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VARIATION IN ESTROGENIC-LIKE SUBSTANCES IN RED CLOVER AND ALFALFA AS RELATED TO ENVIRONMENT, VARIETIES, AND STAGE OF GROWTH

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WALTER DEDIO

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By: Walter Dedio

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ABSTRACT

The isoflavone level in red clover as affected by genotype, stage of development, environmental conditions, and sample preparation methods was investigated. A method has been developed for quantitative estimation of the isoflavones - biochanin A and formononetin in red clover. This involved scanning the paper chromatograms with a densitometer for biochanin A and with a fluorometer for formononetin. Isoflavone level was the same in fresh and oven dried samples, if the temperature did not exceed 80°C. Drying at higher temperatures or exposure to prolonged wet conditions resulted in considerable loss of isoflavones. Some variation in isoflavone content was observed between varieties. Highest isoflavone content (approximately 1% of dry weight of each isoflavone) occurred in the leaves before flowering and then it declined rapidly. Only small amounts of isoflavone were present in stems and petioles throughout the growing season. Genetic variation of each isoflavone was observed in the four clones tested. Long photoperiod and low temperature resulted in higher isoflavone. Highest isoflavone concentration occurred in the folded leaf stage. This was followed by a gradual decline of biochanin A and formononetin until senescence when another isoflavone, daidzein appeared in some clones. The kinins (kinetin and N-6-benzyladenine) administered to the soil resulted in an increase in isoflavone content in red clover seedlings. The isoflavones remained for a longer period in the red clover leaf discs if they were suspended in a solution

containing kinins or benzimidazole than in water.

Coumestrol content increased greatly in alfalfa as the plants matured. White clover and birdsfood trefoil contained a trace amount of coumestrol, while red clover had none. Application of dithane greatly reduced coumestrol content in alfalfa. Variation among samples within a clone was less than within or between varieties. Inoculation with Leptosphaerulina briosiana induced coumestrol synthesis in alfalfa. Coumestrol was present in most annual medic species at maturity.

TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

Estrogen-like substances in plants have been reported for the past fifty years. Among the plants that are known to possess these substances are the forage legumes. Research in these components was stimulated because of reproductive difficulties in livestock grazed on these plants. These substances have been identified to be the isoflavones in red and subterranean clover (3) and coumestrol in the alfalfa and Ladino clover (2).

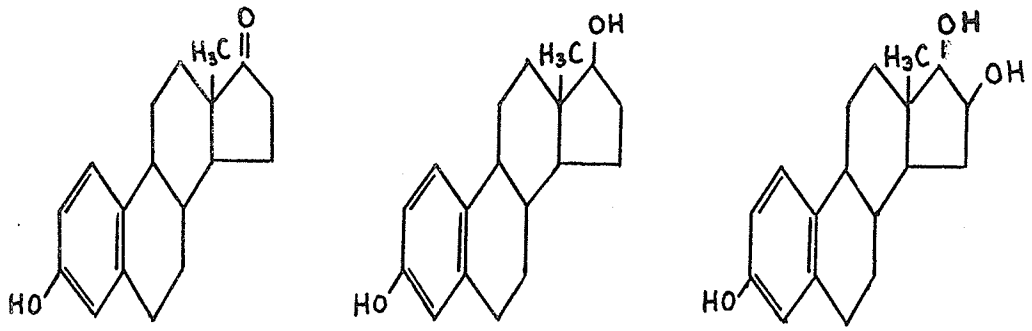
Although a precise and sensitive method has been developed for determination of coumestrol (4), such a method was lacking for isoflavone analysis. The procedures used for isoflavones either involved elutions from paper chromatograms (9) and measuring spectrophotometrically or visually comparing with known amounts on thin-layer chromatograms (1). Therefore, it seemed necessary to look for a simpler method for the quantitative estimation of formononetin and biochanin A in red clover.

Although considerable work has been done on the genetic and environmental factors affecting the isoflavone content of subterranean clover by Australian workers (7, 8) few investigations have been conducted on red clover. Also, little is known about the effects of harvesting procedures on isoflavone levels. Therefore, a series of the experiments were undertaken to determine the effect of sample preparation methods along with genetic and environmental factors and ontogenetic changes in isoflavone content of red clover.

LITERATURE REVIEW

1. Estrogenic plants

In the female animal, the hormone estrogen is produced by the follicles of the ovary at the onset of ovulation and the estrus cycle. This hormone causes a change in the uterus characteristic of the proliferative phase and of cornification of the vagina when absorbed directly into the cells of these tissues. In the animal, estrogens are steroidal in structure. Examples of such substances are estrone, estradiol and estriol (Fig. 1). However certain plants possess substances which are not steroidal but still exhibit estrogenic activity.



Estrone Estradiol Estriol

Fig. 1. Natural animal estrogens.

The estrogenic activity of plant extracts at first was determined on the basis of the Allen-Doisy test in which the onset of estrus was observed by appearance of cornified epithelial cells in vaginal smears from ovariectomized mice. More recently it has been discovered that the increase in weight of the uterine tube in ovariectomized or immature

female mice or rats can be employed to determine estrogenic activity. Later, ovariectomized sheep were used in Australia (31). Material under investigation can either be fed by incorporating it into a diet or it can be injected subcutaneously. Australian workers have measured the increase of teat length of wethers in determining estrogenic activity (24).

Bradbury and White (22) in 1954 reported over 50 different plants that have been found to be estrogenic. High activities were reported for garlic, milkweed and oats. Some activity was found in common plants such as coffee, sunflower, wheat, barley, some fruits, yeast, willow flowers, rye grass, potato tubers, and in a number of forage legumes.

Few reports claiming the isolation of estrogenic steroids identical with the normal animal estrogens have appeared. Butenandt and Jacobi (25) in 1933 have isolated estrone from date palm kernels. This was closely followed with the isolation of estriol from willow flowers by Skarzynski (75).

The presence of estrogenic substances in plants gained importance when it was discovered that they were the cause of infertility in livestock when grazing on forage legumes. Bradbury and White (21) subjected various fractions from an extract of subterranean clover to mouse uterine weight bioassays. They have identified genistein, an isoflavone, as an estrogenic substance. Later other isoflavones have been identified having estrogenic activity in the subterranean clover. Considerable estrogenic activity was reported in red clovers of New Zealand by Flux

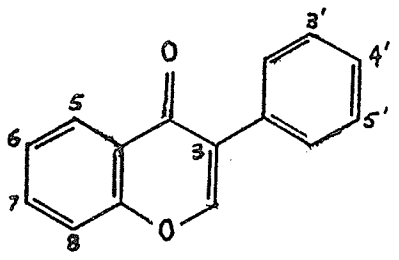
et al. (35). Kitts et al. (45) in studying the affect of maturing on estrogenic activity of certain forages, found high activity in alfalfa and ladino clover at certain stages. Later, the active substance was found to be coumestrol (15), a compound closely related to the isoflavones.

2. Isoflavones

The chemical identification of estrogens in forages was started when the cause of infertility in sheep grazed on subterranean clover in Australia from 1941 to 1951 was traced to the occurrence of these substances in the legume. At first the isoflavone genistein (4',5,7-trihydroxyisoflavone) was isolated (21), being about 1/100,000 as active as the synthetic estrogen, diethylstilbestrol. Shortly after the other isoflavones: formononetin, (4'-methoxy,7-hydroxyisoflavone), biochanin A (4'-methoxy,5,7-dihydroxyisoflavone), and daidzein (4',7-dihydroxyisoflavone) were found as constituents of red and subterranean clover. The chemical structures are presented in Fig. 2. Wong (84) has recently isolated a new estrogenic isoflavone from red clover and named it, pratensein. This compound differs from biochanin A by the addition of a hydroxyl group in the 3' position, having therefore the structural formula 5,7,3'-trihydroxy-4'-methoxyisoflavone.

The isoflavones are usually present in the glycoside form. The source of a number of identified glycosides are listed in Table I.

TABLE I. Glycosides of isoflavones naturally occurring in forage plants.



Isoflavone aglycone	Isoflavone glycoside	Sugar and point of attachment	Source
Genistein			
	Genistin	7 -D-glucose	Soybeans (80)
	Sophoricoside	4'-D-glucose	Sophora japonica, L. (28)
	Sophorabioside	4'-D-glucose and L-rhamnose	"
		6"-malonyl-7-glucose	Subterranean clover (7)
Biochanin A			
		7-glucose	Cicer arietinum (86)
			Subterranean clover (72)
		6"-malonyl-7-glucose	Subterranean clover (7)
			Red clover (7)
Daidzein			
	Daidzin	7-glucose	Soybeans (3)
Formononetin			
	Ononetin	7-glucose	Ononis spinosa (10)
			Subterranean clover (72)
		6"-malonyl-7-glucose	Subterranean clover (7)
			Red clover (7)

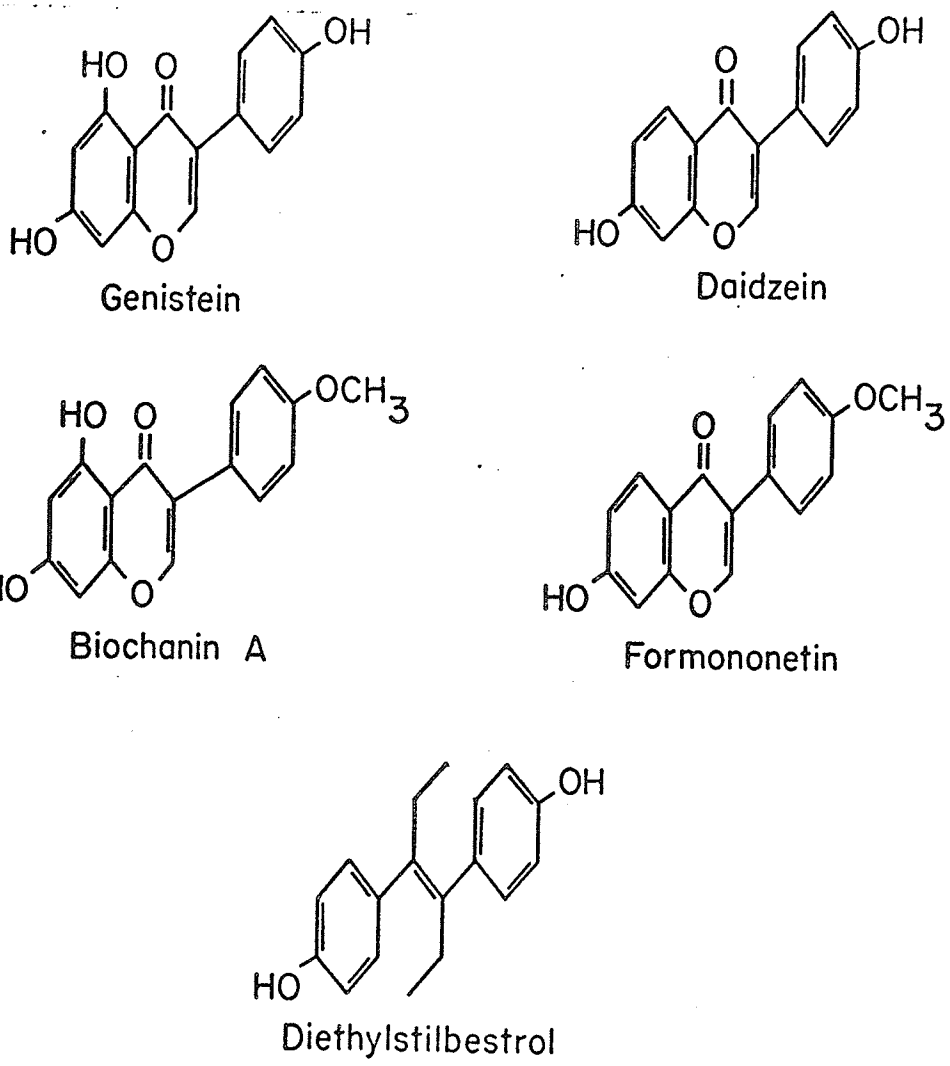


Fig. 2. Structures of estrogenic isoflavones and the synthetic estrogen, diethylstilbestrol.

The isoflavones have weak estrogenic activity. Genistein for example has a potency of only about 1/100,000 that of diethylstilbestrol (Table II). Similar potency values of isoflavones were obtained by Cheng et al. (29) and Wong and Flux (85) using mouse uterine weight bioassay. However, by the teat increase of wether bioassay, of the three isoflavones present in the subterranean clover, only formononetin showed a high correlation between its level and estrogenic activity (56). Biological transformation of these compounds may occur with the production of more active intermediates. For example, demethylation of biochanin A to genistein and formononetin to daidzein occurs in the presence of rumen fluid as shown by Nilsson (58). However, Nilsson (59)

TABLE II. Relative potency of forage estrogens vs. diethylstilbestrol and estrone (11).

Compound	Quantity to produce 25 mg uterus, ug	Relative potency
Diethylstilbestrol	0.083	100,000
Estrone	1.20	6,900
Coumestrol	240	35
Coumestrol diacetate	340	24
Genistein	8,000	1.00
Daidzein	11,000	0.75
Biochanin A	18,000	0.45
Formononetin	32,000	0.26

was not able to show any difference in estrogenic activity by the mouse uterine tube bioassay between biochanin A or red clover extracts incubated with rumen fluid and non-treated samples. Formononetin has been shown to be metabolised to equol, an isoflavan, in chickens (27) and sheep (23). When fed to mice, equol exhibited estrogenic activity equal to one-half of genistein (74).

The stability of estrogenic substances to drying treatments varies with the forage crop. Bickoff et al. (14) using the mouse uterine bioassay, found little if any reduction in estrogenic activity when the red clover was dried prior to assay. Oven drying resulted in considerable loss in estrogenic activity in alfalfa (13), while in Ladino clover slight loss occurred (16).

Isoflavones are present mostly as glycosides in the chloroplasts and thus it is necessary to hydrolyze the glycosides before estimating the isoflavone content. Endogenous enzymes will liberate the isoflavones if the plant material is crushed (6). Generally, isoflavones have been detected and estimated by paper (82) or thin layer (6) chromatography. In the former case, the isoflavones were eluted from the chromatograms and measured spectrophotometrically, while in the latter a semi-quantitative estimate was obtained by visual comparison with known amounts of synthetic isoflavones. Nilsson (60) developed an isotope-dilution method for the quantitative determination of biochanin A in red clover.

Isoflavone content of forage legumes varies greatly with varieties, species and environmental conditions. The isoflavones are predominant in the Trifolium species which includes the economically important subterranean and red clovers. The main isoflavones in red clover are formononetin and biochanin A while in subterranean clover, in addition, genistein occurs in high concentration. In a survey of 100 Trifolium species, Francis et al. (41) discovered that 14 have isoflavone contents comparable with that of subterranean clover.

In the subterranean clover at least, the greatest concentration of isoflavones is found in the leaves, probably in the chloroplasts (62). In the study of ontogenetic changes of isoflavone content of subterranean clover (69), it was found that amounts of the three main isoflavones were highest at leaf emergence and then declined during the remainder of life falling to low levels at death. In red clover, Schultz (72) reported that in the vegetative stage, formononetin content remained constant at 0.4%, while that of biochanin A decreased by 50% during the period of differentiation. Biochanin A content was at least 1% greater than formononetin in the leaves. In New Zealand, Wong (83) found that the isoflavone content was higher during flowering than before flowering in the red clover.

Environmental factors also contribute to substantial variation in isoflavone level. In subterranean clover, Rossiter and Beck (68) reported that long photoperiod and high intensity light favors isoflavone synthesis. Low isoflavone level are associated with low contents of

starch and sugar (64) which in turn are related to light intensity. However after full leaf expansion, isoflavone synthesis ceases and are thus unaffected by light or carbohydrate level. Isoflavone concentrations were much higher at day-night temperatures of $15^{o}C/10^{o}C$ than at lower or higher temperatures (66). In experiments with varying nutrient levels, phosphorus deficiency resulted in higher isoflavone level (67, 71). Although the New Zealand workers (26) found little effect with varying nitrogen supply on isoflavone level in the red clover, Rossiter (63) using young plants of subterranean clover reported that nitrogen deficiency was associated with increased concentrations of isoflavones. Magnesium or sulfate deficiency lowered the isoflavone in red clover cultivated in sand (71), but in a more extensive study sulfur deficiency resulted in as much as double the level of isoflavones in the subterranean clover (65). In addition Schoo et al. (70) found that with increasing rates of phosphorus and sulfur, formononetin level decreased significantly in the leaf blades of subterranean clover. The isoflavone content was not affected by potassium levels (26). Thain and Robinson (77) had reported that red leaves formed in subterranean clover from anthocyanin pigmentation due to water logging or nutrient deficiency contained 5 times as much genistein and twice as much formononetin, but less biochanin A, than normal green leaves.

The formation of isoflavones in subterranean and red clover appears to be determined genetically (55, 57). Morley and Francis (57), in

examining the concentrations of formononetin, genistein and biochanin A in 151 lines of subterranean clover, found considerable differences among varieties in individual isoflavones. However, the differences in total isoflavones were not as great as there were negative correlations between concentrations of genistein and biochanin A. Francis and Millington (38), by the use of the mutagen ethyl methane sulfonate, were able to obtain mutants deficient in the enzymes which methylates daidzein and genistein to formononetin and biochanin A respectively. They also obtained a mutant which was deficient in enzyme that released the isoflavones from their glycosides. They also found that at least two genes were involved in the production of isoflavones.

Growth regulators may indirectly affect the synthesis of flavonoid compounds. These include the auxins, whose prime action is cell elongation and kinins, which have been shown to delay senescence in a number of species (32, 80) as determined by retention of chlorophyll and protein. In the duckweed, Spirodela oligorrhiza anthocyanin formation was inhibited by benzimidazole,2,6-diaminopurine, guanine and kinetin (78), suggesting that a pyrimidine or purine is involved in anthocyanin synthesis. However, in cultured, excised immature petals of Impatiens balsamina, Klein and Hagen (6) found that the addition of kinetin increased anthocyanin content.

3. Coumestrol

In 1957, Bickoff et al. (15) at the U.S.D.A. Western Regional

Research Laboratory, Albany, Calif. isolated a new estrogen from white clover, differing from isoflavones and animal estrogens. The compound has a coumarin-like structure and has been named coumestrol (Fig. 3).

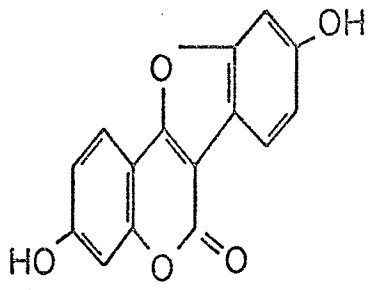


Fig. 3. Structure of Coumestrol.

Coumestrol is about 30 times more potent than genistein, but still considerably less active than the natural animal estrogens or diethylstilbestrol. Later it has been found to occur in alfalfa, subterranean clover (39), Medicago tribuloids (55), and small amounts in M. hispida (53) and most other forage legumes (40, 53).

Cooperative research projects within the United States Department of Agriculture then followed to determine the factors controlling coumestrol content. Samples of alfalfa were collected at different stages in a number of states and then analyzed for coumestrol. They varied from 0 to over 600 parts per million and the variation seems to be due to location, year and stage of growth (43). In most of the states, the U.S.D.A. group found a rise in coumestrol synthesis after the flowering stage. The exception was in the states of California and Utah where

coumestrol content was low throughout.

Further investigations into factors influencing coumestrol content led to the discovery that pathogens act as inducers of coumestrol synthesis. Four fungal infestations; namely, blacketem (Aschochyta), common leaf spot (Pseudopeziza medicaginis), Leptosphaerulina leaf spot and Stemphylium leaf spot (43, 52) have been shown to cause a marked increase in coumestrol. Sherwood et al. (73) after studying the coumestrol synthesis in alfalfa in response to pathogenic fungi reported that the coumestrol accumulation is limited to the infected area. Two aphids (pea and spotted alfalfa) (50) have also shown to induce coumestrol synthesis, while the alfalfa yellow mosaic virus (43) stimulated coumestrol accumulation in some experiments. The low coumestrol content of alfalfa in California and Utah can be explained by the low disease incidence in these states.

Other compounds related to coumestrol known as coumestans and flavones have been isolated from alfalfa (17). The content of these substances in alfalfa was increased when plants were infected with Pseudopeziza medicaginis (18).

In addition to ladino clover and alfalfa, coumestrol has also been found in considerable amounts in subterranean clover (34), Medicago tribuloides (55), and Medicago hispida (53). Francis and Millington (40) found considerable variation among annual medics in coumestrol content, with high amounts in Medicago littoralis and several Medicago truncatula (also referred to as M. tribuloides) strains. The coumestrol

level was higher in the dry annual medic swards than the green pastures. At the burr development stage most coumestrol was found in the mature leaves, with only relatively insignificant quantities in the stems and pods. In dry material however, the stems and pods had very high coumestrol contents. In M. littoralis, Loper (51) observed that coumestrol accumulated with age and was concentrated in the necrotic physiogenic spots.

4. Biosynthesis of isoflavones

The biosynthesis of isoflavones is known in some detail and is illustrated in Figure 4. The synthesis of the skeleton C15-intermediate, which is common for all flavonoid synthesis, is derived from two separate pathways. The A-ring arises from condensation of 2 malonyl CoA units and an acetyl CoA. The remaining C_6 - C_3 portion may come from hydroxycinnamic acid, cinnamic acid or phenylalanine. Grisebach (42) has shown that aryl migration subsequently occurs to form the isoflavone structure. This was based on experiments in which cinnamic acid labelled at each position in the C_3-side chain is correctly incorporated into formononetin by clover seedlings. Glycosylation occurs after this step. In genistein and subsequent biochanin A synthesis the carbon in the 5-position is hydroxylated. Biochanin A and formononetin is finally produced by methylation of genistein and daidzein respectively. Coumestrol is biogenetically related to the isoflavones in that it is derived by arrangement of the isoflavone, daidzein rather synthesis from the coumarin

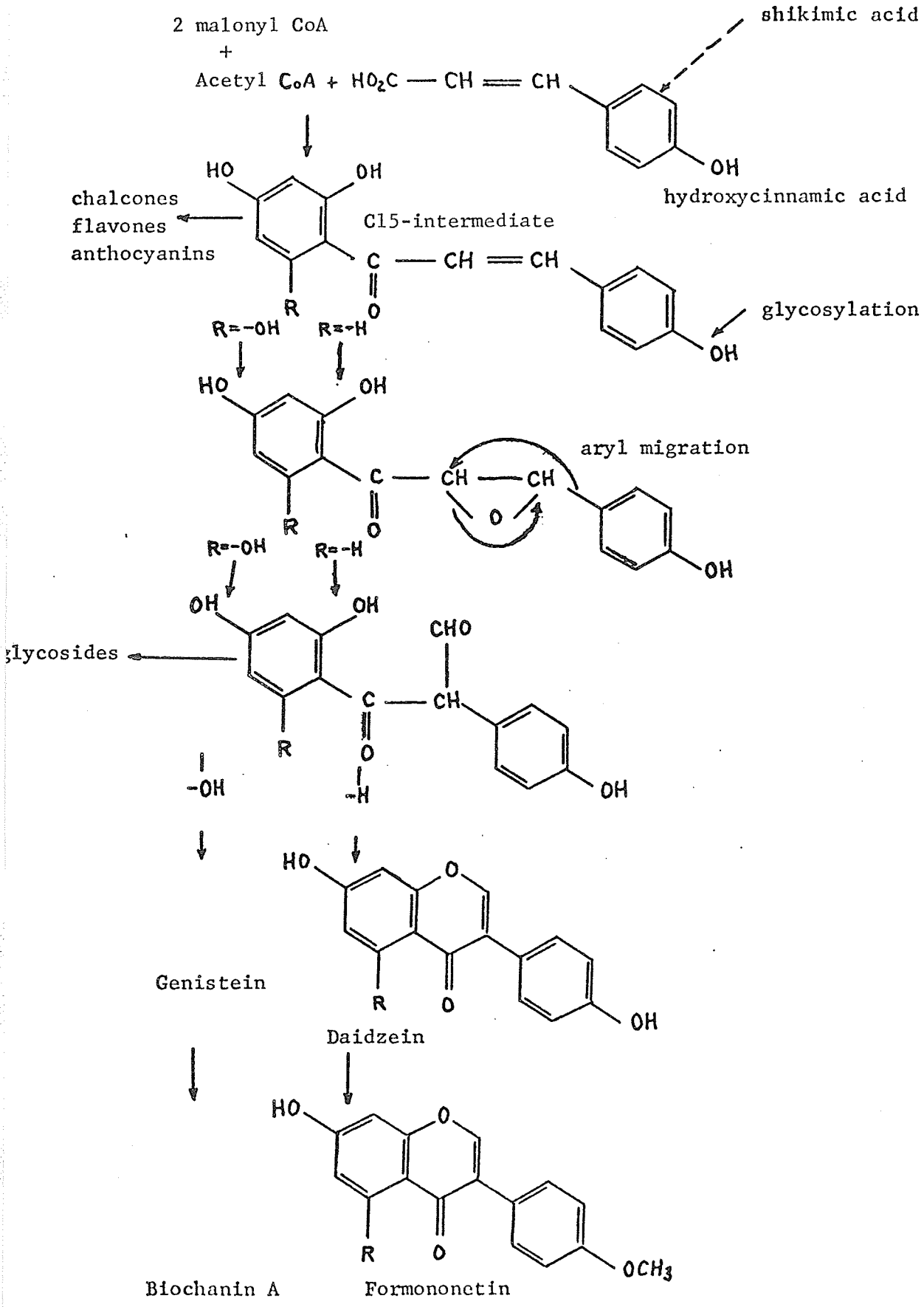


Fig. 4. Biosynthesis of estrogenic isoflavones

nucleus intermediate (20).

5. Physiological effects of phytoestrogens

Infertility from plant estrogens ingested arises because of deviation from the normal levels of estrogens in the female animal. Excessive estrogenic stimulation from ingesting large amounts of estrogen-containing forage results in hyper-estrogenic syndrome. Plant estrogens may reduce the normal endogenous levels (hypo-estrogenic syndrome), but this is not well established. Emmens (33) established that synthetic estrogens may act as estrogens or anti-estrogens depending on the level administered. Competition between a weak plant or synthetic estrogen and a strong endogenous estrogen for a receptor site could explain the anti-estrogen effect of some estrogens. Plants may contain anti-estrogen substances which mask the effects of the estrogen.

Substances other than estrogens may act as infertility agents. Leavitt (48) found that conception did not occur in mice fed certain samples of Ladino white clover, though little estrogenic activity was revealed by oral bioassay.

In the female animal, the ovary under the influence of gonadotrophic hormones secreted by the pituitary gland, produces the gonadol hormones (i.e. estrogens) which regulates the estrus cycle. Leavitt and Wright (44) reported that coumestrol has anti-gonadotrophic activity and its primary effect is to inhibit the release of the gonadotrophin.

Anti-estrogenic activity has been reported in alfalfa (1, 12),

yellow pine (Pinus ponderosa) needles (4), and in a number of other forage crops (19). Adler (1) reported estrogen inhibitors in alfalfa which were specific for coumestrol as well as for estradiol and diethylstilbestrol. Already a triterpene constituent, glycyrrhetinic acid isolated from licorice (Glycyrrhiza glabra) has been shown to possess anti-estrogenic activity (47). Bickoff et al. (12) reported that certain alfalfa samples contain estrogen potentiators which enhanced the effect of coumestrol.

6. Effects of estrogens on livestock

Reproductive difficulties were first noticed in 1941, when an outbreak of infertility in sheep appeared in Western Australia (9). A combination of factors (low rainfall, shortage of fertilizer during wartime) led to a greater than usual intake of subterranean clover. Symptoms included failure of ewes to conceive, stillbirth or early death of lambs and the lambing percentages dropped to as low as 8%. It has been estimated that a chronic reproduction problem of sheep in Australia exists with about a 15% loss in the annual lamb crop.

In the United States, several observations on reproductive difficulty have been reported. Engle et al. (34) observed that ewes grazing on Ladino clover required three weeks longer to conceive than those grazed on grass. Reports from farmers of New Hampshire of reproductive failure in heifers and dairy cows on a diet rich in Ladino clover prompted Wright's (87) research where infertility in rabbits was induced

by feeding them ladino clover. In Oregon, Fox (37) demonstrated that ewes fed on red clover pasture had a considerably longer lambing period than the ewes fed on grass pasture with little Ladina clover, and 20% dry ewes occurred in the former flock. Ranchers in the Western U.S.A. and Canada have noticed that beef cows tend to abort or give birth to weak calves when they consume yellow pine needles (54). In Israel, infertility in cattle was traced to the feeding of alfalfa hay (2).

Interference with fertility of the male animal may occur from estrogens. Infertility in male mice (10) as well as the female (36) has been reported. Bennetts (8) observed changes in prostate and bulbo-urethral glands of castrated male sheep grazed on subterranean clover. The seminal vesicles remained unchanged but in the experiment of Oldfield et al. (61) with wethers on high coumestrol alfalfa there was an eightfold increase in the weight of the seminal vesicle. There was also some increase in the pituitary gland weight.

Estrogens have also been known to increase growth rate and feed efficiency. Until recently steers were fed or implanted with synthetic estrogens such as diethylstilbestrol to promote growth. Because of possible harmful effects in the human, diethylstilbestrol has recently been completely banned in Canada as a growth promoter and may be used only in an ear implant in the U.S.A. Oldfield et al. (61), in tests conducted at Oregon State University, showed significantly faster gains on lambs fed on high coumestrol alfalfa than on low coumestrol alfalfa. Organoleptic tests showed slight but significant difference in tenderness,

juiciness and texture between the two treatments — all in favor of the high estrogen-fed lambs. However, more recent results by Stob et al. (76) did not show an increase in gain or efficiency of feed utilization when coumestrol in pure form or in dehydrated alfalfa pellets was fed to cattle.

There is some evidence that estrogens may have a stimulating effect on lactation. Lush green pastures have been associated with increased milk production, but negative results by recent workers (5, 44) with synthetic estrogens have discouraged further studies of estrogens as lactogenic agents.

Further work should be conducted on the effects of coumestrol on livestock, in particular to determine if the beneficial effects will override the detrimental ones. Otherwise, we may need two types of forages, one, a low estrogen strain for breeding stock and the other, a high estrogen line for fattening steers, wethers and poultry.

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BIOCHANIN A AND FORMONONETIN CONTENT IN RED CLOVER VARIETIES AT SEVERAL MATURITY STAGES

ABSTRACT

A method has been developed for quantitative estimation of the isoflavones — biochanin A and formononetin in red clover. This involved scanning the paper chromatograms with a densitometer for biochanin A and with a fluorimeter for formononetin. Some variation in isoflavone content was observed between varieties. Highest isoflavone content (approximately 1% of dry weight of each isoflavone) occurred in the leaves before flowering and then it fell off rapidly. Only small amounts of isoflavone were present in stems and petioles throughout the growing season.

INTRODUCTION

Substances possessing estrogenic activity and affecting the performance of animals have been known to be present in certain forages, particularly the legumes (3). Coumestrol appears to be an important estrogenic substance in alfalfa (Medicago sativa) and ladino clover (Trifolium repens), while the isoflavones, though possessing much weaker estrogenic activity, are present in large amounts in subterranean (T. subterraneum) and red clovers (T. pratense) (10). The main isoflavones in red clover are formononetin and biochanin A, while in subterranean clover, in addition, genistein occurs in high concentration.

Subterranean clover has been responsible for serious infertility problems of sheep in Australia in 1941 (2), and in Oregon a longer lambing period and decrease in lamb crop has been reported when sheep grazed on red clover pastures (5). Detrimental effects on reproduction from ingestion of clovers at one growth stage may be followed later by a beneficial effect of growth stimulation and increased feed efficiency.

Some work has been done in Australia on the ontogenetic changes of isoflavones in subterranean clover (11). The three main isoflavones were highest at leaf emergence and then declined during the remainder of life. Schultz (12) reported that in the vegetative stage of red clover, formononetin content remained constant, while that of biochanin A decreased to 50% during course of differentiation. In New Zealand, Wong (15) found that the isoflavone content was higher during flowering than before flowering in the same plant. Generally, isoflavones have been detected and estimated by paper (14) or thin layer (1) chromatography. In the former case, the isoflavones were eluted from the chromatograms and measured spectrophotometrically, while in the latter, a semi-quantitative estimate was obtained by visually comparing with known amounts. Both methods have disadvantages in being either tedious or lacking in precision.

The present experiments were undertaken to develop a simpler method of quantitative estimation of formononetin and biochanin A in red clover and to study the variation of these isoflavones among varieties and at different stages of growth.

MATERIALS AND METHODS

Extraction

Red clover samples, either frozen or fresh, 3-5 g, were ground with some sand and water and extracted as described by Beck (1). However, after removal of lipids and chlorophyll with petroleum ether, the ethanol fraction was taken to dryness in a flash evaporator and the residue taken up in 10 ml of benzene-ethanol (50% v/v).

Estimation of Biochanin A

Previously, isoflavones have been separated by paper chromatography (14), and more recently by thin-layer chromatography (1). However, in both cases, only semi-quantitative estimation was made and it appeared that more accurate determination could be obtained by scanning the developed paper chromatograms with a densitometer.

For biochanin A, 2-6 ul of extract were spotted and chromatographed on Whatman No. 1 paper by ascending technique in a formic acid-water (40:60) solvent system for 3½ hours. Together with these, duplicate samples of biochanin A of 1, 2, 3, 4, 5 and 6 ug were spotted to obtain a standard curve. This was done with each batch of chromatograms with red clover extracts. After the chromatograms were dried for at least 2 hours, they were developed by spraying with the salt of diazotized sulfanilic acid in NaOH (13). However, in order to minimize the background a lesser concentration, 0.1 g in 100 ml NaOH, was found to be more suitable.

After final drying, the chromatograms were cut into strips and scanned on Densicord, Model 542. The aperture was fully open and a narrow-band secondary filter having peak transmittance at 445 mu was used. Since the baseline tended to drift, it was more convenient to cut out the area under the peak and weigh it rather than to use the integrator. The amount of biochanin A in extract was determined from the standard curve and expressed on a dry weight basis.

Estimation of Formononetin

Two to six microliters of extract were chromatographed on Whatman No. 1 paper by ascending technique in 2 N NH_3 solvent system overnight (about 16 hours). As with biochanin A, formononetin references of 1, 2, 3, 4, 5 and 6 ug were chromatographed with each batch of unknown extracts. The chromatograms were dried overnight, cut into strips and scanned on the fluorometer. A primary filter, Corning No. 5970, was used to screen out the visible light from the short-wave ultraviolet light, and a narrow-band secondary filter having peak transmittance at 505 mu was employed. The amount of formononetin in extract was determined from the standard curve in the same way as for biochanin A.

Red Clover Samples

(1) Isoflavone content in various varieties. Leaves of 16 varieties of red clover grown in the field were collected in August, 1965, and frozen. Duplicate samples of each variety were taken and at least two aliquots of the sample were chromatographed for formononetin and at

least three for biochanin A.

(2) Isoflavone content at various stages of maturity. Two varieties were used, Tamisto and Svalof 059, a tetraploid variety. Samples were taken in summer of 1966 from the field in their second year's growth and they represented six stages in first cut and two stages in the second cut. These were stored frozen and before analysis, the leaves were separated from the stems and petioles. Isoflavone content determinations were done on both parts. For stems and petioles, a single 5-10 g sample was taken, while for the leaves, duplicate 3-5 g samples were used. At least three aliquots were chromatographed for each isoflavone from each sample.

RESULTS AND DISCUSSION

Isoflavone Determination

For biochanin A estimation, it was necessary to scan the chromatograms within a day after spraying with diazotized sulfanilic acid; otherwise, a dark background develops, particularly if left exposed to light. Formononetin gives a reddish spot which overlaps the yellow spot of biochanin A and therefore a higher-than-actual biochanin A content was obtained. This can be corrected, once the formononetin content is known, by subtracting the adsorption due to formononetin from the total sbsorption. For these isoflavones, Beer's Law was obeyed in the range of 1-6 ug.

In the case of fomononetin, an intense fluorescence was observed initially because an ammonia solvent was used for developing. When the chromatograms were scanned at time intervals, it was found that initially the intensity of the fluorescence would drop sharply, but eventually a stable response was obtained (Fig. 1). It is clear, therefore, that for accurate determinations, the chromatograms should be scanned about 10 hours after development. Lindner (8) however, reported a stable maximum reading could be obtained by reading the fluorescence right after exposure to ammonia vapor.

Although a number of solvent systems have been used in separating formononetin and biochanin A, the formic acid-water (40:60) and 2 N ammonia systems were the most satisfactory. These two resulted in the least tailing, and gave the maximum intensity under ultraviolet light and with diazotized sulfanilic acid for formononetin and biochanin A respectively.

When known amounts of isoflavone were added to plant extracts prior to chromatography, a mean recovery of 96% was obtained for formononetin and individual determinations were usually within 10% of the mean. The mean recovery for biochanin A was 97% but deviations up to 30% from the mean were obtained, and for accurate biochanin A estimation, at least six determinations were required.

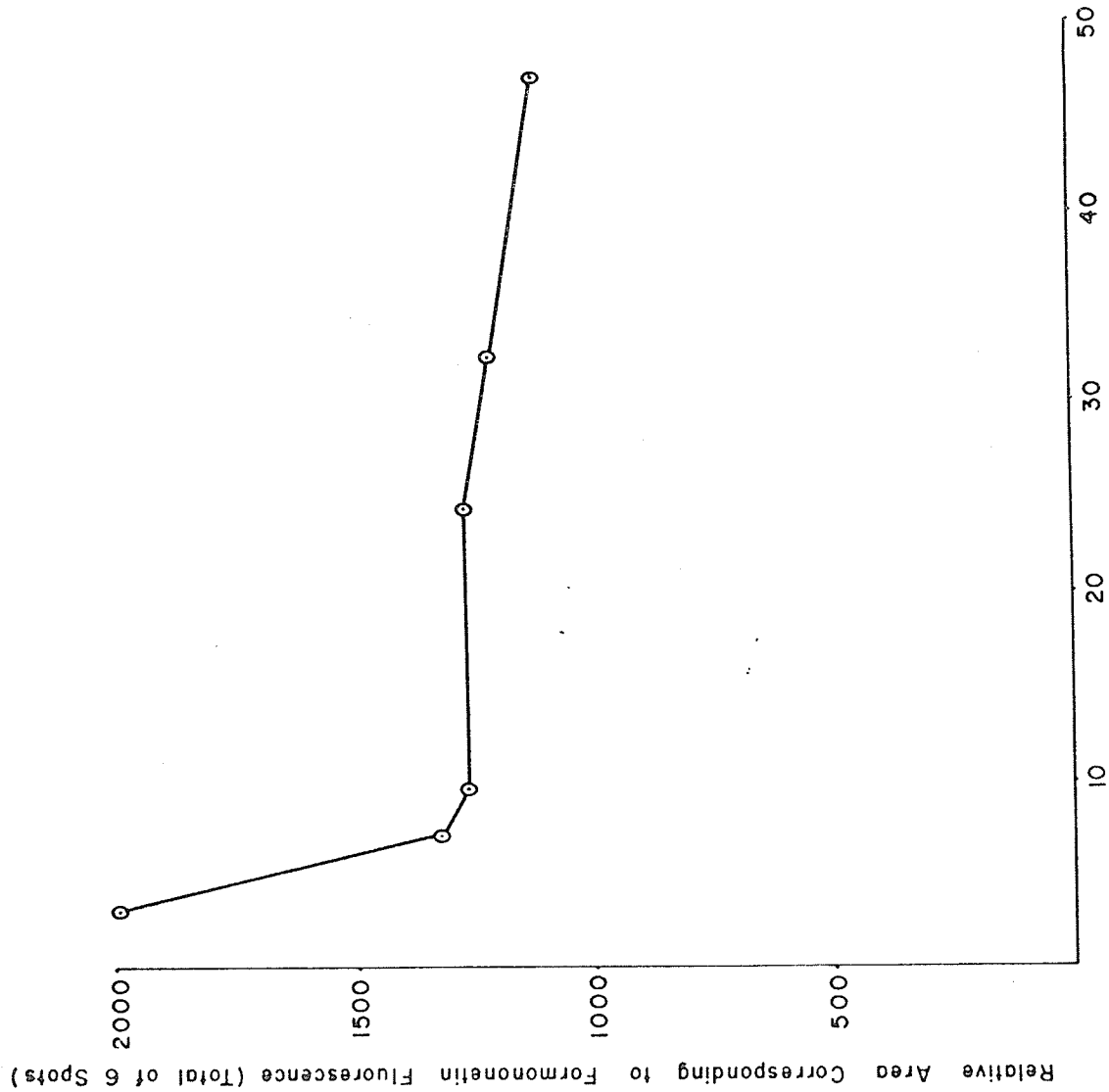

Fig. 1. Fluorescence intensity as a function of time after development in ammonia solvent.

Isoflavone Content in Red Clover

(1) Variation among varieties. Formononetin content ranged from 0.35 to 0.94% while biochanin A range was 0.60 to 1.12% (Table 1). Highest isoflavone concentration was found in the Lakehead variety, while the lowest was in the Manhardy and Heges Hogenheimer varieties. There appeared to be a high correlation between biochanin A and formononetin estimates. In the four varieties that Francis et al. (7) tested, the range for formononetin was 1.00 to 1.20%, and 0.95 to 1.20% for biochanin A.

Since red clover is a cross-pollinating plant, the samples may not be representative of a variety since they were taken from a limited number of plants. Francis et al. (7) observed a variation of 0.1 to 3.0% in isoflavone content of individual plants within a variety. With such a variation, it would appear possible to select for varieties with low and high isoflavone conent.

(2) Isoflavone content at various maturity stages. High isoflavone content was found in the earlier stages, until about flowering (Figs. 2 and 3). This was followed by a rapid decline. No significant difference between a diploid and a tetraploid variety was noticed. The pattern is somewhat like that of subterranean clover (11), where formononetin and genistein synthesis occurs essentially during leaf unfolding. In New Zealand (4), a lower estrogenic activity in red clover was obtained in the more mature stage, as determined by the mouse uterine bioassay.

Table 1. Isoflavone Content of Some Red Clover Varieties
(% on dry weight basis)

Variety	Formononetin	Biochanin A
Tepa	0.82* ± 0.02	0.81* ± 0.04
Merkur	0.75 ± 0.01	0.88 ± 0.05
Altaswede	0.70 ± 0.04	0.70 ± 0.04
Snow Brand	0.83 ± 0.04	0.89 ± 0.04
Robina	0.66 ± 0.01	0.73 ± 0.03
Tammisto	0.67 ± 0.04	0.64 ± 0.03
Manhardy	0.35 ± 0.02	0.66 ± 0.04
LaSalle	0.78 ± 0.05	0.79 ± 0.03
Tsukikei	0.84 ± 0.03	0.92 ± 0.04
Lakeland	0.94 ± 0.04	1.12 ± 0.10
Fetzers Ritzinger	0.60 ± 0.02	0.71 ± 0.06
Oldenwalden	0.49 ± 0.02	0.70 ± 0.05
No. 0294 Double Cut	0.73 ± 0.04	0.85 ± 0.03
Heges Hogenheimer	0.37 ± 0.02	0.60 ± 0.02
Svalöf 059	0.62 ± 0.06	0.74 ± 0.05
Lembkes	0.49 ± 0.02	0.77 ± 0.05

* The mean is the average of at least 4 determinations for formononetin and at least 6 determinations for biochanin A.

The value given after the mean is the standard error.

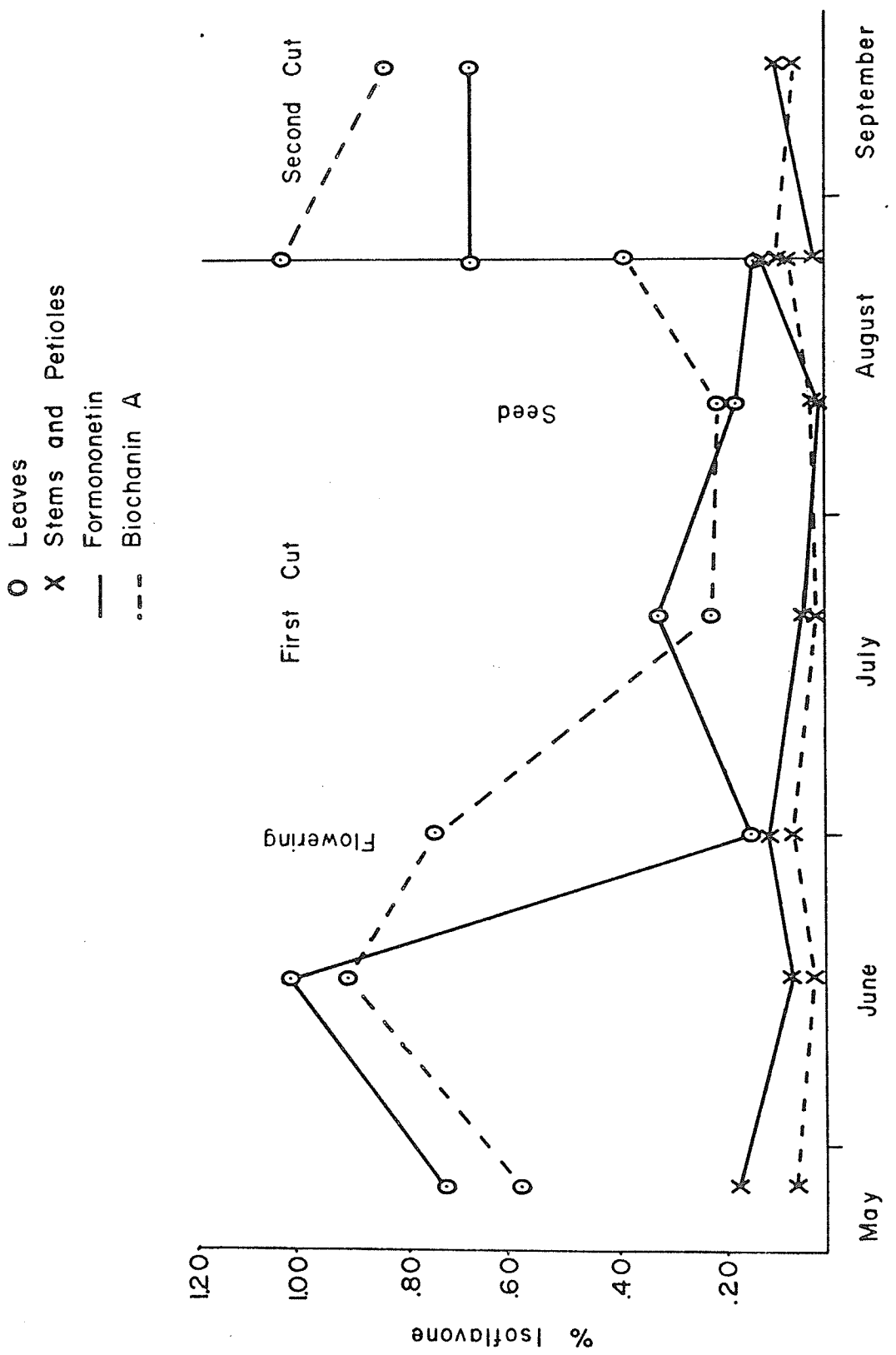

Fig. 2. Isoflavone content at various stages in Tamisto red clover.

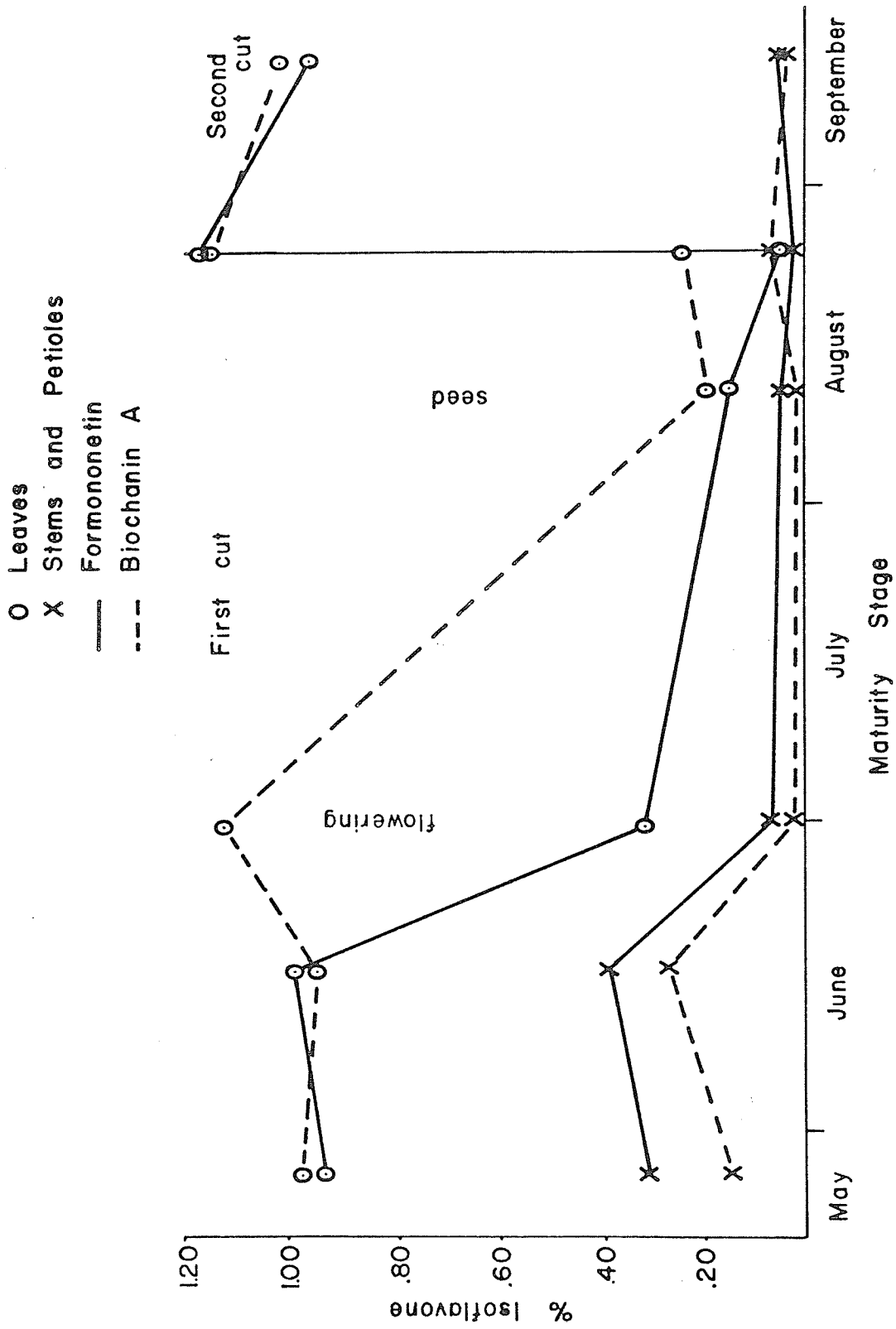

Fig. 3. Isoflavone content at various stages in Svalof 059 red clover.

However, by chemical methods lower isoflavone content was obtained during flowering stage than in a later stage (15).

Biochanin A content remained high two weeks longer than formononetin in both varieties. This is in contrast to the findings of Schultz (12) who stated that formononetin remained constant while biochanin A decreased to 50% in the vegetative stage. Though there is some evidence that a flavonoid compound stimulates floral formation (9), this does not appear to be the case here, since there would be a natural selection toward high isoflavone plants. It appears that the isoflavones are broken down at the time of flowering, so that their metabolites would be available for the flowering process.

Isoflavone content in stems and petioles were low at all stages and in both varieties. However, the extraction method used estimated only the free isoflavones in the stems and petioles, since it relies on endogenous enzymes to hydrolyze the bound isoflavones. These enzymes are present only in the leaf blades (1).

When stem and petiole extracts were chromatographed, a fluorescent spot and a spot colored with diazotized sulfanilic acid could be detected at higher Rf's than those corresponding to formononetin and biochanin A. These likely were the glycosides of the respective isoflavones. Nevertheless, the glycosides appeared to be in small quantities. This is in agreement with other workers who have reported lower isoflavone content in stems and petioles by chemical assay in red and subterranean clover (11, 12), and low estrogenic activity by biological tests in

subterranean clover (6).

This study shows the importance of sampling time if one is interested in the variation between the different genotypes. For breeding programs, when selections are made for low- or high-isoflavone varieties, samples should be taken before flowering. Environmental factors may be important in determining isoflavone content. The higher temperatures in the summer result in higher respiration rate and metabolism of the isoflavones. The different photo-periods in New Zealand may explain the discrepancy in isoflavone content estimated in that country.

A biological test, particularly with sheep, should be useful in confirming the changes in estrogenic activity at various stages. If formononetin and biochanin A are substances mainly responsible for estrogenic activity, this study would indicate that red clover after the flowering stage would be suitable for livestock in reducing reproductive difficulties. However, the early vegetative growth may be beneficial for fattening stock or for stimulating milk production.

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EFFECTS OF SAMPLE PREPARATION METHODS ON THE ISOFLAVONES OF RED CLOVER

ABSTRACT

Isoflavone levels in red clover samples, which were oven-dried at temperatures below 80°C or frozen, did not differ from fresh clover samples. Quantitative estimation of isoflavones can be made on dry red clover samples provided they are first moistened to hydrolyze the isoflavone glycosides. Partial hydrolysis of glycosides resulted from drying at high temperatures or from freezing treatments.

INTRODUCTION

Red clover (Trifolium pratense) has been shown to contain high levels of the isoflavones, biochanin A and formononetin, and trace amounts of daidzein and genistein which are estrogenically active (8, 10). The isoflavones are bound as glycosides and it is necessary to liberate the isoflavones before they can be quantitatively estimated. Beck (1) obtained satisfactory results only when fresh clover leaves were crushed with water to release the hydrolytic enzymes prior to extraction with boiling ethanol.

The purpose of this study was to investigate the effects of sample preparation methods on biochanin A and formononetin levels in red clover.

MATERIALS AND METHODS

The isoflavones, biochanin A and formononetin, were analyzed by paper chromatography as described previously (7). The developing time was increased to at least 6 hours from 3½ hours to completely separate the two isoflavones.

In the study on the effect of sample preparation methods on isoflavone content, red clover leaves of Svalof 059 and Hermes varieties were collected in July and August, 1967, respectively. The Svalof 059 leaves (about 400 g) were rendered homogeneous and divided into seven portions. Moisture content was determined on one portion. On another portion, triplicate 5 g samples were crushed with sand and water and analyzed for isoflavones. The other portions were frozen in a deep freezer, freeze-dried, dried at room temperature (3 days) and oven-dried at 80°C overnight, respectively, before triplicate 5 g samples (fresh weight equivalent) were taken for isoflavone analysis. The remaining portion was subjected to 5 days of soaking in water to simulate wet haying conditions before it was dried, and triplicate 5 g samples were taken for isoflavone analysis. Dry samples were first moistened overnight, in 1 ml water for each g sample, before extraction twice with 70% ethanol. "Free" isoflavones (non-glycosides) were determined in duplicate from each portion by immersing the samples in boiling 95% ethanol.

The effect of drying temperatures and hydration time was studied

on a batch of Hermes variety leaves in a similar manner. Only formononetin was analyzed in these samples.

RESULTS

Small but insignificant differences in the isoflavone content were observed between various sample preparation methods except where samples were soaked for 5 days (Table 2). Oven-drying at 80°C or freezing samples resulted in some liberation of isoflavones from their glycosides (Table 3). Drying at room temperature did not increase the free isoflavone content.

No appreciable loss of formononetin occurred when samples were dried at room temperature or at 60°C (Table 4). However, at 92°C there was a significant decrease in formononetin content and a visible change in the color of the sample. There was no difference in formononetin content when samples were hydrated from ½ hour to 24 hours (Table 4).

DISCUSSION

The experiments demonstrated that it is not essential to use fresh clover samples for isoflavone analysis. Beck (1) was able to obtain satisfactory results only by crushing fresh clover leaves to liberate hydrolytic enzymes, which in turn release the isoflavones from their glycosides. No appreciable loss of isoflavones occurred when samples were dried at temperatures below 80°C. It is conceivable that the isoflavones and the glycosides are in separate, adjacent compartments, and

Table 2. Isoflavone Content (% dry weight) of Svalof 059 Red Clover as Affected by Methods of Sample Preparation; Values are Means of Triplicates[+]

Preparation Method	Biochanin A	Formononetin	Total
Fresh clover	0.74	0.54	1.28
Dried at room temperature	0.53	0.62	1.15
Dried at 80°C	0.63	0.58	1.21
Dried at room temperature after soaking in water for 5 days	0.28**	0.32**	0.60**
Frozen	0.62	0.58	1.20
Freeze dried	0.65	0.46	1.11*
Average C.V.	16.6	12.4	6.7

+ Comparison was made with fresh clover samples by Dunnett's procedure.

* Significant at 5% level.

** Significant at 1% level.

Table 3. "Free" isoflavone content (% dry weight) of Svalof 059 Red Clover as Affected by Methods of Sample Preparation; Values are Means of Duplicates+

Preparation method	Biochanin A	Formononetin	Total
Fresh clover	0.29	trace	0.29
Dried at room temperature	0.11**	trace	0.11*
Dried at 80°C	0.29	0.30**	0.59**
Dried at room temperature after soaking for 5 days in water	0.12**	0.10**	0.22
Frozen	0.45**	0.15**	0.60**
Freeze dried	0.40*	0.23**	0.63**

+ Comparison was made with fresh clover samples by Dunnett's procedure.

* Significant at 5% level.

** Significant at 1% level.

Table 4. Formononetin Content of Hermes Red Clover (% dry weight) as Affected by Drying Temperatures and Hydration Time; Values are Means of Duplicates

Drying temperature	Hydration time	Formononetin
Room temperature	½ hour	0.67
	2 hours	0.68
	8 hours	0.67
	24 hours	0.67
	Boiling ethanol	0.13
60°C	24 hours	0.70
	Boiling ethanol	0.08
92°C	24 hours	0.43
	Boiling ethanol	0.10

treatments such as freezing and drying cause partial disruption of the separating wall. It was necessary to maintain drying temperatures below 80°C to prevent decomposition of isoflavones. Over 90°C, there was a change in the color of the sample with a decline in isoflavone content.

Other workers using mouse uterine weight bioassays detected a considerable loss in the estrogenic activity in alfalfa (3) on drying, as much as 75% in some samples, while in Ladino clover slight losses occurred (5) when samples were oven-dried. The chemical results presented here, however, are in agreement with bioassay analyses in subterranean and red clover (4, 6), in that little or no loss in estrogenically active compounds occurred when samples were rapidly dried. Leaching or destruction of isoflavones could occur, particularly in unfavorable, wet haying periods.

Two reasons have been suggested for the different effects of drying on estrogenic activity of red clovers and alfalfa. Swierstra (9) has suggested that the greater retention of the activity of red and subterranean clovers during prolonged storage of the meal reflects a greater stability of the isoflavones over coumestrol. Increased activity after drying of red and subterranean clovers compared with alfalfa and white clover may also be due to a less stable estrogen inhibitor or a more stable estrogen potentiator (2).

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INFLUENCE OF CYTOKININS ON ISOFLAVONE AND ANTHOCYANIN SYNTHESIS IN RED CLOVER SEEDLINGS

ABSTRACT

Kinetin and N-6-benzyladenine stimulated isoflavone production in red clover, particularly if applied at an early stage in the nutrient solution. The cytokinins induced a reduction in yield and moisture content and an increase in anthocyanin synthesis. Anthocyanin also appeared in mature red clover leaves when petioles were girdled but isoflavone level remained unchanged, suggesting that certain enzymes synthesising isoflavones may become inactive during maturation of the leaf. The anthocyanin was tentatively identified as cyanidin-3-bioside. Isoflavone leaching was retarded from red clover discs when these were floated on kinetin, benzyladenine or benzimidazole solution.

INTRODUCTION

Various plant growth regulators are known which evoke morphological changes and alter metabolic pathways. Auxins, such a indole-3-acetic acid (IAA), enhance water uptake resulting in cell elongation, while kinetin and related compounds, by inhibiting water uptake, cause stem shortening and stimulation of cell division. It has become accepted, however, that the basic action of cytokinins (9), as well as IAA and gibberellin (5), is a stimulation of ribonucleic acid (RNA) and protein

synthesis. In addition, the cytokinins have been shown to delay senescence in detached leaves of a number of species (3, 17) as determined by chlorophyll and protein loss.

This paper describes an investigation into the effects of auxins, cytokinins and a growth retardant on the oestrogenic isoflavones which are present in large quantities (10, 16) in red clover (Trifolium pratense). An attempt is made to correlate isoflavone synthesis with anthocyanin production and other metabolic changes in this species.

EXPERIMENTAL

Preliminary experiments were conducted to test various growth regulators that may affect isoflavone synthesis in red clover. Batches of 100 seeds of Robina and Altaswede varieties were planted in vermiculite in a Petri dish in the glasshouse and watered every second day. After about 10 days, each batch of seedlings was watered with 20 ml of Hoagland solution and 20 ml of various growth regulator solutions. These included indole-3-acetic acid (IAA), 2,4-dichlorophenoxyacetic acid (2,4-D), potassium salt of gibberellic acid (GA), chlormequat chloride (CCC), benzimidazole, kinetin and N-6-benzyladenine, in concentrations of 10^{-5} - 10^{-3}M. Application of the Hoagland growth regulator mixture was repeated every second day for 7 days. The unifoliate and trifoliate leaves were then harvested, dried at 70°C, weighed and analysed for the isoflavones, biochanin A and formononetin.

After it was established that only the cytokinins markedly affected isoflavone synthesis, an experiment was designed to study the effect of time of application of benzyladenine and kinetin on isoflavone synthesis, yield, chlorophyll content and anthocyanin production. Batches of 250 seeds were planted in vermiculite in 12.5 x 13 cm plastic trays and watered every second day. These seeds were grown in a Coldstream growth cabinet at 70°F, with illumination provided by 32 General Electric F96T12/CW fluorescent lamps supplemented with eight 60 W incandescent lamps with a light intensity of 1200 ft candle (1 ft candle = 10.76 lx). After 7 days, each batch of seedlings was watered with 50 ml of Hoagland solution, and either 50 ml of water or a cytokinin solution. This was repeated every second day. Three batches were used as control. The cytokinins were applied twice, two days apart, the first application being 7, 12 and 17 days after seeding. After 24 days, the leaves were harvested from all trays. A portion of the leaves (0.1 - 0.25 g) was analysed for chlorophyll content by acetone extraction, as described by MacKinney (7). Yield of dry matter and moisture content were determined on the remainder. Isoflavone content and anthocyanin estimation were determined on dried leaves.

The isoflavones were analysed by scanning the paper chromatograms as described previously (1). The extraction procedure was modified slightly because dry material was used, which was moistened with water before extraction with ethanol (2). The anthocyanin was extracted over-

night with 1% HCl in methanol and chromatographed on Whatman No. 3 chromatography paper in four solvent systems: butanol-acetic acid-water (BAW) (4:1:5 by vol.), upper phase; butanol/2 N-HCl (BuHCl) (1:1 by vol.), upper phase; acetic acid-HCl-water (HAc-HCl) (15:3:82 by vol.); and 1% HCl. The aglycone was obtained by hydrolysing the methanol extract with 2 N-HCl for 15 min and extracting it with isoamyl alcohol. It was identified by chromatographing it with known anthocyanidins, obtained from blueberry skins, on cellulose coated thin-layer plates using solvent systems of formic acid-HCl-water (10:1:3 by vol.) and n-amyl alcohol-acetic acid-water (2:1:1 by vol.) as described by Nybom (8).

A study was also made on the effect of plant growth regulators on leaf discs. The discs, 1 cm in diameter, were cut with a cork borer from red clover leaves in a quantity sufficient to almost completely cover a 10 cm Petri dish. They were floated, with adaxial surface uppermost, on 10 ml of water or solution in Petri dishes. The solutions used were IAA, 2,4-D, GA, CCC, benzimidazole, kinetin and N-6-benzyladenine in concentrations of 10^{-5} - 10^{-3}M. The solutions were changed every second day and the Petri dishes were kept in darkness at room temperature. After 7 days, the leaf discs were dried, weighed and analysed for isoflavones.

RESULTS

After one week of treatment, kinetin and benzyladenine, at concentrations of 10^{-3} and 10^{-4}M, retarded growth of red clover seedlings (Table 5). Benzyladenine was more effective than kinetin in inhibiting growth. At a concentration of 10^{-4} or 10^{-5}M, benzyladenine stimulated isoflavone synthesis; a concentration of 10^{-4}M kinetin was required for a stimulative effect. 2,4-D inhibited growth at concentrations of 10^{-5} - 10^{-2}M but only concentrations of 10^{-4} and 10^{-5}M resulted in an increase in isoflavone level. IAA, GA, CCC and benzimidazole did not have a significant effect on isoflavone level or growth of seedlings.

The time of application of the cytokinins was critical. When the 10^{-4}M kinetin or benzyladenine solution was applied early (i.e. with the appearance of cotyledons only), severe retardation of growth occurred (Table 6). The cytokinins resulted also in a considerable reduction in moisture content and shorter petioles of leaves. This had been frequently observed with cytokinin treatments. Although the cytokinins appeared to induce darker green coloration of leaves, no increase in chlorophyll content on a dry weight basis was found (Table 6). The appearance of darker green coloration was probably due to concentration of chlorophyll through loss of water.

Increased isoflavone level and growth retardation were associated with anthocyanin production (Table 7). Kinetin and benzyladenine, at 10^{-4}M concentration, resulted in anthocyanin synthesis, especially when

Table 5

Effect of Growth Regulators in Robina Red Clover Seedlings on Isoflavone Content and Yield.

Values are means of duplicates and expressed as % of control

Growth regulator	Concn., M	Yield	Biochanin A	Formononetin	Total
Control	-	100	100 (0.11+)	100 (0.30+)	100 (0.41+)
IAA	10^{-3}	69	92	109	104
	10^{-4}	84	178**	114	128
GA	10^{-3}	86	69	116	102
	10^{-4}	100	144	79	95
2,4-D	10^{-3}	50	131	109	116
	10^{-4}	51	278**	176**	200**
	10^{-5}	57	211**	141**	158**
CCC	10^{-3}	89	154**	119	129**
	10^{-4}	89	144	93	105
Benzimidazole	10^{-3}	73	100	122	116
Kinetin	10^{-3}	43	256**	83	124
	10^{-4}	75	311**	134*	176**
	10^{-5}	89	189*	103	124
N-6-Benzyladenine	10^{-3}	40	167*	117	129*
	10^{-4}	67	456**	148**	221**
	10^{-5}	82	400**	172**	226**

** Significant at 1% level according to L.S.D. procedure

* Significant at 5% level

+ % on dry weight basis

Table 6

Effect of Cytokinins in Altaswede Red Clover Seedlings on Yield, Moisture and Chlorophyll Content

Cytokinin	Time of application, days after seeding	Yield, g dry wt.	Moisture, % dry wt.	Chlorophyll, % of dry wt. a	b	total
Control		0.97	20.5	0.50	0.40	0.90
Kinetin:						
10^{-4}M	7	0.39	27.9	0.46	0.35	0.81
	12	0.60	29.6	0.33	0.31	0.64
	17	0.91	23.7	0.39	0.35	0.74
N-6-Benzyladenine:						
10^{-4}M	7	0.23	29.3	0.47	0.31	0.78
	12	0.57	30.0	0.29	0.26	0.55
	17	0.58	27.5	0.35	0.30	0.65
10^{-5}M	7	0.84	23.9	0.49	0.40	0.89
	12	0.95	22.3	0.46	0.39	0.85
	17	0.98	22.2	0.44	0.37	0.81

Table 7

Effect of Cytokinins in Altaswede Red Clover Seedlings on Isoflavone and Anthocyanin Content

Cytokinin	Time of application, days after seeding	Isoflavone content, % of dry wt. Biochanin A	Formononetin	Total	Anthocyanin*
Control		0.68	0.67	1.35	-
Kinetin:					
10^{-4}M	7	1.07	0.89	1.96	++
	12	1.03	0.91	1.94	+
	17	0.62	0.68	1.30	+
N-6-Benzyladenine:					
10^{-4}M	7	1.06	1.03	2.09	+++
	12	0.74	0.71	1.45	+++
	17	0.51	0.58	1.09	+
10^{-5}M	7	0.81	0.79	1.60	-
	12	0.91	0.84	1.75	-
	17	0.61	0.70	1.31	+

* Expressed as relative concentration: - (absent) to +++ (large amounts)

applied at an early stage.

Since isoflavone production increased in conditions which favoured anthocyanin synthesis, a study was made on the effect of girdling of petioles on the isoflavones in red clover leaves. This was done to selected leaves in four plants, as the cortex could be easily scraped off with a fingernail or razor blade. After a period of one week, girdled leaves showed considerable anthocyanin coloration, mostly in the veins, but there was no significant increase in either one or total isoflavone level (Table 8).

Partial identification of the anthocyanin was carried out. The anthocyanin, when chromatographed on cellulose thin-layer plates, had an R_f value similar to that of cyanidin and co-chromatographed with it. Chromatographic data strongly suggest that it is a cyanidin bioside. The R_f values from paper chromatography (Table 9) are close to the two common cyanidin biosides — cyanidin-3-sambubioside and cyanidin-3-sphoroside (6). No authentic compounds were available for co-chromatography.

In experiments with leaf discs, kinetin and benzyladenine very effectively retarded the loss of chlorophyll, even at concentrations of 10^{-5}M, and consequently the isoflavone level was higher than in the control after 7 days (Table 10). Conversely, benzyladenine at 10^{-3}M concentration resulted in necrosis and a considerable loss in isoflavones. 2,4-D, at 10^{-3}M concentration, had a similar effect. Benzimidazole

Table 8

Effect of Petiole Girdling of Red Clover Leaves on Isoflavone Content and Anthocyanin Production

Plant No.	Isoflavone content, % of dry wt.			Anthocyanin
	Biochanin A	Formononetin	Total	
1	0.22	0.66	0.88	present
Control	0.18	0.70	0.88	absent
2	0.70	0.98	1.68	present
Control	0.47	0.97	1.44	absent
3	0.38	0.57	0.95	present
Control	0.29	0.55	0.84	absent
4	0.30	0.45	0.75	present
Control	0.37	0.54	0.91	absent

Table 9

Chromatographic data of Red Clover Anthocyanin in Different Solvents

Anthocyanin	R_f			
	BAW	BuHCl	HAc-HCl	1% HCl
Red clover anthocyanin	0.27	0.22	0.52	0.26
Cy3-sambubioside*	0.36	0.24	0.51	0.24
Cy3-sphoroside*	0.33	0.22	0.61	0.34

* R_f values of these anthocyanins are from Harborne (6).

Table 10

Effect of Growth Regulators in Red Clover Leaf Discs on Isoflavone Level

Values are means of duplicates, except for benzimidazole which are means of quadruplicates, and are expressed as % of control

Growth regulator	Concn., M	Biochanin A	Formononetin	Total
Control	-	100 (0.40+)	100 (0.58+)	100 (0.98+)
IAA	10^{-3}	106	58**	75**
	10^{-4}	102	104	103
GA	10^{-3}	74	97	90
	10^{-4}	90	109	100
2,4-D	10^{-3}	72*	63**	66**
CCC	10^{-3}	118	83	96
Benzimidazole	10^{-3}	128**	121*	124**
	10^{-4}	115	102	108
	10^{-5}	128**	120*	124**
Kinetin	10^{-3}	140**	133**	136**
	10^{-4}	119*	121*	120**
	10^{-5}	117	137**	128**
N-6-Benzyladenine	10^{-3}	44**	51**	48**
	10^{-4}	138**	132**	134**
	10^{-5}	140**	121*	130**

** Significant at 1% level according to L.S.D. procedure
* Significant at 5% level
+ % on dry weight basis

retarded chlorophyll loss to a much lesser extent, but it had the same effect as the cytokinins in maintaining a higher isoflavone level. An unusual observation was that benzimidazole was more effective at a concentration of 10^{-3} or 10^{-5}M than at 10^{-4}M in preventing isoflavone loss.

DISCUSSION

Benzyladenine and kinetin retarded growth of red clover seedlings when applied in concentrations of 10^{-3} and 10^{-4}M; benzyladenine was more effective than kinetin in inhibiting growth. Wittwer and Dedolph (15) also found that benzyladenine was a better growth inhibitor of tomatoes and cucumbers. They reported that, after application of the cytokinins in a nutrient solution, in concentration range 10^{-5} - 10^{-7}M, the root, stem and leaf growth of cucumbers and tomatoes were inhibited several weeks. At concentrations higher than 10^{-5}M, the plants were killed. It is likely that red clover seedlings would have been killed at 10^{-4}M and possibly at 10^{-5}M cytokinin concentration if they had been allowed to grow for several weeks.

It should be noted that the yield of dry matter usually decreased with a corresponding increase in isoflavone level with cytokinin treatment. This indicates a possible suppression of synthesis of plant products other than isoflavones. However, this shift could not account for the significant increase in isoflavone level with 10^{-5}M benzyladenine, while the yield reduction was small, if any. Coupled with the fact that

anthocyanin production occurs with cytokinin treatment, it would appear that cytokinins result in an accumulation of carbohydrates which serve as precursors for flavonoid compounds. Thain and Robinson (12) have also reported the association of anthocyanin with higher isoflavone level in red pigmented leaves of subterranean clover when the plants were grown in abnormal conditions.

The same situation does not appear to exist in matured leaves. Girdling of petioles probably resulted in phloem damage and blockage of basipetal translocations of carbohydrates. The fact that this resulted in anthocyanin synthesis but did not affect isoflavone level suggests that the availability of enzymes and not of carbohydrates is the limiting factor in isoflavone synthesis in matured leaves. Decline of the isoflavone level with age in red clover (1) and in subterranean clover (11) may be further evidence that the enzymes become inoperative as the leaf matures and no de novo synthesis occurs.

The effect of the cytokinins on the synthesis of isoflavones and anthocyanins in red clover differs from the inhibitory effect on anthocyanin synthesis caused by benzimidazole, 2,6-diaminopurine, guanine and kinetin in the duckweed, Spirodela oligorrhiza (13). The synthetic pathway of flavonoids thus appeared to be different in the two organisms.

Retardation of isoflavone loss by the cytokinins and benzimidazole is probably due to preservation of the membranes of the organelles in which the isoflavones are located. Benzyladenine and kinetin have been

found to preserve the chloroplasts in mature Brussels sprout leaves (4), while benzimidazole maintained chloroplast structure in wheat leaves (14). The isoflavones have been presumed to be located in the chloroplasts and the results would indicate that the cytokinins may retard isoflavone leaching from the chloroplasts.

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VARIETY, STAGE OF DEVELOPMENT AND ENVIRONMENTAL EFFECTS ON ISOFLAVONE CONTENT

ABSTRACT

Genetic variation of the isoflavones, biochanin A and formononetin in the red clover was observed. The combined level however did not significantly differ in the four clones tested. Long photoperiod and low temperature favored isoflavone synthesis. Highest isoflavone concentration occurred in the folded leaf stage. This was followed by a gradual decline of biochanin A and formononetin until senescence when another isoflavone, daidzein appeared in some clones.

INTRODUCTION

Red clover (Trifolium pratense) has been shown to contain high levels of the isoflavones, biochanin A and formononetin and trace amounts of daidzein and genistein which are estrogenically active on the animals that ingest the forage.

Although considerable work has been done on the genetic and environmental factors affecting the isoflavone content of subterranean clover (T. subterraneum), few studies have been conducted on red clover. The formation of isoflavones in subterranean and red clover appears to be determined genetically (5, 6). However, substantial variation due to environmental factors and ontogenetic changes occurs. In subterranean clover, high intensity light favors isoflavone synthesis (10). Higher

isoflavone level was found in subterranean clover when grown in below optimum temperatures (9). As the leaf unfolds and develops, there is a decline in the isoflavone concentration (11).

The purpose of this experiment was to study the genetic and environmental factors and ontogenetic changes in isoflavone level of red clover varieties.

MATERIALS AND METHODS

Four red clover plants from three varieties, Altaswede, La Salle and Manhardy were cloned by splitting the crown as described by Barrales and Ludwig (1) and transferred into pots. The varieties used are recommended Canadian varieties. La Salle is an early maturing double-cut variety, while Altaswede and Manhardy are single-cut varieties. After two weeks growth in the greenhouse, the clonal plants were transferred to two Coldstream growth cabinets. Illumination was provided by fluorescent and incandescent lamps at an intensity of 1200 f.c. at pot level.

A factorial arrangement of duplicate plants of the four selections at two photoperiods (8 and 16 hours light) and at two temperatures (60^{o}F and 80^{o}F) was employed. The illumination period was set for 16 hours in each growth cabinet and in order to obtain an 8 hour photoperiod, boxes made with black polyethylene were placed over the plants for 16 hours. After growing under these conditions for a month, about half of the leaves representing all stages of development were collected from each plant. These were dried to obtain moisture content and analyzed for isoflavones

(2, 3).

Since growth was poor in the 60°F growth cabinet, after one month the temperature was raised to 70°F, while the 80°F cabinet remained at the same temperature. After a month's growth at these conditions, the samples were again taken and analyzed. An analysis of variance was conducted on the isoflavones of samples collected after one month's growth in the growth cabinet.

To study the isoflavone level at various stages of development, leaves at three different maturity stages from three selections were collected and analyzed for biochanin A and formononetin. In addition, daidzein, a fluorescent isoflavone was estimated fluorometrically in paper chromatograms developed in 2 N ammonia similar to formononetin. It was well separated from formononetin.

RESULTS AND DISCUSSION

Each isoflavone and the total isoflavone content was affected by photoperiod and temperature (Tables 11 and 12). Higher concentrations were found with longer photoperiods and lower temperatures. The quantity of each isoflavone varied significantly among the clones, but the total isoflavone content was not significantly different in the four clones tested. Statistical analysis was conducted on the interaction of the factors but they were not significant and therefore included in the error.

Table 11. Isoflavone Content of 4 Red Clover Selections After One Month's Growth under 2 Photoperiods and 2 Temperatures. Values are Means of duplicates (% dry weight).

	Biochanin A		Formononetin		Total	
Temperature 60°F						
	Photoperiod (hours)					
	8	16	8	16	8	16
Altaswede	0.90	1.22	0.46	0.48	1.36	1.80
Altaswede	0.84	1.35	0.62	0.82	1.46	2.17
La Salle	0.73	0.98	1.04	1.50	1.77	2.48
Manhardy	0.88	0.72	1.04	1.40	1.92	2.12
Temperature 80°F						
	Photoperiod (hours)					
	8	16	8	16	8	16
Altaswede 1	1.16	1.26	0.42	0.37	1.58	1.63
Altaswede 2	0.47	1.27	0.49	0.65	0.96	1.92
La Salle	0.38	0.51	0.73	0.90	1.11	1.41
Manhardy	0.29	0.40	0.90	1.00	1.19	1.40

Table 12. Analysis of Variance for Isoflavone Content (% dry weight) After One Month's Growth at two Photoperiods and two Temperatures.

		Mean square		
Source	D.F.	Biochanin A	Formononetin	Total
Selections	3	0.5730*	0.7508**	0.0143
Photoperiod	1	0.5304*	0.2926*	1.6110**
Temperature	1	0.4278*	0.5050**	1.8625**
Error	26	0.0767	.0621	0.1849

** Significant at 1% level
* Significant at 5% level

After one month's growth, only one plant (a La Salle selection) was flowering and that was at 80°F and 16 hours light. However, after two months at these conditions, all the plants were flowering, though there was little change in the isoflavone concentration (Table 13). Since at 60°F, the red clover plants made little growth (Table 14) in one month, the temperature of the growth cabinet was raised to 70°F, a more favorable growing temperature. After growing at these conditions for a month the La Salle and Manhardy selections reached the flowering stage. There was also a decline in isoflavone concentration and the level was similar to that of the plants grown at 80°F (Table 13).

After one month's growth, a significant negative correlation coefficient (-.82) was obtained between the biochanin A and formononetin content, while after two month's growth, the correlation coefficient (-.07) was not significant.

Higher isoflavone concentrations occurred when red clover plants were grown under a 16 hour photoperiod. This was also reported by Rossiter and Beck (10) in subterranean clover and it is generally believed that flavonoid synthesis depends on photosynthesis to provide the carbohydrates. Light is the most important external factor for the full development of anthocyanin colour in fruits such as the apple and strawberry and in most garden flowers.

Growth at 60°F was slow and anthocyanin coloration occurred particularly with a 16-hour photoperiod. Since isoflavone level was higher at

Table 13. Comparison of Isoflavone Content After One and Two Month's Growth under Photoperiods of 8 Hours and 16 Hours (in brackets). Values are mean of duplicates and expressed on % of dry weight basis.

a) After one month's growth.

Cabinet and Temperature	Biochanin A	Formononetin	Total
1 (60°F)	0.83 (1.06)	0.79 (1.08)	1.62 (2.14)
2 (80°F)	0.58 (0.86)	0.64 (0.73)	1.22 (1.59)

b) After two month's growth. The 60° growth cabinet was raised to 70°F after one month. The 80°F cabinet remained at the same temperature.

1 (70°F)	0.38 (0.60)	0.66 (0.83)	1.04 (1.43)
2 (80°F)	0.43 (0.62)	0.84 (0.85)	1.27 (1.47)

Table 14. Comparison of Isoflavone Content and Yield (g. dry weight) under Different Growing Conditions.

	60°F		80°F		Total
Photoperiod	Yield	Isoflavone	Yield	Isoflavone	Yield
8 hours	0.731	1.62	0.996	1.22	1.727
16 hours	0.824	2.14	1.547	1.59	2.371
Total	1.555		2.543		

60°F, it would appear that a temperature which favors anthocyanin production also favors isoflavone synthesis. Rossiter and Beck (9) reported that in subterranean clover a higher isoflavone level occurred when plants were grown at 15°/10°C day-night temperatures than at 36°/31°, but at 9°/4° diurnal temperatures there was a decrease in isoflavone level. However, it should be recognized that it may not be justifiable to make comparisons here since Rossiter and Beck used diurnal fluctuations. Since low temperature delays maturity, the high isoflavone concentration could be due to slower ontogenetic changes in the red or subterranean clovers.

The optimum growing conditions found for red clover were unlike those of Gist and Mott (4) in that they reported optimum growth at a lower temperature, 60°F and 1200 f.c. light for 12 hours. However, they worked with seedlings while the plant material used here had just completed a summer's growth before they were transferred to the growth cabinets. On per plant basis highest isoflavone production was obtained at 16 hour photoperiods (Table 14). It would be interesting to determine isoflavone production under conditions in the Gist and Mott experiments since their optimum growth conditions also appears to be favorable for isoflavone production.

Since variation in the individual isoflavones, formononetin and biochanin A, was observed, it should be possible to select for a strain high or low in a particular isoflavone. However, in the four clones

studied the total isoflavone level was constant (Table 12). A negative correlation found between formononetin and biochanin A in red clover parallels that of subterranean clover where a negative correlation between biochanin A and genistein was reported by Morley and Francis (6). It would appear that a genetic mechanism controls the relative amount of the two isoflavones, while environmental factors which influence the carbohydrate pool affects the isoflavone level. This seems to hold true in red clover before flowering.

The isoflavone concentration was highest in folded leaves (Table 15). Both biochanin A and formononetin levels decreased with age, while another isoflavone, daidzein, appeared in senescent leaves, with one selection (La Salle) in particular having larger amounts. The pattern of isoflavone level in red clover followed closely that of subterranean clover (11) in that isoflavone content of leaf declined with age.

The appearance of daidzein at maturity was also reported by Rossiter and Beck (11) in subterranean clover. The daidzein level was high in the clone which was high in formononetin content. This would infer that formononetin is demethylated to daidzein as it is in the rumen (8). The immediate conversion of daidzein to equol in the rumen (7) could reflect the good correlation between formononetin content of subterranean clover and estrogenic activity in sheep.

Table 15. Isoflavone Content of Leaves at Three Stages of Maturity.

(B - biochanin A, F - formononetin, D - daidzein)
(% dry weight)

	Stage of Leaves										
	Folded			Fully expanded				Senescent			
Clone	B	F	Total	B	D	F	Total	B	D	F	Total
Altaswede 1	0.91	0.89	1.80	0.67	-	0.35	1.02	0.70	0.07	0.34	1.11
Altaswede 2	0.64	0.84	1.48	0.54	-	0.60	1.14	0.30	0.03	0.23	.56
La Salle	0.41	1.88	2.29	0.27	0.02	0.83	1.12	0.22	0.34	0.44	1.00

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COUMESTROL CONTENT OF FORAGE LEGUMES AS RELATED TO VARIETIES, STAGE OF MATURITY AND FUNGAL PATHOGENS

ABSTRACT

Coumestrol content was low in recommended Canadian alfalfa varieties at normal harvesting time. However, the level rose sharply during the late stages of maturity due to fungal pathogens. No coumestrol was detected in red clover, while white clover and birdsfoot trefoil had slight amounts. Inoculation with L. briosiana resulted only in slight increase in coumestrol content. Spraying with dithane M-45 reduced considerably but did not eliminate coumestrol synthesis in alfalfa. Varying amounts of coumestrol were detected in annual medics at late stages grown in the field.

INTRODUCTION

Coumestrol has been established to be the primary estrogen-like compound in alfalfa, ladino clover and other legumes (2, 9). In addition, high levels of coumestrol in field grown plants of M. littoralis, M. truncatula Gaertn., M. scutellata (L.) Mill., and M. polymorpha were reached at advanced maturity (5). Foliar pathogens were found to be the principal agents associated with accumulation of coumestrol in alfalfa (3, 7).

The purpose of this study was to investigate coumestrol levels of locally grown alfalfa, and other forage legume varieties. The influence of local environment on coumestrol of annual medics and the feasibility of foliar spray in controlling coumestrol level was also investigated.

MATERIALS AND METHODS

(a) Analytical Procedure for Coumestrol Determination

Samples were analyzed for coumestrol using modifications of the procedure of Livingston et al. (6). The samples were dried, rehydrated with water and methanol was added (20 ml for 1 g sample). After storage in the dark for two days, the solvent was filtered off and the chlorophyll and lipids were removed with petroleum ether. The methanol was removed in a flash evaporator and the coumestrol was extracted with ethyl ether twice. The ether extractions were combined and the ether was removed in a water bath. The residue was taken up in acetone (1 ml representing 1 g). 1 - 10 ul was spotted on Whatman No. 1 paper and the paper was developed for 16 hours in acetic acid-water (50/50% v/v) solvent.

Samples with high coumestrol content were soaked in 70% methanol (5 ml for 1 g) for two days, filtered and chromatographed directly. 20 ul was spotted on chromatographic paper.

Along with unknown extracts, coumestrol references of 0.05 to 0.5 ug were spotted and chromatographed. The chromatograms were dried,

cut into strips and scanned on the fluorometer. A primary filter, Corning No. 5970, was used to screen out the visible light from the ultraviolet light and a narrow band secondary filter having peak transmittance at 465 mu was employed. The amount of coumestrol in the extract was determined from the standard curve, which was obtained by plotting the amount of coumestrol spotted against peak height.

A sample of coumestrol was obtained from Dr. Bickoff, Albany, Cal. A coumestrol solution of 0.05 ug/ul was prepared in ethanol-benzene as a standard. Since a small amount of coumestrol was available which may be impure, it was not possible to prepare an accurate standard by weighing the sample. The final concentration was, therefore, checked spectrophotometrically at 343 mu and calculated using the molecular extinction coefficient of 26,950.

(b) Samples for coumestrol determination.

(1) Coumestrol content of Canadian alfalfa varieties.

Four recommended Canadian alfalfa varieties, Beaver, Ladak, Rambler and Vernal were grown at Carman, Manitoba in 1966. First and second cut samples were collected at flowering stage on June 30 and August 15, respectively. The samples were dried and analyzed for coumestrol.

(2) Stage of maturity of various forage legumes.

Samples of six varieties of alfalfa (M. sativa), two varieties of red clover (T. pratense), Svalof 059 and Tamisto, and one each of early type white clover (T. repens) variety, S100 white clover and birdsfoot

trefoil (Lotus corniculatus), USDA P.I. 234786, were collected at eight stages ranging from bud to seed stage of first cut and bud and flower stage of second cut in the summer of 1966. The samples were oven-dried at 70°C and duplicate 5 g samples were used for coumestrol determination. Coumestrol content was also determined on matured alfalfa plants grown in the greenhouse.

(3) Variability and effect of fungicide on coumestrol at various stages.

Five strains of alfalfa, grown in the field were used to determine the effect of fungicidal control of foliar diseases on coumestrol: The strains and their origin were:

	P.I.	Origin
Q 81	199280	Portugal
Q 115	220808	"Tuma", Sweden selection
Q 167	256004	France
Q 192	237722S	Germany
Q 191	236605S	France (leaf spot resistance)

3 plants from each of the strains Q 81, Q 115, Q 167 and Q 192 were sprayed and a similar number were left unsprayed as checks. Also five plants from a single clone selected from the Q 191 strain was sprayed and five were left unsprayed to study variation within a clone. The sprayed and unsprayed plants were alternated. An aqueous solution of Dithane M-45 was applied with a hand sprayer. Spray applications contained 2 g Dithane M-45 per gallon of water with 1 ml Tween 20 to promote wetting and were made once a week at a rate of 1½ gallons for 17

plants. Spraying was begun on June 2 at bud stage and discontinued on August 17. Also 3 plants from the same Q 191 clone were grown in the greenhouse to study changes in coumestrol content at these conditions. Samples were collected every three weeks and stored frozen. At the time of analysis they were oven-dried and analyzed for coumestrol content. Single determinations were made on each sample.

Coefficients of variation were calculated on sprayed and unsprayed plants, for variation within a clone, within varieties and between varieties at the latter two stages.

(4) Inoculation of alfalfa plants with Leptosphaerulina briosiana (Poll.).

Sporulating cultures of L. briosiana were produced on V-8 agar medium as described by Martinez and Hanson (10). The inverted plates were then suspended over individual Rambler alfalfa plants by taping the plants to steel supports and covering the pots with plastic bags for 3 days to maintain humid conditions. Leaf samples were collected after 6 - 12 days and analyzed for coumestrol. Samples from check plants were also taken.

(5) Coumestrol content in annual Medicago spp.

Various annual medics were grown to maturity in the greenhouse and in the field. Samples collected at maturity (pod stage) from the greenhouse included M. auriculata, M. hispida, M. intertexta, M. littoralis, M. lupulina, M. maculata, M. tribuloides and M. turbinata. Samples harvested in the field were from two stages - flowering and pod, and

were collected on July 27 and August 22, 1967 respectively. The species included M. arabica, M. auriculata, M. intertexta, M. littoralis, M. lupulina, M. maculata, M. murex, M. orbicularis, M. scutella, M. tornato, and M. tribuloides. The samples were dried and analyzed for coumestrol.

RESULTS AND DISCUSSION

(1) Analytical Method

Fluorescence from as little as 10^{-2} ug coumestrol could be observed in paper chromatograms under ultraviolet light. This made it possible to prepare a calibration curve ranging from 0 to 0.4 ug coumestrol. This is more sensitive than that reported by Livingston et al. (6) on the fluorometer designed by Bailey (1) with a range of 0.2 to 1.0 ug. However, Loper (private communication) was also able to improve the sensitivity of Bailey's fluorometer. A linear relationship was obtained between coumestrol content and peak height. As coumestrol did not tail in chromatograms, it was not necessary to determine the area under the peak as it was for formononetin. Some overlapping of other fluorescent spots, presumably coumestans with coumestrol occurred particularly in samples high in coumestrol content.

(2) Coumestrol content at various stages of maturity of forage legumes and alfalfa varieties

Considerable rise in coumestrol level in alfalfa varieties was noted as the plants matured, which began from about the flowering stage (Fig. 4). Coumestrol content was again low in young leaves in the

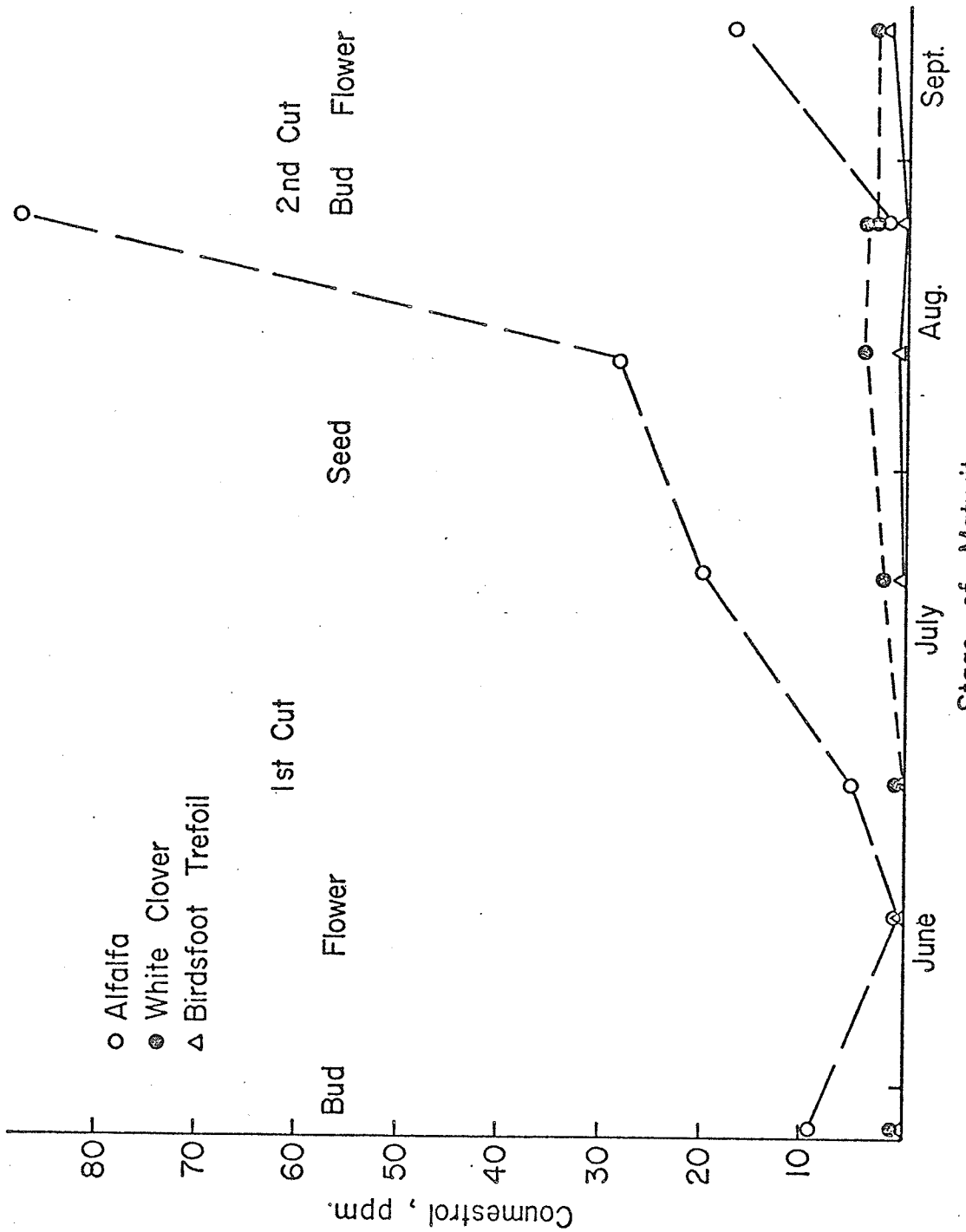

Fig. 4. Coumestrol content at various stages of forage legumes.

second cut. A coumestrol content of 3-4 ppm was detected in white clover sampled collected in the latter part of summer. A trace amount of coumestrol was present in only two birdsfoot trefoil samples. Coumestrol was not detected at any stage in red clover. In samples of 20 Rambler matured alfalfa plants grown in the greenhouse, coumestrol content ranged from 0 to 5.7 ppm with a mean of 2.1 ppm.

The high coumestrol level in matured alfalfa plants was expected as they were heavily infested with leaf spots. Further proof that infection induces coumestrol synthesis was evident from greenhouse alfalfa plants which were apparently free of leaf spots at maturity and contained little coumestrol. Coumestrol content was again low in the second cut. This confirms the general belief that young leaves are more resistant to fungal infestation since a heavy inoculum has been built up by the time the second cut was made. The low coumestrol content in the early type white clover was interesting in view of the high coumestrol levels reported in the Ladino variety. Although some coumestrol has been reported in subterranean clover (4), no coumestrol was detected in the red clover.

Coumestrol content of the recommended alfalfa varieties for Canada were low at flowering stage (Table 16) which is the normal harvesting time. A slight increase in coumestrol level occurred in the second cut presumably due to a buildup of inoculum during the summer. However, coumestrol level was too low at both harvests to be of concern regarding reproduction difficulty.

Table 16. Coumestrol Content (ppm) of four Alfalfa Varieties at Flowering Stages from Two Cuts.

Variety	Cut	
	1st	2nd
Beaver	1.5	10.2
Ladak	1.8	6.8
Rambler	3.0	8.8
Vernal	1.7	6.1

(3) Variability and effect of fungicide on coumestrol at various stages

In all alfalfa varieties, coumestrol content was low or absent at flowering or pre-flowering stage (June 2, June 23, and July 14) in dithane-sprayed or unsprayed plants (Table 17). After this period there was a considerable increase in coumestrol level with over 200 ppm in some samples. Application of a fungicide caused a substantial decrease in coumestrol content. The fungicide did not completely prevent fungal infection because it frequently was washed away by rains and consequently some leaf spots appeared. This was reflected by a substantial coumestrol content of 32.5 to 86.3 ppm in dithane-sprayed samples collected on August 24. Therefore, it could not be determined if factors other than pathogens would be responsible for coumestrol synthesis in the field, particularly at late stages of maturity. Plants from a selection of Q191 strain appeared free of pathogens if grown in the greenhouse and thus coumestrol level was low.

Coefficients of variability were high in dithane-sprayed and control plants in the pod stage (August 4) samples (Table 18). At this stage the plants were in various degrees of fungal infestation and therefore, not much significance can be attached to the high CV. However, in the more matured seed stage (August 24) infestation was heavy and more uniform and the CV was lower. At this stage the higher CV between and within varieties than within a clone in the unsprayed plants implies that there is a variation in susceptibility to leaf spots among alfalfa plants.

Table 17. Coumestrol Content (ppm) of Four Alfalfa Varieties and One Clone, Unsprayed and Sprayed (in brackets) with Dithane at Five Stages.

Variety of Clone	Coumestrol content (ppm)				
	June 2	June 23	July 14	Aug. 4	Aug. 24
Q 81	0 (0)	0 (0)	4.8 (1.6)	54.1 (6.2)	234.5 (32.5)
Q 115	0 (0)	0 (0)	0.9 (0.3)	44.0 (4.5)	209.9 (53.5)
Q 167	0 (0)	0 (0)	1.4 (0.3)	32.0 (2.0)	165.1 (39.8)
Q 192	0 (0)	0 (0)	1.6 (1.9)	29.2 (10.3)	186.4 (86.3)
Q 191 clone*	0.6 (0.3)	0 (0)	0.6 (0)	11.6 (0.5)	135.7 (38.9)
Q 191 clone in greenhouse	2.7	0.6	4.5	5.9	2.0

* Mean of 5 plants. All others are means of 3 plants.

Table 18. Coefficient of Variability of Coumestrol Content in Unsprayed and Dithane-sprayed (in brackets) Alfalfa Plants at the Two Late Stages of Maturity

	Coefficient of variability	
	Aug. 4	Aug. 24
Within Q 191 clone	101 (71)	24 (68)
Within varieties	82 (66)	49 (39)
Among varieties	83 (91)	54 (55)

Table 19. Coumestrol Content of Annual Medic Species at Maturity Grown in the Greenhouse

Species	Coumestrol content (p.p.m.)
M. auriculata	0
M. hispida	10.6
M. intertexta	15.2
M. littoralis	0
M. lupulina	0
M. maculata	17.7
M. tribuloides	7.6
M. turbinata	12.5

(4) Coumestrol content of alfalfa plants inoculated with Leptosphaerulina briosiana

Rather discordant results were obtained when Rambler alfalfa plants were inoculated with L. briosiana. Generally, the fungus induced coumestrol synthesis but in some inoculated plants no coumestrol was detected even though all plants showed minute leaf spots. The mean coumestrol content of 10 plants was 8.9 ppm after 6-12 days, while the 3 control plants averaged 0.9 ppm. In the inoculated plants, the range was from 0 to 28.0 ppm coumestrol and other fluorescent spots probably coumestans, appeared on the paper chromatograms.

The rise in coumestrol content was not as great as in Loper's experiments (7), who reported 71.7 ppm in heavily infected leaves. This was probably due to a lighter infection obtained in this study as the lesions were small. A longer period of incubation with re-inoculation would probably result in a higher coumestrol content. No work was done with Pseudopeziza medicaginis which was responsible for the common leaf spot and was a better inducer of coumestrol synthesis (7). It appears possible to develop low coumestrol alfalfa strains by selecting for P. medicaginis resistance (8).

(5) Coumestrol content in annual Medicago species

M. auriculata, M. lupulina, and M. littoralis did not contain any coumestrol when grown to maturity in the greenhouse. Other annual medic species varied from 7.6 to 17.7 ppm coumestrol (Table 19). The annual

medics contained more coumestrol than alfalfa in the pod stage when in the greenhouse. Greenhouse grown plants were obviously free from fungal pathogens. It cannot be said that the coumestrol content of the various species was typical since only one strain of each species was studied.

Little or no coumestrol was detected in the annual medics at the flowering stage grown in the field (Table 20). However, there was a dramatic rise in coumestrol content as the plants reached the pod stage. Highest coumestrol concentration was in M. tribuloides with 63.0 ppm. Francis and Millington (5) obtained a range of 5 to 120 ppm coumestrol in the 8 strains of M. tribuloides that they investigated. The climatic conditions here, probably the photoperiod, was not favorable for flowering in M. intertexta and M. maculata and thus they remained in a vegetative state. However, there was some increase in coumestrol synthesis in these species as the leaves approached senescence.

The distribution of coumestrol in the various parts of the plant was not studied but it is of interest in that Francis and Millington (5) reported that field drying dramatically increased the coumestrol content of stems and burrs in annual medics but not in alfalfa. A small reduction in coumestrol content occurred in the leaves. A study of the mechanism of coumestrol synthesis in this situation would be interesting.

Table 20. Coumestrol Content (ppm) of Field-grown Medicago Species at Two Stages of Maturity.

	Flower (July)	Pod+ (August)
M. arabica	0	14.0
M. auriculata	0	
M. intertexta*	6.0	23.4
M. littoralis	0.8	
M. lupulina	0	29.6
M. maculata*	0	17.3
M. murex	0	2.8
M. orbicularis	0.7	
M. scutella	0	
M. tornato	0	1.9
M. tribuloides	2.5	63.0

* Did not flower under field conditions

+ Late germination prevented some species to reach seed stage.

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SUMMARY AND GENERAL DISCUSSIONS

A simpler and more precise method has been developed for quantitative estimation of isoflavones in red clover by paper chromatography. Although more time is required for the development of the paper chromatograms than for thin-layer chromatograms, the former method eliminates the time and expense of preparing thin-layer plates. An improvement might be made to find a more suitable solvent system since two different solvent systems were necessary for each of the two isoflavones. Considerable time could be saved if a single solvent system can be found which separates the two isoflavones and the spots are suitable for densitometric estimations. An improvement in the accuracy of biochanin A estimation can be obtained if a colorimetric reagent which does not require NaOH can be discovered since the latter results in an uneven background.

It was discovered that the extraction procedure could be simplified by using dried material. Crushing of fresh leaves to liberate the glycoside was not necessary since hydration of dried material appeared sufficient to activate the hydrolytic enzymes. Extensive purification of red clover extracts was not necessary as the high isoflavone concentration did not require spotting large amounts of the extract.

Like the situation in subterranean clover highest isoflavone content appeared in the metabolically active cells in the red clover. It should be interesting to study the biochemical mechanism of this decline.

The isoflavones may be the first compounds to be degraded as the plant reaches maturity by production of degradative enzymes or there may be a cessation of isoflavone production at senescence.

The study indicated that environmental factors have a similar effect on isoflavones in red clover as they have in subterranean clover in that light and cool temperatures favored isoflavone synthesis. However, this study on the effects of environmental and genetic factors on the isoflavone content in red clover should be considered a preliminary one in that a small number of plants were used in experiments of short duration. Because red clover is a cross pollinating plant it is necessary to obtain adequate samples to properly represent them. Another problem in sampling is the fact that isoflavone concentration varied considerably with stage of development of the leaves. Although an attempt was made to collect leaves representing the whole plant, the plants were not at the same stage of development when grown under various light and temperature conditions. Difficulty was also encountered in maintaining one factor while varying another. For instance, shielding the plants from light with black polyethylene boxes likely raised the temperature within them. Temperature can affect some edaphic factors such as soil moisture, aeration and even available nutrients.

Variation in the individual isoflavones was considerable among individual plants. However, there were some indications of a negative correlation between the two isoflavones. If such is the case, diffi-

culty will be experienced in selecting for strains low or high in total isoflavone content.

It should be fruitful to investigate the effects of herbicides used for weed control in red clover in view of the fact that 2,4-D resulted in a higher isoflavone content in the seedlings. Similar effect may occur with recommended herbicides for forage legumes such as MCPA (methylchlorophenoxyacetic acid) and 2,4-DB (2,4-dichlorophenoxybutyric acid). The study was carried out on soil applications on early stages of development and the effect may be different with foliar applications.

More work should be conducted on establishing better correlations between isoflavone content and estrogenic activity in livestock as some confusion still exists. Probably a multiple regression equation could be formulated on the individual isoflavones and other unknown factors. These unknown factors in red clover may be estrogen inhibitors or potentiators as appear to be present in alfalfa. Also consideration should be given to long term effects since it has been discovered that chemical estimates were not correlated with biological effects in ewes until after four years of grazing (2).

It has been well established that coumestrol level in alfalfa is chiefly influenced by infestation with fungi or aphids. In some of the annual medics, limited investigations indicated large variation among strains in apparent disease free plants. More work should be conducted to determine how large the genetic factor is in determining coumestrol level in other Medicago spp. Little is known about factors

affecting coumestrol synthesis in the Ladino clover. If suitable germ plasm can be found, they may be transferred to other species to obtain crops with the desired coumestrol level.

The role of the isoflavones in red clover and coumestrol in alfalfa has still not been fully established. Isoflavones may have some fungicidal properties or be insect repellants. Since Virtanen and Hietala (3) have reported fungal inhibitory effects of red clover isoflavone, it should be worthwhile to investigate the relationship between resistance to fungal diseases and isoflavone content.

Nothing is known about the mechanism that triggers synthesis of coumestrol and other coumestans. Although some coumarin-like compounds with structures similar to coumestrol such as pisatin in peas and trifoliorhizin in roots of red clover have been found to impart immunity to the plant, this phenomenon has not been shown to exist with coumestrol in alfalfa. These antibodies in plants of which synthesis has been stimulated in response to a fungal attack have been called phytoalexins (1). The phytoalexins are synthesized rapidly after an attack usually within a few hours, but in alfalfa indications are that the response of coumestrol synthesis to a fungal infection is much slower.

In view of the recent ban on use of diethylstilbestrol for fattening livestock, it may be profitable to investigate the growth promoting effects of the isoflavones, if strains with a high isoflavone level can be obtained. Some studies have been conducted on high coumestrol alfalfa as a growth promoting substance in sheep. High yield alfalfa

with a high coumestrol content may not be possible because of extensive defoliation that occurs when leaves are infested with fungal diseases. In addition, if an alfalfa stand is allowed to grow to maturity, a reduction in nutritional value occurs and this may explain some of the discouraging results obtained.

Since environment and genetic factors have different effects on estrogenic substances of red clover and alfalfa, different management practices are in order to obtain hay low in estrogenic substances in these two crops. Little can be done in red clover to reduce the level of estrogenic substances except harvesting the crop in an advanced stage of maturity when seed has set. In alfalfa, however, low coumestrol forage can be obtained if it is cut at the normal harvesting time, that is before it passes the flowering stage. In addition, coumestrol appears to be less stable than the isoflavones and treatments such as high temperatures, prolonged storaged or ensiling could reduce the level.

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