of red clover grown in unfertilized mixed swards, it is possible to draw conclusions as to the effect of plant composition in the swards on the oestrogenity of red clover.

It appears that red clover growing in unfertilized mixed swards on the average develops more plant oestrogens than does clover in pure red clover swards. The difference in aftermath is also statistically significant. A possible explanation could be the vigorous growth and great vitality of red clover which increase in autumn due to advantageous growth conditions, especially when competing with timothy for living space. This is also reflected in increased relative crop shares of red clover.

The effect of nitrogen fertilizer on plant oestrogen content

Nitrogen fertilizer had a strong effect on the clover yields and plant oestrogen contents of the mixed swards. Nitrogen fertilizer is known to favour the growth of timothy at the expense of red clover, which clearly became evident in the present investigation also, especially in the yields of the spring crops. In the early summer yield of the mixed swards, a higher level of fertilizing decreased the summer yield of red clover in the mixed swards as well as the plant oestrogen content. The subsequent effect of nitrogenous fertilization on mown aftermath was not as obvious. Nitrogen fertilization also decreased the plant oestrogen content of pure red clover. In unfertilized red clover the average plant oestrogen content (1.562 % ± 0.330 %) is notably higher than in red clover (1.352 % ± 0.275 %) treated with 100 kg N/ha, which for its part, is markedly lower than the plant oestrogen content of red clover fertilized with 50 kg N/ha (1.537 % ± 0.372 %). In addition, when separately examining the plant oestrogen contents of the spring and aftermath crops, it can be proved that the plant oestrogen content of red clover regularly decreased with an increasing rate of nitrogen fertilizer. At the rate of 100 kg N/ha this difference is statistically significant in all groups, with the exception of the clover-poor one.

The plant oestrogen and crude protein content of red clover and harvest yields

In addition to plant oestrogen content, the present investigation examined the crude protein content of red clover and harvest yields of the swards. These investigations resulted in the observation that the changes with regard to the plant oestrogen and crude protein content of red clover are generally in the same direction. Instead, a negative correlation was discovered between the plant oestrogen content of red clover and the red clover and total harvest yield, and between crude protein in red clover and corresponding harvest yields as well. Positive and negative correlations usually were more complete in spring swards compared to aftermath, especially concerning plant oestrogens and crude protein in heavy fertilized swards. It is possible in these cases that in autumn there are more factors effecting the oestrogen content but not necessarily the crude protein content. In an earlier published study it has been shown that among others, the plant oestrogen content can alter subsequent to cold nights (*Petterson et al.* 1984), by the effect of plant diseases (*Shutt* 1976), and also as a consequence of storage (*Ludewig* 1973, *Kallela* 1980). Such factors, however, hardly have the same effects on the crude protein content of red clover.

On the basis of this investigation, it seems apparent that the variations in the plant oestrogen content of red clover parallel with those of the crude protein content and probably owing to the same reasons, despite that chemical difference of these substances.

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Sammanfattning

Växlingar i växtöstrogenhalten i rödklövertimotejvall under vegetationsperioden.

Undersökningen utreder förändringarna i östrogenhalten i rödklöver-timotej-valler med samma grundgödsling men med olika kvävenivå och växtkomposition.

Östrogenhalten i rödklövern från ren klövervall och vall med timotej-inblandning var hög på våren och minskade framemot högsommaren. Östrogenhalten i rödklöver från efterslåttern låg konstant på hög nivå, högre än i vårväxten. Återväxtens ålder räknad från föregående avmejning påverkade mängden av östrogen, liksom också tidpunkten för slåttern.

Rödklöver från blandvall innehöll i genomsnitt något mera östrogen än rödklöver från ren klövervall. Östrogenhalten i ren timotejvall var generellt mycket låg.

Kvävegödsling minskade rödklöverns andel i blandvall och sålunda också vallens totala östrogenhalt. Denna effekt var speciellt betydande i vårväxten. I efterslåttern från blandvall var östrogenhalten regelbundet högre än i vårväxten.

Kvävegödsling sänkte östrogenhalten i ren rödklöver och också råproteinhalten minskade. En stark korrelation förelåg mellan växtöstrogenhalten och halten av råprotein i rödklöver. Uppenbarligen är båda beroende av samma faktorer trots olikheten i kemisk komposition.

Förändringarna i östrogenhalten bestäms i huvudsak av formononetin och biochanin-A, vilkas växlingar går i samma riktning. I jämförelse med dem var daidzein- och genisteinmängderna små, liksom också växlingarna i deras koncentration.

(Received January 12, 1987).

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