

**Factors Affecting Isoflavone Concentration
in Red Clover (*Trifolium pratense* L.)**

By

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Abstract

Red clover (*Trifolium pratense* L.) contains isoflavones, which are of interest because of their benefits for human health as well as their adverse effects on the fertility of farm animals. Isoflavone concentration in 10 cultivars ranged from 8923 to 12753 $\mu\text{g g}^{-1}$ DM averaged across sites, harvests, and years. One cultivar, 'Start', distinguished itself with particularly low isoflavone levels. Concentrations varied according to plant part with leaves having the highest levels followed by stems and inflorescences, when averaged across maturity levels and cultivars (11970, 4896 and 3297 $\mu\text{g g}^{-1}$ DM, respectively). Greatest content was found in leaves and stems during vegetative stages. Fresh herbage contained higher isoflavone content than either silage or hay (14464, 12200 and 11604 $\mu\text{g g}^{-1}$ DM, respectively). Foliar application of yeast extract, chitosan, and acetic acid elicitors overall showed modest (12-15%) increases in isoflavone concentration over untreated control plants. Differences were not observed between elicitor concentrations used.

Résumé

Le trèfle rouge (*Trifolium pratense* L.) contient des isoflavones, qui ont des effets bénéfiques sur la santé humaine mais aussi des effets néfastes sur la fécondité des animaux de ferme. La concentration en isoflavones de 10 cultivars a varié entre 8923 et 12753 $\mu\text{g g}^{-1}$ MS à travers différents sites, coupes, et années. Un cultivar, 'Start', se distingue des autres, ayant de faibles concentrations d'isoflavone. Les concentrations observées variaient selon les parties de la plante; les feuilles ayant les concentrations les plus élevées, suivies des tiges et des fleurs (11970, 4896 et 3297 $\mu\text{g g}^{-1}$ MS, respectivement). Les concentrations varient aussi selon le stade de maturité; les concentrations les plus élevées furent observées dans les feuilles et les tiges aux stades végétatifs. L'herbe fraîche contient des concentrations d'isoflavone plus élevées que l'ensilage ou le foin sec (14464, 12200 et 11604 $\mu\text{g g}^{-1}$ MS, respectivement). Des applications d'extrait de levure, de chitosan et d'acide acétique sur les feuilles résultèrent en des augmentations modestes (12-15%) de la concentration en isoflavone lorsque comparé à des plantes non traitées. Pas de différences furent observées entre les concentrations d'elliciteurs utilisées.

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Contributions of the authors

This document contains manuscripts which were co-authored by the candidate and Dr. Philippe Seguin, Department of Plant Science, Macdonald Campus, McGill University. The candidate carried out the experiments and data analyses and was the primary author of the manuscripts. Dr. P. Seguin provided funds and assistance for the research, including supervisory guidance and the reviewing of the manuscripts.

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1.0 General Introduction

Red clover (*Trifolium pratense* L.) is a perennial legume grown in temperate areas throughout the world. It is grown for forage as well as part of crop rotations for soil improvement. Red clover is valued as a forage crop due to its high protein content and digestibility. As a legume, red clover also adds important nitrogen to the soil. Red clover reaches maturity quickly, and thus is able to be harvested multiple times a year; the number of harvests dependent upon the area in which it is grown. In Québec, red clover is typically harvested twice per growing season, whereas in warmer areas of the United States three times per year is common. Despite being a perennial, it behaves as a biennial, as yields are significantly reduced by the second post-seeding year (Speer and Allinson, 1985, Marten et al. 1990, Sheaffer and Marten, 1991).

Isoflavones are phenolic compounds primarily found in the legume family. Red clover contains high concentrations of isoflavones, compounds that are involved in numerous plant processes and have been shown to be biologically active in mammals. Flavonoids are involved in the plant defense response to pathogens, UV light and act as a deterrent to herbivory (Stafford 1997, Olsson et al. 1998). Flavonoids are also important in nitrogen fixation in legumes, being secreted into the soil and acting as signals to rhizobacteria in the soil, leading ultimately to nodule formation (Long 1989, Olsson et al. 1998). Recently, isoflavones have received a lot of attention due to their proposed health benefits and thus value as nutraceuticals.

Isoflavone content of red clover has implications for animal producers as well. Isoflavones have been reported to cause reproductive problems when consumed by ruminant animals (Adams 1995). Formononetin may also be of benefit to animals when

impact on fertility is not an issue, as it has been reported that high levels of formononetin in feed may increase weight gain in lambs (Moorby et al. 2004).

Isoflavone concentration in legumes is influenced by a number of factors. Genetic control of isoflavone concentration has been widely demonstrated in soybean (Choi 2000, Seguin et al. 2004) and to a lesser extent in red clover (Wong 1963, Dedio and Clark 1968). Environmental factors have been shown to strongly affect isoflavone concentrations as well (Eldridge and Kwolek 1983, Lee et al. 2000). The effects light (Rossiter 1967), temperature (Rossiter and Beck 1966, Tsukamoto et al. 1995), and fertilization levels (Rossiter 1969, McMurray et al. 1986) have on isoflavone concentrations in legumes have all been investigated. In addition, a number of researchers have been able to increase production of isoflavones and other compounds through the application of elicitors, compounds that can induce plants' defense responses (Kneer et al. 1999, Al-Tawaha et al. 2005).

There is some evidence to suggest that isoflavone concentration in red clover is not consistent between plant parts (Dedio and Clark 1968, McMurray et al. 1986, Vetter 1995) and over maturity levels (Dedio and Clark 1968, McMurray et al. 1986). Although isoflavones have generally been shown to be quite stable (Vetter 1995), the method of storage and preservation may also have an affect on total and individual isoflavone concentrations present, as has been shown in red clover (Sarelli et al. 2002) and subterranean clover (*Trifolium subterraneum* L.) (Smith et al. 1986).

Despite being widely grown by producers, red clover currently has little or no direct economic value, as it is harvested for hay or silage for on-site animal consumption or grown as a pasture component for grazing. One of the primary goals of this project is

to develop red clover as a source of isoflavones for the nutraceutical industry. This would help to diversify the cash crops available to producers in Québec while fulfilling the demands of a growing niche market.

The goals of this research project are to:

1. Identify red clover cultivars currently recommended for use in Québec that contain high and low levels of isoflavones.
2. Assess how isoflavone concentration fluctuates over a range of identifiable stages of maturity in leaves, stems, and flowers of red clover.
3. Test methods of preservation and storage for observable effects on isoflavone concentration in red clover.
4. To test the possibility of using elicitor compounds to increase isoflavone concentration in red clover.

Research hypotheses:

1. Isoflavone concentration in red clover is under genetic control and cultivars with inherently high and low levels can be identified.
2. Isoflavone concentration in red clover fluctuates as the plant matures to varying degrees in different plant parts.
3. Preservation and storage techniques can influence the amount of isoflavones present in harvested herbage.
4. Exogenous application of chemical compounds can induce plant defense response and associated compounds, including isoflavones, in

red clover. Increases will be dependent on the nature and concentration of the elicitor.

2.0 Literature Review

2.1 Red clover agronomy

Red clover is a perennial legume native to South-East Eurasia, but has been widely distributed and is grown in temperate areas across the world. It is grown for forage as well as a part of crop rotations for soil improvement. Red clover is often grown as part of a rotation where it is harvested and later dried into hay or ensiled, or as a pasture component along with grasses. Red clover is valued as a forage crop due to its high protein content and digestibility. As a legume, red clover also adds important nitrogen to the soil. Red clover reaches maturity quickly, and thus can be harvested multiple times a year; the number of harvests depending on the area in which it is grown. In Québec, red clover is typically harvested twice per growing season, whereas in warmer areas of the United States three times per year is common. Despite being a perennial, it behaves as a biennial, as yields are significantly reduced by the second post-seeding year (Speer and Allinson, 1985, Marten et al. 1990, Sheaffer and Marten, 1991).

Despite its numerous beneficial qualities, consumption of large amounts of red clover can cause bloating in cattle, which can be avoided by keeping red clover to not more than 50% of feed. In addition, red clover contains large amounts of isoflavones, compounds that have been shown to have negative effects on the reproductive health of ruminant animals (Bennetts et al. 1946).

2.2 Impact of isoflavones on animal health

There are problems associated with consumption of large amounts of isoflavones by animals. Bennetts et al. (1946) reported on a set of symptoms observed in sheep fed

subterranean clover termed 'clover disease'. Clover disease was characterized by a number of symptoms detrimental to animal reproduction, often occurring independently of one another, but ultimately resulting in decreased fertility. Clover disease was found to be the result of the ingestion of phytoestrogens present in subterranean clover, specifically isoflavones. The main isoflavones in subterranean clover are genistein, biochanin A, and formononetin. Formononetin, itself possessing low oestrogenicity, is metabolized in the gut of ruminants to the isoflavan equol, which has oestrogenic properties (Cox and Braden 1974). Red clover has also been demonstrated to contain high levels of biochanin A and formononetin, as well as much lower levels of daidzein and genistein, and their conjugates. Fertility problems have also been observed in sheep due to the consumption of red clover (Morley et al. 1966).

Infertility caused by the ingestion of phytoestrogens can be either permanent or temporary. Permanent infertility has only been documented in sheep (Bennetts et al. 1946). Temporary infertility due to consumption of phytoestrogens has been reported in a number of animals, including sheep (Adams 1995) and cattle (Kallela et al. 1984). In sheep, normal fertility returns approximately 6-8 weeks after animals are removed from the oestrogenic pasture (Morley et al. 1966). It was observed in 1962 that the level of oestrogenic effect on sheep varied in different clover cultivars (Lloyd-Davies and Bennett 1962). In addition to observed clinical infertility, which is relatively rare at the present time, subclinical effects due to the consumption of phytoestrogens is thought to cause widespread economic loss (Adams 1998).

Alternatively, if fertility problems are not a consideration, as in lambs destined for slaughter, high formononetin levels in red clover feed may have some beneficial aspects.

It has been reported that finishing lambs fed red clover high in formononetin showed quicker weight gain and finished with a carcass that was of equal or better quality than control grass-fed lambs (Moorby et al. 2004).

2.3 Effects of isoflavones on human health

The effects observed in animals due to the consumption of isoflavones generated a lot of interest into how these compounds would affect human health. Diets rich in soy, which contains high levels of isoflavones, such as the traditional diets in Japan and Korea, have been correlated with lowered incidence of certain hormone-dependent cancers such as breast and prostate cancer, as well as decreased incidence of osteoporosis and cardiovascular disease when compared to Western societies (Wang and Murphy 1994, Aussenac et al. 1998, Choi 2000). This has particular importance to those who live in North America, where cancer and heart disease are among the leading causes of death (Parr and Bolwell 2000). Isoflavones present in soy foods have been targeted as possible candidates for being involved in the lower incidence of these diseases. One way isoflavones may provide health benefits is by interacting with the oestrogen receptor, which could be related to the lower risk of breast cancer and reduction in menopausal symptoms associated with isoflavones (Parr and Bolwell 2000).

Isoflavones have also been suggested to play a role in reducing some of the negative effects associated with menopause. A dietary supplement derived from red clover, Promensil® (Novogen Ltd., Australia), has been demonstrated to reduce the occurrence of hot flashes in post menopausal women (van der Weijer and Barentsen 2002). Although much of the available epidemiological evidence concerning human

health benefits are related to isoflavones obtained from soybean, it has been demonstrated that isoflavones from soy-products and those from red clover are absorbed at approximately the same rate (Tsunoda et al. 2002).

Flavonoids, including the isoflavones, are a part of a larger group of phenolic compounds. Phenolics possess antioxidant activity, which helps protect the body against the harmful effects associated with free radicals (CAST 2003). Free radicals have been implicated in several major diseases, including atherosclerosis, arthritis, cancer, and diabetes, as well as in radiation damage, infection, and aging (Kehrer and Smith 1994; Shahidi 1997). Antioxidants are thought to lower the risks of these diseases by reducing the number of free radicals present (Parr and Boswell 2000).

The effects flavonoids have on human health are not yet fully understood. Cassidy et al. (1995) reported that ingestion of isoflavones resulted in a reduction in total and LDL cholesterol concentration in young females. However, Nestel et al. (1997) reported no effect on blood lipid concentrations due to the ingestion of the isoflavone genistein. Perhaps more imperative, is the possibility of negative health effects due to isoflavone consumption. Indeed, as isoflavones possess estrogenic properties, the danger of isoflavones promoting breast cancer has been raised as well (Boue et al. 2003).

2.4 Nutraceuticals

Interest in isoflavone and phytonutrients has fuelled the demand for nutraceutical products. Nutraceuticals can be defined as “nutrients and non-nutrient compounds in food that have health-promoting, disease-preventative, or medical properties” (CAST, 2003). Use and acceptance of nutraceuticals has grown as many people are becoming

more interested in preventative and alternative health care. The value of the U.S. nutraceutical and functional foods segment was \$20.6 billion US in 2002, a 9.1% increase over 2001. This was a faster increase than either the dietary supplement or conventional food industries (CAST, 2003). There is much interest surrounding nutraceuticals recently due to their proposed role in fighting and preventing many dietary-related diseases. Nutraceuticals can be taken in the form of purified or concentrated supplements, or by consuming the foods in which they are found naturally.

2.5 Isoflavone description and synthesis

Isoflavones are phenolic compounds found primarily in the *Fabaceae*, including red clover. All phenolics share the common feature of the hydroxyl-substituted benzene ring (Parr and Bolwell 2000). Isoflavones have in common a 1,2-diarylpropane structure (Cassidy et al. 2000). Phytoestrogens must have a structure that allows binding to the estrogen receptor in mammals (Adams 1995). Phytoestrogens have similar features to oestradiol, such as two hydroxyl groups separated by a similar distance (Miksicek 1995) and the phenolic ring which is vital for estrogen receptor binding (Metzger et al. 1995). Isoflavones are synthesized from phenylalanine by way of the shikimic acid pathway (Parr and Bolwell 2000). Phenylalanine is converted to cinnamic acid through deamination by phenylalanine ammonia-lyase (PAL). After additional reactions, p-coumaroyl-CoA enters the flavonoid pathway where it is combined with three molecules of malonyl-CoA by chalcone synthase to form tetrahydroxychalcone, which is then converted by chalcone isomerase to a flavanone. Upon entry into the isoflavonoid pathway, the flavanone (naringenin for genistein, liquiritigenin for daidzein) undergoes

abstraction of a hydrogen radical at C-3 followed by B-ring migration from C-2 to C-3 and subsequent hydroxylation of the resulting C-2 radical (Dixon, 2004). Formononetin and biochanin A are then derived upon methylation of daidzein and genistein, respectively.

2.6 Functions of isoflavones in plants

Flavonoids, including the isoflavones, are involved in a number of important plant processes. They play a key role in plant defense and responses to a variety of stressors. Flavonoids act as a deterrent to herbivory, and are produced as a response to attack by pathogens (Stafford 1997), or physical damage caused by chemicals or UV light (Olsson et al. 1998). Flavonoids are also involved in the plant-microbe interactions associated with nitrogen fixation in legumes (Long, 1989, Olsson et. al. 1998). Flavonoids are released into the soil as part of the communication process between legumes and rhizobia. In this way, flavonoids act as chemoattractants to rhizobia bacteria, which in turn emit lipochitooligosaccharides (LCOs), and ultimately leading to root hair infection and thus nodule formation.

2.7 Factors affecting isoflavone levels in plants

2.7.1 Genetic factors

There is evidence to suggest that isoflavone content in red clover and other species is heritable, with differences being observed between populations and cultivars. In one early study, Wong (1963) reported that differences existed in isoflavone concentration between 4 varieties tested, with isoflavone concentrations varying by a

factor of 10 between cultivars. In a more extensive study, it was reported that formononetin concentration varied from 3500 to 9400 $\mu\text{g g}^{-1}$ DM, and biochanin A from 6000 to 11200 $\mu\text{g g}^{-1}$ DM among 16 field-grown cultivars tested (Dedio and Clark, 1968). In both of these studies, evaluations for isoflavone concentrations in red clover cultivars relied on samples collected during a single collection in one field.

However, evaluating cultivars in a single environment may not give an accurate picture of how isoflavone concentrations will vary in different environmental conditions. Only by measuring isoflavone concentrations over a range of environments can cultivars be confidently assessed for differences. It has been noted that differences in isoflavone content in soybean can only be detected through multi-site, multi-year trials (Lee et al. 2003). Evaluations in multiple environments are necessary in order to test how genotype by environment interactions, as well as environmental main effects influence isoflavone concentration in legumes. Although there is little information regarding the evaluation of red clover cultivars for isoflavone concentration in multi-year, multi-site trials, studies of this kind have been performed with soybean.

Soybean contains the isoflavones daidzein, genistein, and glycitein. Isoflavone concentrations in soybean have been reported to vary according to cultivar (Choi 2000, Hoeck 2000, Lee 2003, Wang and Murphy 1994, Eldridge and Kwolek 1983). In one study, isoflavone concentrations of seven American soybean cultivars grown in 1989 were reported to range from 2053 to 4216 $\mu\text{g g}^{-1}$ DM (Wang and Murphy 1994). Additionally, when one soybean cultivar, Vinton 81, was grown in 3 consecutive seasons in 1989-1991, total isoflavone content in the seed ranged from 1176 to 3309 $\mu\text{g g}^{-1}$ DM. Also, total isoflavone concentration ranged from 1176 to 1749 when Vinton 81 was

grown in 4 different locations in 1991 (Wang and Murphy 1994). As is demonstrated in this study, isoflavone concentrations in soybean genotypes fall in a broad range. The isoflavone concentration in specific cultivars can vary to a large degree in separate environments as well. In another study, 4 varieties of soybean grown in Urbana, IL in 1980 were found to contain isoflavone levels ranging from 1159 to 3093 $\mu\text{g g}^{-1}$ DM (Eldridge and Kwolek 1983). Additionally, total isoflavone concentrations in two soybean cultivars, 'Hardin' and 'Corsoy-79', grown in 4 locations in the same year varied from 469-1708 and 799-1951 $\mu\text{g g}^{-1}$ DM (Eldridge and Kwolek 1983). Despite the wide range of concentrations observed for each cultivar, the rankings of the cultivar did not change from site to site; Corsoy-79 had higher isoflavone concentration at each site, though the magnitude of the difference to vary from site to site. In a third experiment, the soybean cultivar 'Clark' was grown in the same location for 4 consecutive years. Total isoflavone concentrations were found to range from 2450-3620 $\mu\text{g g}^{-1}$ DM (Eldridge and Kwolek 1983). From these studies, it can be seen that both genetic and environmental effects can strongly influence the isoflavone concentration in soybean. Lee et al. (2002) reported that main genotype effects accounted for less than 5% of total variation observed; genotype x environment interactions accounted for 61.6% of observed variation. Therefore, they concluded that selection for isoflavones in a single environment or at multiple sites in a single year would not likely be effective. They suggest that in order to confidently assess isoflavone concentration in soybean cultivars, multi-year, multi-site trials are necessary.

As demonstrated in these studies, the interaction of genotypes with the environment is necessary to be taken into consideration when evaluating cultivars for

isoflavone concentration. Genotypic effects on isoflavone concentration are considerable, as are environmental effects and genotype by environment interactions. Therefore, it is necessary to evaluate cultivars in multiple environments in order to accurately assess isoflavone concentration. There is no such information available for red clover. Unpredictable environmental influence is not accounted for in previous studies evaluating red clover cultivars for differences in isoflavone concentration performed in a single site and year.

2.7.2 Isoflavone partitioning within red clover

Isoflavones are not uniformly distributed throughout the herbage of red clover. It has been reported that the highest isoflavone concentrations were found in the leaves just prior to flowering (Dedio and Clark 1968). Stems and petioles were found to contain low amounts throughout the growing season. In a greenhouse study, formononetin concentration was reported to be higher in leaves than in either petioles or stems in 8 of 9 weekly harvests. Over the course of the study, average formononetin concentrations were 9730, 7830, and 7100 $\mu\text{g g}^{-1}$ DM for leaves, petioles, and stems, respectively (McMurray et al. 1986). In a similar study, Dedio and Clark (1968) collected two samples, one of leaves, the other of stems and petioles, from field-grown red clover plants of two varieties at six times throughout the first cut of the season. They reported that leaves contained higher concentrations of both biochanin A and formononetin throughout the course of the study. Leaves were reported to contain between 1800 and 10000 $\mu\text{g g}^{-1}$ DM formononetin, and 1600 and 11,000 $\mu\text{g g}^{-1}$ DM biochanin A, depending on the sampling date and cultivar. Stems and petioles, though, contained much lower

amounts, with formononetin and biochanin A concentrations always lower than 4000 $\mu\text{g g}^{-1}$ DM.

Vetter (1995) carried out perhaps the only published report concerning the isoflavone concentrations of red clover flowers. In this study, plants of seven *Trifolium* species, *T. alpestre*, *T. fragiferum*, *T. incarnatum*, *T. montanum*, *T. pratense*, *T. repens*, and *T. subterraneum*, were collected during the flower phase at a single site and divided into leaf, stem, and flower fractions. Of the species tested, *T. alpestre*, *T. pratense*, and *T. subterraneum* were found to contain much higher concentrations of isoflavones than the others. In *T. pratense* (red clover), flowers were found to have the highest isoflavone concentration at 1209 $\mu\text{g g}^{-1}$ DM, as compared to 1067 $\mu\text{g g}^{-1}$ DM in leaves, and 744 $\mu\text{g g}^{-1}$ DM in stems. It is interesting to note that the concentrations reported in this study are considerably lower than what is usually reported for red clover, being approximately 5-20 times lower than in many other studies, as well as having very different ratios of individual isoflavones. Although sampling and extraction methods vary, the isoflavone concentrations reported in this study are quite incongruent with those reported in most other studies.

2.7.3 Effect of maturity

As with many compounds, the production and breakdown of isoflavones can be expected to change as the plant goes through its life cycle. Isoflavones, functioning in a number of capacities in plant defense and nitrogen fixation, vary in concentration as they are needed by the plant.

Isoflavone concentrations, in general, are reported to decrease as red clover matures. It was observed in field grown plants that when the first harvest was delayed by 21 days, formononetin concentration decreased by 39%. For the second harvest, plots that were allowed the shortest regrowth had the highest formononetin concentration. Differences between harvest dates might be attributable to differences in plant maturity at harvest (McMurray et al. 1986). Sarelli et al. (2003) reported that in wilted field-grown red clover, formononetin and biochanin A concentrations decreased when harvested during flowering rather than at the budding stage. In a greenhouse experiment formononetin concentration was analyzed in leaves, petioles, and stems in weekly samplings over a 9-week period from the late-vegetative stage through the dying inflorescence stage (McMurray et al. 1986). All parts analyzed followed a general decline in formononetin concentration as maturity increased, decreasing by 48, 54, and 67% in leaves, petioles, and stems, respectively, from weeks 1 to 8, though fluctuations were observed. The authors reported an unexplained increase in formononetin concentration during the final week of the study. This study, by taking place in a greenhouse, was able to control for variables that are difficult to remove from maturity trials that occur in the field, such as temperature, water conditions, and other environmental stresses. Therefore, observations made in this study could be more confidently attributed to the effects of maturity.

A similar study was carried out using field-grown red clover and had similar results. Two red clover varieties were sampled at six times during the first cut, divided into leaves or stems and petioles, and analyzed for formononetin and biochanin A concentrations. Isoflavone concentrations were greatest during the vegetative stage, and

reached maximum levels at the second sampling, a few weeks prior to the onset of flowering (Dedio and Clark, 1968).

2.7.4 Effect of temperature

Temperature has been shown to affect isoflavone content in red clover (MacMurray et al. 1986) and other species, including subterranean clover (Rossiter and Beck 1966) and soybean (Tsukamoto et al. 1995).

McMurray et al. (1986) tested the effects of two temperature schedules on formononetin concentration in red clover. In that experiment, field grown red clover (cv. 'Hungaropoly') were transplanted into pots and grown in a growth cabinet at 20°C for 20 days, then transferred to either a 23/17 or 17/13°C day/night temperature schedule. The lower temperature schedule resulted in a 28% increase in formononetin concentration in expanded leaves and a 17% increase in expanding leaves. Differences in formononetin concentration between temperature schedules were not observed in stems and petioles.

Rossiter and Beck (1966) carried out similar studies on the effects of temperature on isoflavone levels on subterranean clover. They grew subterranean clover in growth chambers at one of five day/night temperature regimens: 36/31, 30/25, 27/22, 21/16, 15/10, 12/7, and 9/4°C. Isoflavone concentrations were reported to be highest for plants grown under in the 15/10°C day/night temperature schedule. It was also found, however, that plant growth in the 15/10°C degree temperature schedule was only 30 to 50% of that of plants grown at 27/22 or 21/16°C. Thus, it can be proposed that cooler temperatures, such as the 15/10°C temperature schedule used in this experiment, would be optimal insofar as isoflavone concentration is concerned, whereas a somewhat higher temperature

schedule would be preferred if total isoflavone per area of sown subterranean clover is sought.

Soybean was found to have decreased isoflavone content when exposed to high temperatures during seed development in the field (Tsukamoto et al. 1995). These effects were determined to be the result of high temperatures, and not due to variation of sowing date, another factor tested in this study. The effect of temperature was further studied in growth chambers. Isoflavone concentrations were lower when soybean was exposed to a 38/28°C day/night temperature schedule as compared to a 25/10°C day/night temperature schedule. Observations from the field studies were thus supported by the experiments evaluating the effect of temperature on soybean isoflavone concentration in controlled environments.

2.7.5 Effect of fertilization

Mineral deficiency has been demonstrated to raise isoflavone levels in legumes. In 23 day old subterranean clover plants, nitrogen deficiency led to increased concentrations of isoflavones in leaves (Rossiter 1969). Isoflavone concentration was highest in nitrogen deficient plants and decreased with increasing nitrogen fertilization. Nitrogen levels tested were 0, 5.6, 22.4, and 89.6 kg ha⁻¹. Plants that did not receive nitrogen had approximately twice the isoflavone concentration than plants that received 22.4 kg ha⁻¹. Total isoflavone content per plant was maximized at 22.4 kg N ha⁻¹, whereas maximum dry weight per plant was achieved at 89.6 kg N ha⁻¹. Nitrogen deficiency was reported to be clearly visible 3 weeks after sowing.

The amount of available phosphorus has also been shown to affect isoflavone content in red clover. Phosphorus deficiency increased the formononetin concentration by 43% over plants receiving 23 and 96 kg P ha⁻¹. There was no significant difference in formononetin concentration between plants receiving 23 or 96 kg P ha⁻¹. Plants receiving no phosphorus had a 15% higher formononetin concentration when harvested after 73 days as opposed to 58 days (McMurray 1986). Formononetin concentration was however only increased when phosphorus deficiency was great enough to dramatically affect growth rate. Dry weight of shoots with phosphorus deficiency was only 14% of that of plants grown at 23 kg P ha⁻¹ and 11% of plants receiving 96 kg P ha⁻¹. Thus, controlling the level of phosphorus fertilization could only be used as a means to increase formononetin concentration if a dramatic reduction in total formononetin production per area is not an issue.

2.7.6 Preservation and Storage

Isoflavone concentrations of harvested herbage can be affected by the method in which it is preserved and stored. The effects preservation and storage have on isoflavone concentration of harvested material has important implications for researchers, as well as for those involved in the end use of the material such as agricultural producers and nutraceutical companies. The ability of preservation and storage techniques to raise or lower isoflavone concentrations in harvested plant material is important when material is used as feed for animals; both in order to increase isoflavone levels for producers of finishing lambs, and decrease levels when fertility problems are to be avoided. Red clover silage has been reported to contain 18% higher isoflavone concentration than non-

ensiled herbage previously wilted to the same degree (Sarelli et al. 2002). Also, in subterranean clover, it was observed that isoflavone concentrations in dried samples were up to 50% lower than in fresh or frozen material (Smith et al. 1986).

2.7.7 Natural elicitors

Attack by pathogens causes plants to induce defense mechanisms, including the production of secondary metabolites (He 1996). Certain compounds can be applied to plants and induce defense responses as well (Benhamou et al. 1994, Pitta-Alvarez and Giulietti 1999). These compounds are known as elicitors. Elicitors can come from a variety of sources and can be either biotic or abiotic in origin.

A wide range of elicitors has been successfully used for various purposes in many plant species. Some elicitors that have shown promise are salicylic acid, citric acid, yeast extract, chitosan, acetic acid, methyl jasmonate, lipo-chitoooligosaccharides (LCO's), actinomycetes, plant growth promoting rhizobacteria (PGPR), jasmonic acid, and copper chloride. Al-Tawaha et al. (2005) carried out studies on the response of isoflavone levels in soybean to foliar application of a number of elicitors. In greenhouse studies, isoflavone concentrations increased with foliar application of all elicitors evaluated: yeast extract, chitosan, acitomycetes, and lipo-chitoooligosaccharides (LCOs). In subsequent field trials, however, results were much more modest. The biggest increase in soybean isoflavone levels in the field was due to application of yeast extract (Al-Tawaha, unpublished data).

Chitosan is a major component of the cell walls in pathogenic fungi. Chitosan has been shown to increase phenylalanine ammonia lyase (PAL) activity, a key enzyme in the

flavonoid biochemical pathway (Hadwiger 1989). Chitosan has been effectively used to induce the accumulation of the phytoalexin pisatin in pea pods (Hadwiger and Beckman 1980, Walker-Simmons et. al. 1984). Production of isoflavones in white lupin roots has been induced due to the application of chitosan to the growth medium (Gagnon and Ibrahim 1997). Leaves of *Oryza sativa* seedlings have shown a dramatic increase in the production of the phytoalexins sakuranetin and momilactone A when treated with chitosan (Agrawal et. al. 2002). Both chitosan and yeast extract have also been used to increase synthesis of plumbagin in suspension cultures of *Plumbago rosea* L. (Komaraiah 2002). Acetic acid has been shown to increase the accumulation and release of the alkaloids scopolamine and hyoscyamine in roots of *Brugmansia candida* when applied to the growth medium (Pitta-Alvarez and Giulietti 1999, Raskin and Poulev 2002). Yeast extract has been used to increase production of isoflavonoids in white lupin roots (Gagnon and Ibrahim 1997). Lu et al. (2001) were able to increase saponin production in ginseng cell suspensions by as much as 20-fold with the application of 3 g/L of yeast extract. Cultured cells of *Glycyrrhiza echinata* produced higher levels of the isoflavonoid phytoalexin medicarpin upon elicitation with yeast extract. Pterocarpan, which includes medicarpin, are isoflavone-derived phytoalexins. Interestingly, the levels of formononetin, normally found at a high constitutive level, were seen to drop in cells from one cultivar (Nakamura et al. 1999). It is noted that different responses to yeast extract elicitation were observed, and that the induction of this particular biochemical pathway, at least, may be cultivar specific.

2.8 Conclusion

Isoflavone content in red clover is important due to their effects on animals as well as their potential for use in humans as nutraceuticals. Isoflavone concentration has been shown to be affected by a range of genetic and environmental factors; however, the extent to which these factors control concentrations is not yet completely understood. There is a lack of a comprehensive study as to how genetic and environmental factors interact and influence isoflavone concentration in red clover.

2.9 Connecting text

The following chapter was submitted for publication in the Journal of Agricultural and Food Chemistry. The manuscript is co-authored by the candidate and Dr. Philippe Seguin, Department of Plant Science, Macdonald Campus of McGill University. The candidate carried out the experiments and data analyses and was the primary author of the manuscript. Dr. P. Seguin provided funds and assistance for this research, including supervisory guidance and the reviewing of the manuscript.

3.0 Effects of the Environment, Cultivar, Maturity, and Preservation Method on Red Clover Isoflavone Concentration

3.1 Abstract

Red clover (*Trifolium pratense* L.) contains isoflavones, which are of interest because of their benefits for human health as well as their adverse effects on the fertility of farm animals. A series of field experiments was conducted in Sainte-Anne-de-Bellevue, QC, Canada to determine the effects of the environment, cultivar, plant maturity, plant part, and preservation method on the concentration of the two predominant isoflavones in red clover, formononetin and biochanin A. In a multi-year, multi-site trial total isoflavone concentration in 10 cultivars ranged between 8923 and 12753 $\mu\text{g g}^{-1}$ DM averaged across sites, harvests, and years. Despite strong environmental effects, the cultivar 'Start' consistently had the lowest isoflavone concentrations, with few differences observed among other cultivars. Across stages of maturity leaves were found to have the highest isoflavone concentration followed by stems and inflorescences (11970, 4896 and 3297 $\mu\text{g g}^{-1}$ DM, respectively). Changes in isoflavone concentrations with increasing maturity varied depending on the plant part. Overall, highest isoflavone concentration was found in leaves and stems during the vegetative stages, with formononetin concentration declining as plants initiated flowering, especially in stems. Upon initiation, inflorescences contained similar isoflavone concentrations than leaves, but concentrations decreased rapidly during flower development to fall even below those observed in stems. Fresh herbage contained higher formononetin and total isoflavone concentrations than did silage and hay (14464, 12200

and 11604 $\mu\text{g g}^{-1}$ DM, respectively). Isoflavone concentration in field-grown red clover is thus high but can be affected by a range of agronomic factors.

3.2 Introduction

Studies have shown that isoflavones may lower blood cholesterol levels (Cassidy et al. 1995, Nestel et al. 2004) and be useful in the prevention and treatment of cancer (Jarred et al. 2002), bone loss (Atkinson et al. 2000), and symptoms associated with menopause (van der Weijer and Barentsen 2002). Red clover is a legume species that contains the isoflavones daidzein, genistein, formononetin and biochanin A, the latter two being found in especially high concentrations (Dedio and Clark 1968, McMurray et al. 1986, Vetter 1995). Isoflavone concentration in red clover herbage has been reported to be 2 to 10 times more than in soybean seeds, the more common source of isoflavones (Institute for Environment and Health 2000). Products containing extracts or powdered red clover material are currently being sold as non-prescription food supplements.

Isoflavones are also of interest due to their biological activity in farm animals. Consumption of forages with high isoflavone concentrations has been demonstrated to cause reproductive problems in sheep and cows (Beck 1964, Braden et al. 1970). Alternatively, isoflavones may have some desirable effects on animals for slaughter. Finishing lambs fed red clover with high levels of the isoflavone formononetin gain weight more quickly than lambs fed low formononetin red clover or ryegrass (Moorby et al. 2004).

Isoflavone concentration in legumes is controlled by both genetic and environmental factors. Early studies reported differences in isoflavone concentration

among red clover cultivars (Wong 1963, Dedio and Clark 1968). Dedio and Clark (1968) reported 2.7- and 2-fold variation in formononetin and biochanin A, respectively among 16 field-grown cultivars sampled at a single date. Environmental factors have also been reported to affect red clover isoflavone concentration. In a greenhouse experiment, formononetin concentration in expanded leaves was reported to be 28% higher in red clover plants grown in a 17/13°C temperature regimen as opposed to 23/15°C (McMurray et al. 1986).

Management may also affect red clover isoflavone concentration (McMurray et al., 1986). It was observed that when the first harvest was delayed by 21 days, formononetin concentration decreased by 39%. For the second harvest, the plots that were allowed the shortest regrowth had the highest formononetin concentration. Differences between harvest dates might be attributable to differences in plant maturity at harvest. A decrease of 57% in formononetin concentration was reported between the late vegetative and the dying inflorescence stages in greenhouse grown plants (McMurray et al., 1986).

Isoflavones do not exist in uniform concentrations throughout the plant. It has been reported that stems and petioles contain lower concentrations of formononetin and biochanin A than leaves when sampled various times during the growing season (Dedio and Clark, 1968). However, it has also been reported that flowers contain the highest total isoflavone concentration, followed by leaves and stems when measured at a single sampling date (Vetter 1995). It is not known how isoflavone concentration evolves with maturity in inflorescences, which are most commonly used part by the nutraceutical industry (Filière des Plantes Médicinales Biologique du Québec 2004).

Finally, preservation and storage may have an effect on the isoflavone concentration in red clover. This is especially relevant for herbage to be used as an animal feed. Red clover silage has been reported to contain 18% higher isoflavone concentration than non-ensiled wilted herbage (Sarelli et al. 2002). Also, in subterranean clover (*Trifolium subterraneum* L.), it was observed that isoflavone concentrations in dried samples were 30 to 50% lower than in fresh or frozen material (Smith et al. 1986).

Despite the current importance of red clover as a source of isoflavone for the nutraceutical industry, as well as its widespread use as a feed for farm animals, there has been to date no comprehensive study looking at factors affecting isoflavone concentration in field-grown red clover. We thus designed a set of three field experiments to determine how the environment, cultivar, plant maturity, plant part, and storage method affect red clover isoflavone concentration.

3.3 Materials and methods

3.3.1 General site description and management

Three different experiments were conducted in four red clover fields established in either 2002 (field A) or 2003 (field B, C, D) in Sainte-Anne-de-Bellevue, QC, Canada (45°25'45" N, 73°56'00" W). Soil types were as follows: field A was a Chicot fine sandy loam, field B a Bearbrook clay, field C a Chateauguay clay loam, and field D a St. Bernard clay loam. All plots (5 x 1.35 m) were seeded in early May at a rate of 10 kg ha⁻¹ using a disk-drill (Fabro, Swift Current, SK, Canada). Seeds were inoculated prior seeding with a peat-based rhizobial inoculant (Nitragin, Milwaukee, WI). Cultivars of red clover seeded varied depending on the experiment. In the seeding year, all fields received

400 kg ha⁻¹ of 5-20-20 (N-P₂O₅-K₂O) fertilizer in May just prior seeding, and 225 kg ha⁻¹ of 0-15-30 the first week of September. In post-seeding years, fields received 250 kg ha⁻¹ of 0-15-30 in the spring, and 250 kg ha⁻¹ of 0-18-36 after the first harvest and in the first week of September. Fertilization was done according to local recommendations for red clover forage production (CRAAQ, 2003).

3.3.2 Cultivar evaluation experiment

Ten red clover cultivars recommended for forage production in Québec at the onset of experimentation (AC Charlie, Azur, Belle, Concorde, Prima, Ram, Scarlett, Start, Tempus, and Walter) were seeded on May 15, 2002 in field A, and May 12, 2003 in field B. Each cultivar was replicated four times in a randomized complete block design. Cultivars were grown for two consecutive years, the seeding year and the first post-seeding year (2002-03 for field A, 2003-04 for field B), and were harvested twice in each year. A 0.6 by 4.4 m area was cut in the center of each plot at each harvest to a 7 cm stubble height using a flail forage harvester (Swift Machine & Welding, Swift Current, SK, Canada), when 10% of the plants produced flowers. Field A was harvested on August 2 and October 25, 2002, and June 20 and July 29, 2003. Field B was harvested on July 30 and August 29, 2003, and June 11 and July 21, 2004. At the time of harvest, herbage yield was recorded for each plot, and 30 g sub-samples of mixed, chopped fresh plant material were frozen immediately on dry ice and stored at -20 °C for subsequent isoflavone extraction in the laboratory. Representative 500 g samples of harvested herbage were obtained from each plot, dried in a forced-air oven at 60 °C for 48 hr, and weighted to determine dry matter (DM) content.

3.3.3 Maturity and plant parts experiment

Two cultivars, 'Azur' and 'Start', were seeded on May 12, 2003 in field C. Each cultivar was replicated four times in a randomized complete block design. Beginning in May 2004, plants were harvested by hand at specific growth stages described in Ohlsson and Weding (1989), ranging from early vegetative through late flowering. Plant samples were collected at 8 stages as follows: stage 1 (vegetative plants with 3 trifoliate leaves), 2 (vegetative plants with 4 trifoliate leaves), 3 (vegetative plants with 5 trifoliate leaves), 4 (inflorescence of main stem palpable), 5 (single buds on main stem discernable), 6 (at least one open flower on inflorescence of main stem), 7 (inflorescence of main stem at least halfway past flower), and 8 (inflorescence of main stem past flower, sepals still green). At each sampling, samples were further divided into leaves, stems, and inflorescences fractions. Thirty g sub-samples from each part were finely chopped and placed on dry ice and stored at -20°C until isoflavone extraction. At each sampling representative samples were also dried in a forced-air oven at 60°C for 48 hr and weighted to determine DM content.

3.3.4 Preservation method experiment

Plots of the cultivar 'Concorde' were established on May 12, 2003 in field D. On July 21, 2004, at the second harvest of the post-seeding year, herbage was harvested using a flail forage harvester (Swift Machine & Welding, Swift Current, SK, Canada) as previously described, and received one of three preservation treatments: i) as hay after two days of field-curing to a DM content of 90%, ii) as silage after ensiling wilted

herbage (40% DM) in mini-silos for 50 days as described by Seguin and Mustafa (2003), or iii) none (fresh herbage). Upon treatment, 30 g sub-samples were then kept frozen at – 20 °C until isoflavone extraction. Each treatment was replicated four times. Dry matter content of each sample was determined by drying representative samples in a forced-air oven at 60 °C for 48 hr.

3.3.5 Isoflavone extraction

Sample extraction was performed using a slightly modified version of the protocol of Petterson and Kiessling (1984). Briefly, 1 g of plant material was ground with sand with a mortar and pestle, mixed with 2 mL of distilled water, and incubated at 37 °C for 30 min in a water bath. Sixteen mL of ethanol and 2 mL of 3 M HCl were added, samples were mixed, and then heated to boiling. Extracts were allowed to cool, and then 2 mL of extract was removed and centrifuged at 8000 rpm for 8 min. The supernatant was loaded onto SepPak C-18 cartridges (Waters Canada, Mississauga, ON, Canada) as follows: cartridges were first equilibrated with 5 mL of methanol, and washed with 5 mL of deionized water. One mL of extract was then mixed with 3 mL of deionized water and allowed to enter the cartridge. Columns were then washed with 2 mL of 20% methanol and eluted with 2 mL of 80% methanol. Samples were then stored at -20 °C until HPLC analysis.

3.3.6 HPLC analyses

HPLC analyses were performed on a Waters chromatograph system (Waters, Milford, MA, USA) consisting of two pumps (model 510), a WISP autosampler (model

712) and a UV absorbance detector (model 441). The system was equipped with a C18 reverse-phase column (Bondapak, 10 μm , 3.9 x 300 mm; Millipore, Milford, MA, USA). One hundred μL of filtered extract was injected for each analysis. Separation and elution of isoflavones was achieved with the following gradient method using a flow-rate of 1 mL min^{-1} . Elution of isoflavones was performed using a linear gradient system from 20% methanol and 80% water, to 80% methanol and 20% water over the course of 30 min, following an initial 5 minutes of steady elution with 20:80% methanol:water. Isoflavones were detected at 254 nm (Wang et al. 2000; Seguin et al. 2004). Purified chemical standards [formononetin and biochanin A, (Sigma-Aldrich, Mississauga, ON, Canada)] were used to identify isoflavones and determine their concentrations on a DM basis. The term 'total isoflavone' in this study refers to the sum of formononetin and biochanin A concentrations, which together constituted approximately 97% of isoflavones detected in our studies.

3.3.7 Statistical analyses

All data were subjected to analysis of variance (ANOVA) using the GLM procedure of the Statistical Analysis System (SAS, 1989) to identify significant treatment effects and interactions. Data from the cultivar evaluation experiment were analyzed in a combined analysis (McIntosh 1983) regrouping sites, stand ages, cultivars, and harvests in a combined randomized complete block design with strip-plot restriction with cultivars and harvests as spatial and temporal strips, respectively (Gomez and Gomez 1984). Stand ages, cultivars, and harvests were considered fixed effects, while site was considered random. Appropriate *F*-tests in each case were calculated following McIntosh (1983).

Data from the maturity and plant parts experiment were analyzed using a randomized complete block design with split-split-plot restriction with cultivars as main plot, plant parts as split-plots, and stage of maturity as split-split-plots. Finally for the preservation method experiment, data were analyzed using a randomized complete block design. In all experiments, differences between treatments were ascertained using least significant differences (LSD) and *F*-tests.

3.4 Results and discussion

3.4.1 Cultivar evaluation

Total isoflavone concentrations ranged between 1525 and 16756 $\mu\text{g g}^{-1}$ DM depending on cultivar, site, stand age and harvest, and averaged 8844 $\mu\text{g g}^{-1}$ DM with overall 55% formononetin. Isoflavone concentrations were affected by site, stand age, and cultivar main effects as well as interactions between these factors and harvest (**Table 3.4.1**). Results suggest that despite environmental effects differences in isoflavone concentration between red clover cultivars are overall stable across environments and time, and remain relatively unaffected by other factors evaluated. Cultivars affected isoflavone concentrations mainly through main effects. Averaged over site, stand age, and harvest, total isoflavone concentrations in the ten cultivars evaluated ranged from 8923 to 12753 $\mu\text{g g}^{-1}$ DM (**Figure 3.4.1**). One cultivar (i.e., ‘Start’) consistently had the lowest isoflavone concentrations (4842, 4081, and 8923 $\mu\text{g g}^{-1}$ DM for formononetin, biochanin A, and total isoflavones, respectively) ranking 9th or 10th in total isoflavone concentration in all 8 site \times stand age \times harvest combinations. Differences between the

other cultivars were generally limited, with more variation observed for biochanin A concentration.

There was a 4-way interaction between site, stand age, cultivar, and harvest for formononetin, biochanin A, and total isoflavone concentrations. This interaction reflects a three-way interaction between site, stand age, and harvest in three of the ten cultivars evaluated, namely AC Charlie (formononetin, biochanin A, and total isoflavone), Scarlett (biochanin A and total isoflavone), and Start (biochanin A). There were no site \times age \times cut interaction for the other 7 cultivars evaluated. The only other effect implicating cultivars was a cultivar \times site interaction observed for formononetin. This interaction reflected that while greater formononetin concentrations were observed for all cultivars in field B compared to field A, differences were significant only for five cultivars (i.e., AC Charlie, Concorde, Prima, Ram, and Start).

Site main effects were observed for all isoflavones. Overall, concentrations were 17, 30, and 22% greater in plants grown in field B than A for formononetin, biochanin A, and total isoflavone, respectively. Differences between sites might be attributable to inherent differences in soil types and characteristics between the two fields. While soil fertilization was similar in both fields and made according to local recommendations for red clover forage production, it is possible that fields differed for some elements. Soil fertility and concentration of certain elements were previously reported to affect isoflavone concentrations of other legumes (Rossiter and Beck 1966). In addition to this main effect, site was however involved in interactions with stand age, harvest, and cultivar. The site \times stand age interaction for biochanin A and total isoflavone concentrations is attributable to much greater seeding year concentrations in field B

compared to field A. In the seeding year, plants grown in field B had 72 and 39% greater biochanin A and total isoflavone concentrations, respectively, than those grown in field A. No differences were observed between fields in the post-seeding year. A site \times harvest interaction for formononetin and total isoflavone concentrations reflected the lower concentrations for the second harvest in field A, compared to all other site \times harvest combinations.

Stand age overall had limited effects on isoflavone concentrations, affecting isoflavone concentrations mainly through interactions with other factors. A stand age main effect was observed only for formononetin, its concentration being 15% higher in the post-seeding year than in the seeding year when averaged across cultivars, sites, and harvests. A stand age \times harvest interaction for formononetin reflected that differences between harvests differed depending on stand age. During the seeding year, across cultivars and sites, formononetin concentration dropped by an average of 61% (7571 to 4673 $\mu\text{g g}^{-1}$ DM) from the first to the second harvest, while in the post-seeding year, it rose by 11% (6710 to 7460 $\mu\text{g g}^{-1}$ DM). In addition, a three-way interaction between site, stand age, and harvest affected biochanin A concentration. The low biochanin A concentration at the first harvest of the post-seeding year in field B contributed strongly to this interaction. During the post-seeding year, biochanin A concentrations were similar in field A at both harvests (avg. of 4624 $\mu\text{g g}^{-1}$ DM), but increased from 3217 to 5747 $\mu\text{g g}^{-1}$ DM from the first to the second harvest in field B. No differences between harvests were observed at both sites in the seeding year.

Results suggest that cultivar choice can have a noticeable impact on isoflavone concentrations in red clover. Although other factors evaluated will impact isoflavone

concentrations, differences between cultivars will remain relatively constant at different sites, harvest, and for stands of different age. The cultivar 'Start' distinguished itself from other cultivars containing low concentrations of both formononetin and biochanin A; few differences were observed between other cultivars. Therefore, 'Start' would be a cultivar of choice for producers aiming at reducing isoflavone intake of farm animals; options would be greater if red clover is to be produced as a source of isoflavone for the nutraceutical industry. The large site main effects and interactions between factors we observed suggest that environmental factors will also have profound impacts on isoflavone concentrations in red clover, confirming similar observations made with other species (Smith et al. 1986, Seguin et al. 2004, Seguin and Zheng 2005). This underlines the need for further research to understand the impact of specific biotic and abiotic factors on isoflavone concentration in legumes. Furthermore it points out the need for nutraceutical manufacturers to regularly test purchased material for isoflavone concentration, and reinforce the need for strict quality control practices.

3.4.2 Maturity and plant parts

Isoflavone concentrations were affected by stage of maturity, plant part, and cultivar, as well as interactions between them. These interactions were largely due to changes, which differed depending on the isoflavone, of concentrations in leaves, stems and inflorescences as plants mature, the trend of change varying according to parts and cultivars. Relationships between plant parts and stage of maturity were although remarkably constant for both cultivars evaluated; differences in the magnitude of the response generated interactions implicating cultivars (**Figure 3.4.2**). Differences

between cultivars were consistent with results of the cultivar evaluation experiment; when compared with ‘Start’, ‘Azur’ had 69, 44, and 59% greater formononetin, biochanin A, and total isoflavone concentrations, respectively, across stages of maturity and plant parts.

Across all stage of maturity and cultivars average isoflavone concentrations were greatest in leaves ($11970 \mu\text{g g}^{-1} \text{DM}$), intermediate in stems ($4896 \mu\text{g g}^{-1} \text{DM}$), and lowest in inflorescences ($3297 \mu\text{g g}^{-1} \text{DM}$). Differences between plant parts however varied significantly depending on the stage of maturity. Before the onset of flowering (stages 1 to 3), there was little or no difference in leaves and stems formononetin concentration for both cultivars. Concentration of biochanin A was although 2 to 3 times greater in leaves than stems, with greater differences observed with ‘Start’, for which differences were also reflected in total isoflavone concentrations. In the earliest stage of flowering (stage 4), leaves and inflorescences had comparable isoflavone concentrations, which were greater than in stems. However, as flowering progressed formononetin and total isoflavone concentrations differed in the three parts, being greatest in leaves, intermediate in stems and lowest in inflorescences. Biochanin A concentrations of stems and inflorescences were similarly lower than in leaves.

While change in isoflavone concentrations of leaves and stems differed depending on the isoflavone, trends for inflorescences were similar for all isoflavones. Concentrations of formononetin, biochanin A, and total isoflavone in inflorescences were high at the onset of flowering, but decreased sharply by 92% on average by the next stage, to later stabilize. Leaves and stems had their highest formononetin concentration at early maturity stages, with a gradual decrease observed until the onset of flowering. At

that point, formononetin concentration in leaves and stems were only 75 and 32%, respectively of those observed at the first stage sampled. Upon the initiation of flowering, formononetin concentrations in leaves and stem stabilized, with no difference observed between later stages, except for an increase in leaves at the last stage sampled (i.e., stage 8, inflorescence of main stem past flower with sepals still green). Biochanin A concentration was not affected by stage of maturity in leaves, but in stems concentrations were slightly greater before the onset of flowering than all subsequent stages.

This experiment illustrates that as plant maturity progress, variations in isoflavone concentrations depend on the plant part; concentrations decreasing in stems and flowers, while being relatively constant in leaves. These differences in trends resulted in leaves having much greater isoflavone concentrations than either flowers or stems by the time flower buds have become visible. These results are generally in agreement with the results of a greenhouse experiment reported by McMurray et al. (1986). In both studies, formononetin concentration decreased from early harvests during the vegetative stage through late flower in both leaves and stems. Interestingly, both experiments also observed an increase in formononetin concentration in leaves at approximately the onset of flower desiccation. Differences between studies were although observed, for example we observed that stems initially had comparable formononetin concentrations as leaves, while in the first two sampling in the experiment performed by McMurray et al. (1986) leaves had higher concentrations even then. It is difficult to accurately compare studies as stages given by McMurray et al. are only vaguely described.

The results observed here are in contrast to those reported by Vetter (1995), who found that flowers had higher concentrations of biochanin A and slightly higher

concentrations of formononetin than did leaves. It is difficult to confidently compare between Vetter (1995) and our study, as the plant material used for analysis in Vetter (1995) is simply reported as being collected during the 'flowering stage', and as can be seen in the current study, isoflavone levels can vary significantly during the growth of red clover. However, it can be assumed that the results are in sharp contrast nonetheless, as flowers in the current study were found to have much lower concentrations of both formononetin and biochanin A by the time buds are distinctly visible, well before coloring of flowers has occurred.

The low isoflavone concentrations we observed are of importance for the nutraceutical industry, as manufacturers are currently often only using flowers for the extraction of red clover isoflavone. Consequently, most of the production and demand is current for flowers (Filière des Plantes Médicinales Biologique du Québec 2004). This study suggests that this strategy may be inappropriate and need to be re-evaluated, especially given the difficulty to harvest flowers only, requiring manual labour, which increase production costs. Harvesting vegetative material would maximize the isoflavone concentration of red clover. Our results, however, indicate that current recommendations for the harvest of red clover as a source of forage for ruminants, which are to harvest herbage at the early flowering stage, would minimize isoflavone concentrations. Harvesting red clover at this stage would reduce the adverse affect isoflavone may have an animal reproduction.

3.4.3 Preservation method

Differences were observed between preservation methods evaluated in formononetin and total isoflavone concentrations (**Figure 3.4.3**). Total isoflavone concentration was 22% higher in fresh material ($14464 \mu\text{g g}^{-1} \text{DM}$) than either silage or hay (12200 and $11604 \mu\text{g g}^{-1} \text{DM}$, respectively), while formononetin concentration was highest in fresh material, intermediate in silage, and lowest in hay (9021 , 7220 , and $6468 \mu\text{g g}^{-1} \text{DM}$, respectively). No differences were observed between treatments in biochanin A concentration. Conversely, Sarelli et al. (2003) found isoflavone concentrations to be 18% higher in ensiled red clover than in wilted herbage before ensiling. Differences observed between studies and treatments might be due to differences in DM content of material evaluated. In the present study, DM content was of 28, 42, and 90% in fresh herbage, silage, and hay respectively. Sarelli et al. (2003) found that when DM was increased from 25 to 40% by wilting plants longer, isoflavone concentration decreased. In their study, if 40% DM silage is compared to fresh material wilted to 25% DM, the silage contains slightly less total isoflavone concentration. Alternatively, differences between studies could be due to differences in the ensiling process. Ensiling is a dynamic process, dependent on many factors, including initial herbage composition, microbial population, pH, temperature, and time, all of which fluctuate throughout the ensiling process (Charmley 2001). These factors were not measured in our experiment; it is possible that any or all of these factors have an impact on isoflavone concentration in red clover silage. The effect preservation method has on red clover isoflavone concentration is important for producers looking to control isoflavone intake of sheep and cattle. Feeding hay, or

alternatively silage, rather than fresh herbage could minimize risks associated with red clover feeding.

3.5 Conclusion

The present study suggests that environmental factors have a large effect on isoflavone concentrations of red clover. Cultivars however consistently differ in their isoflavone concentration, with one of them ('Start') having especially low concentrations. Specific cultivar recommendations could thus be made depending on if concentrations are to be maximized or minimized depending on the intended use. The large differences observed between plant parts and stages of maturity, underline the need to elaborate new recommendations specific for the production of red clover used for isoflavone extraction. The use of foliage from plants before the onset of flowering should be considered as an alternative to the current predominant practice of using flowers. In the case of red clover to be fed to ruminant animals, current recommendations and practices appear appropriate if producers intend to minimize isoflavone consumption by animals. Producers may however want to consider, when possible, feeding red clover hay rather than fresh herbage to minimize possible reproductive problems.

Table 3.1. Analysis of variance of isoflavone concentration of red clover cultivars grown for two consecutive years at two sites in Sainte-Anne-de-Bellevue, QC, Canada in 2002-2004 and harvested twice per year.

Source of Variation	Formononetin	Biochanin A	Total isoflavone
	----- P value -----		
Site (S)	<0.0001	<0.0001	<0.0001
Stand age (A)	0.0488	0.7662	0.8139
S × A	0.6463	<0.0001	0.0006
Cultivar (C)	0.0045	0.0251	0.0058
A × C	0.3638	0.7892	0.6869
S × C	0.0387	0.2918	0.1731
S × A × C	0.3598	0.4025	0.3641
Harvest (H)	0.4799	0.2807	0.3897
A × H	0.0285	0.1795	0.1161
S × H	0.0044	0.0787	0.0157
S × A × H	0.7805	0.0232	0.1734
C × H	0.0594	0.1378	0.071
S × C × H	0.2116	0.1038	0.1578
A × C × H	0.9716	0.9897	0.8026
S × A × C × H	0.0011	0.0002	0.0002

Figure 3.1. Isoflavone concentration of ten red clover cultivars grown in Sainte-Anne-de-Bellevue, QC, Canada. Results are the average of two harvests done at two sites in two consecutive growing seasons for four replicates (n= 32). Vertical bars indicate 1 SD.

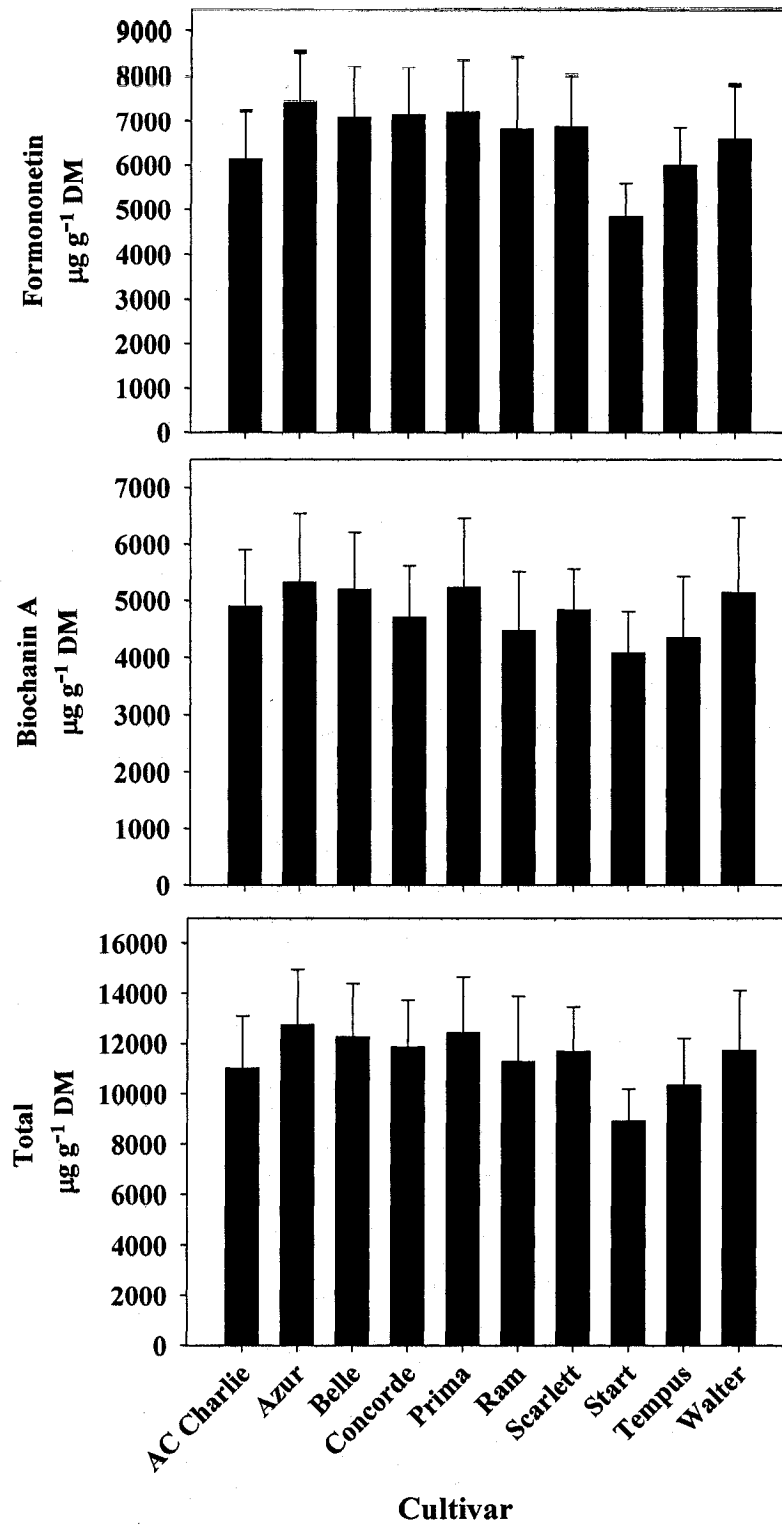


Figure 3.2. Isoflavone concentration in leaves (●), stems (○), and inflorescences (▼) of two field-grown red clover cultivars ('Azur' and 'Start') sampled at eight different stages of maturity. Stage 1 (vegetative plants with 3 trifoliate leaves), 2 (vegetative plants with 4 trifoliate leaves), 3 (vegetative plants with 5 trifoliate leaves), 4 (inflorescence of main stem palpable), 5 (single buds on main stem discernable), 6 (at least one open flower on inflorescence of main stem), 7 (inflorescence of main stem at least halfway past flower), and 8 (inflorescence of main stem past flower, sepals still green). Data are the mean of four replicates \pm SD.

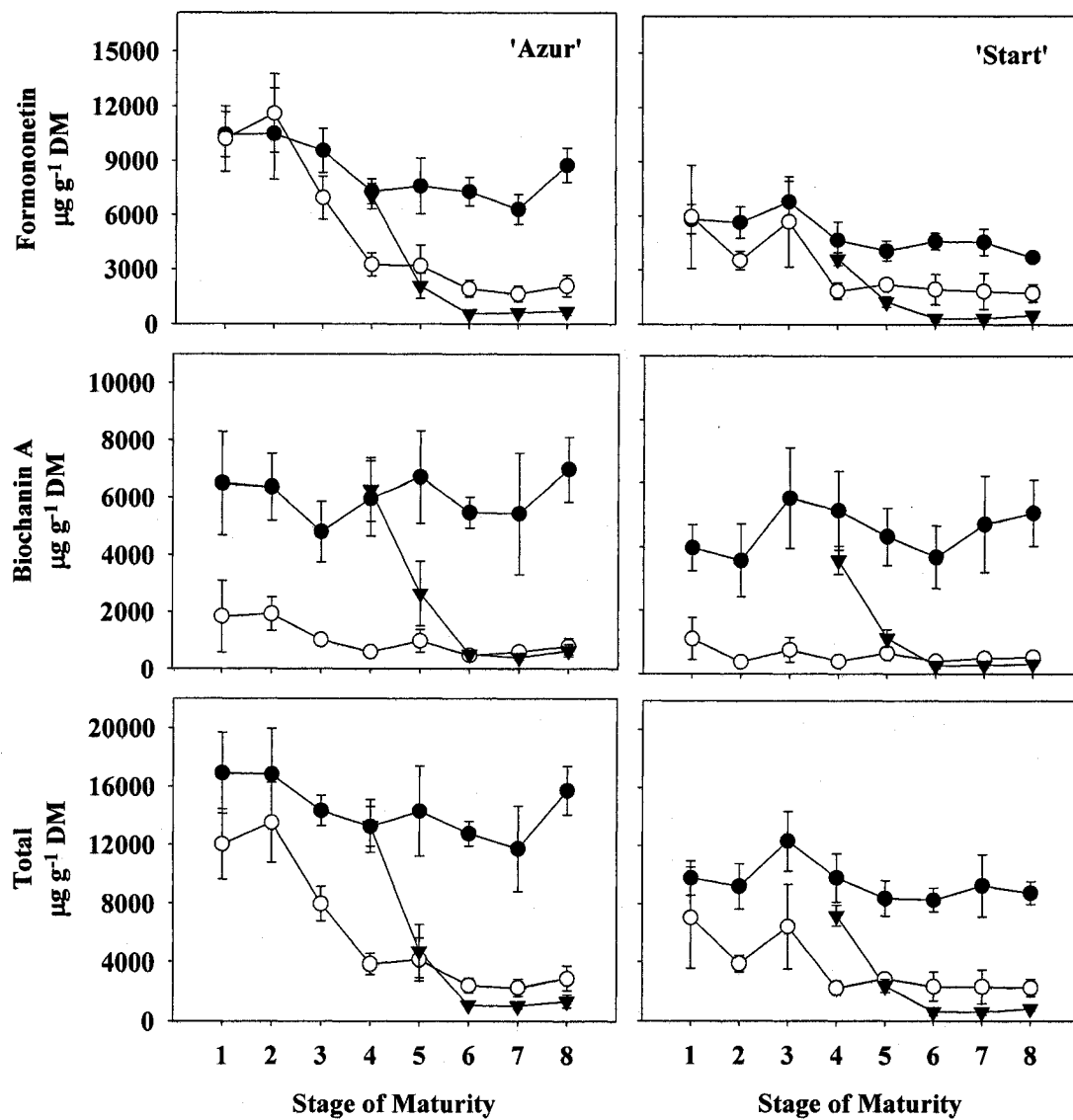
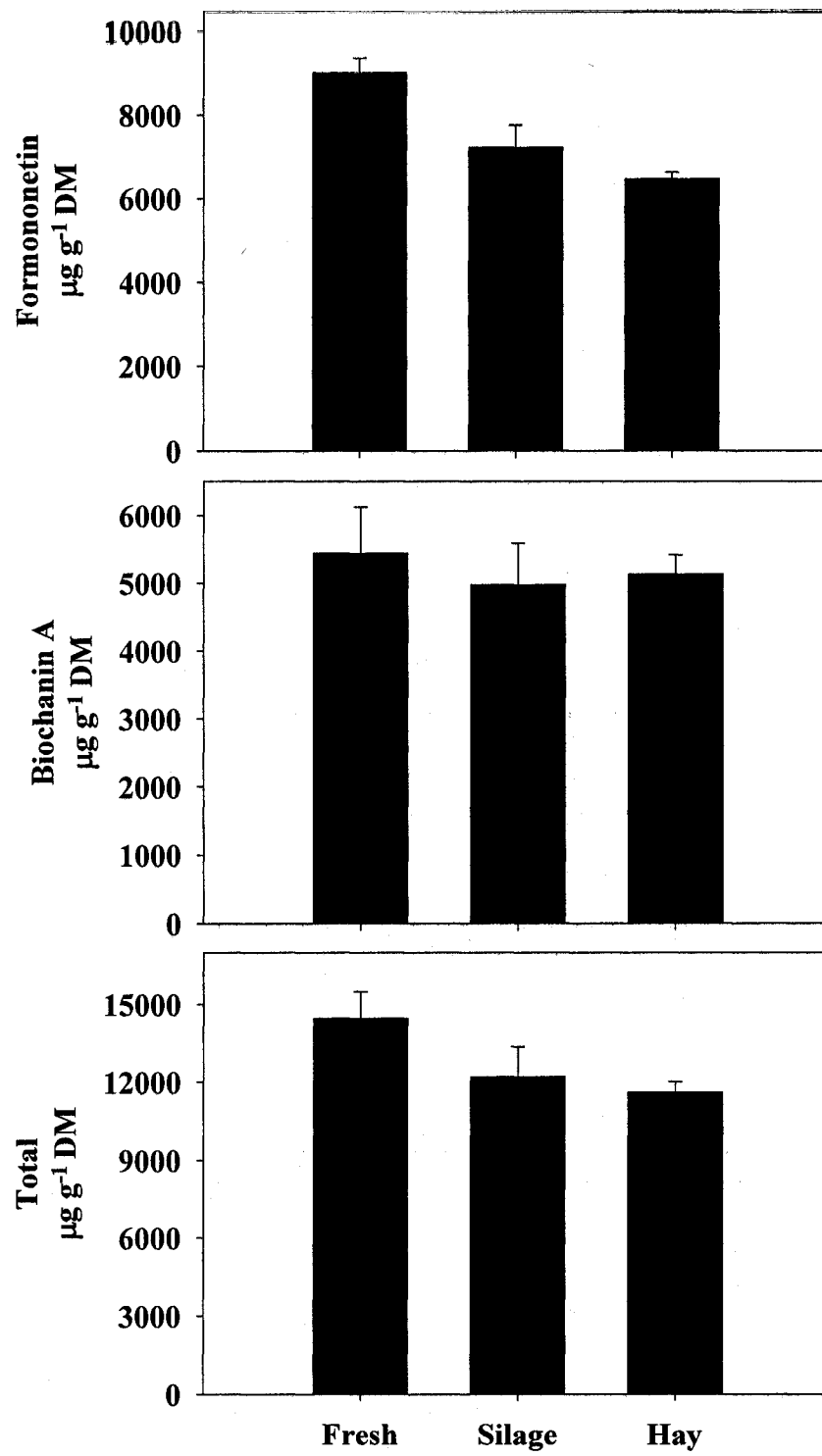


Figure 3.3. Isoflavone concentration of fresh red clover (28% DM) or red clover preserved as silage (42% DM) or hay (90% DM). Data are the mean of four replicates \pm SD.



3.6 Connecting text

In the previous series of experiments, we tested for the presence and stability of differences in isoflavone concentration between red clover cultivars in a multi-year, multi-site trial that also tested for the influence of other factors including harvest, stand age, and year. In addition, we investigated the effects of maturity, plant part, and storage and preservation techniques have on isoflavone concentration in red clover.

In the following study, we decided to look beyond more basic conventional practices, to try to increase isoflavone production in red clover through the exogenous application of elicitor compounds. This approach could provide a quick and inexpensive way to further increase isoflavone concentration beyond what is currently available.

The following chapter was submitted for publication in the Journal of Agronomy and Crop Science. It is co-authored by the candidate and Dr. Philippe Seguin, Department of Plant Science, Macdonald Campus, McGill University. The candidate carried out the experiments and data analyses and was the primary author of the manuscript. Dr. P. Seguin provided funds and assistance for this research, including supervisory guidance and the reviewing of the manuscript.

4.0 Effects of Elicitors Foliar Application on Red Clover Isoflavone Content

4.1 Abstract

Red clover (*Trifolium pratense* L.) contains high concentrations of isoflavones, compounds that have received much interest lately due to their proposed benefits for human health. In this experiment we tested the possibility to induce isoflavone production in the foliage of two greenhouse grown red clover cultivars ('Azur' and 'Start') through the application of elicitor compounds. Foliar applications of different concentrations of acetic acid (50, 100, 250, and 500 mM), yeast extract (1, 2, 3, and 4 g L⁻¹), and chitosan (125, 250, 500, and 1000 mg L⁻¹) were done on plants at the late vegetative stage, which were harvested 2 or 8 days after spraying. Concentrations of genistein, daidzein, formononetin, and biochanin A were determined by HPLC. The two cultivars tested differed in isoflavone concentrations, 'Azur' having on average 36% higher biochanin A, formononetin and total isoflavone concentrations than 'Start' ($P < 0.05$). A cultivar by sampling date interaction ($P < 0.1$) reflected a 20% increase over time in total isoflavone concentration with 'Azur', which was not observed with 'Start'. Effects of elicitors were limited, contrasts indicating overall, 12, 14, and 15% greater total isoflavone concentration in yeast extract ($P < 0.1$), chitosan ($P < 0.05$) and acetic acid ($P < 0.05$) treated plants, respectively, than in untreated control plants. There were few differences between the various elicitors and none between concentrations of each elicitor.

4.2 Introduction

Isoflavones are phenolic compounds found in a number of plant species, though predominately in legumes. Studies have shown that isoflavones may lower cholesterol levels (Cassidy et al. 1995, Nestel et al. 2004) and may be useful in the prevention or treatment of cancer (Jarred et al. 2002), bone loss (Atkinson et al. 2000), and symptoms associated with menopause (van der Weijer and Barentsen 2002). Red clover has been demonstrated to contain high levels of the isoflavones daidzein, genistein, formononetin, and biochanin A (**Figure 4.2**) and their conjugates (McMurray et al. 1986, Vetter 1995). Red clover extracts are currently being sold as herbal supplements to treat or prevent a variety of diseases and physical ailments.

Isoflavones are constitutively found in legumes, but their concentrations often increase in response to biotic and abiotic stresses. They play a role in plant defense responses to a number of stresses, including attack by pathogens, UV light, and physical and chemical damage, as well as being involved in symbiotic plant-microbe interactions (e.g., Long 1989, Stafford 1997, Olsson et al. 1998). Elicitor compounds are substances that can cause or mimic naturally occurring stresses and therefore have the ability to provoke plant defense response and production of associated compounds. Elicitors have been used in a number of plant systems, including soybean [*Glycine max* (L.) Merr] (Al-Tawaha et al. 2005), pea (*Pisum sativum* L.), and tomato (*Lycopersicon esculentum* L.) (Walker-Simmons et al. 1984) to enhance production of various compounds of interest in growing plants, excised tissue, and cell culture.

In one of the only reports on the use of elicitor compounds with red clover, it was found that formononetin aglycones were increased in the roots of red clover seedlings

after application of chito-oligosaccharides and CuCl_2 (Tebayashi et al. 2001). It was also found that these elicitors affected enzymes involved in isoflavone metabolism. With other species, various elicitor compounds have been used successfully to increase isoflavone concentrations. Yeast extract and chitosan have been shown to increase isoflavonoid production in roots of white lupin (*Lupinus albus* L.) seedlings (Gagnon and Ibrahim 1997). Chitosan has also been used to increase the production of the isoflavonoid pisatin in pea (*Pisum sativum* L.) pods (Walker-Simmons et al. 1984), and has been shown to activate defense response in rice (*Oryza sativa* L.) seedlings (Agrawal et al. 2002). In soybean, foliar application of chitosan, lipo-chitooligosaccharides, and actinomycete spores caused a 21 to 84% increase in individual and total isoflavone concentrations in mature seeds of plants treated at a range of growth stages when compared to untreated control plants (Al-Tawaha et al. 2005). Acetic acid has been used to elicit the production of various compounds, including isoflavones, when applied to intact, living plants or plant parts, including roots, in a number of species (Raskin and Poulev 2002).

There is little information available on the use of elicitors to induce isoflavone production in red clover plants. In this experiment we evaluated the potential to induce the production of isoflavones in red clover through foliar application of three elicitors: acetic acid, chitosan, and yeast extract.

4.3 Materials and methods

4.3.1 Plant growth conditions

The experiment was initiated in February 2004 in greenhouses of the Macdonald Campus of McGill University, Sainte-Anne-de-Bellevue, QC, Canada. Ten seeds of one

of two red clover cultivars, 'Azur' and 'Start', were planted per 13 x 13 cm pot, in a 6:1 soil:perlite growing medium, with seedlings thinned to 6 plants per pot at the first trifoliate leaf stage. The growing medium was inoculated with a peat based *Rhizobium leguminosarum* biovar *trifolii* inoculum (Liphatech Inc., Milwaukee, WI, USA). Plants were grown with a 12 h photoperiod and air temperature held constant at 25 °C. The 12 h photoperiod was achieved using Philips 430-W high-pressure sodium lamps (Philips Electronics, Ontario, Canada) with lighting level of 470 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were watered every two days with tap water. Elicitors were applied through foliar spray application at late vegetative stage, just prior to flowering (Stage HH1 7, Ohlsson and Wedin 1989). The foliage of plants was then harvested at either two or eight days following elicitor application. Harvested plants were chopped with scissors and immediately frozen at -80 °C.

4.3.2 Preparation of elicitors

Yeast extract (Sigma-Aldrich, Mississauga, ON, Canada) was weighed into 1, 2, 3, and 4 g samples, each dissolved in 1 L of distilled water. Acetic acid (Fisher Scientific Limited, Nipean, ON, Canada) was prepared to concentrations of 50, 100, 250, and 500 mM in distilled water and brought to a pH of 6.0 with 1 N KOH. Chitosan (Sigma-Aldrich, Mississauga, ON, Canada) was prepared according to a previously described procedure (Benhamou et al. 1994), dissolved in 500 mM acetic solution prepared as described above, and subsequently diluted to concentrations of 125, 250, 500, and 1000 mg L⁻¹. Each pot was sprayed with 150 mL of the appropriate elicitor solution with a

hand sprayer from above; untreated control plants were sprayed with an equivalent volume of distilled water.

4.3.3 Sample extraction

Samples were extracted using a slightly modified version of the protocol of Petterson and Kiessling (1984). Briefly, 1 g of frozen plant material was ground with sand with a mortar and pestle, mixed with 2 mL of distilled water, and incubated at 37 °C for 30 min. in a water bath. Sixteen mL of ethanol and 2 mL of 3M HCl were then added, samples mixed, and then heated to boiling. Extracts were allowed to cool, then 2 mL of extract were centrifuged at 1000 g to pellet large particulates prior to filtering. Extracts were filtered on SepPak C-18 cartridges (Waters Canada, Mississauga, ON, Canada) as follows: cartridges were first equilibrated with 5 mL of methanol, and washed with 5 mL of deionized water. One mL of extract was then mixed with 3 mL of deionized water and allowed to enter the cartridge. Columns were then washed with 2 mL of 20% methanol, and isoflavones eluted with 2 mL of 80% methanol. Extracted samples were stored at -20 °C until HPLC analysis.

4.3.4 HPLC analyses

HPLC analyses were performed on a Waters chromatograph system (Waters, Milford, MA, USA) consisting of two pumps (model 510), a WISP autosampler (model 712) and a UV absorbance detector (model 441). The system was equipped with a C18 reverse-phase column (Bondapak, 10 µm, 3.9 x 300 mm; Millipore, Milford, MA, USA). One hundred µl of filtered extract was injected for each analysis. Separation and elution

of isoflavones was achieved with the following gradient method using a flow-rate of 1 mL minute⁻¹. Five minutes of 20% methanol in water was increased to 80% methanol in water over the next 25 min, the methanol concentration of the mobile phase was then reduced back to 20% over the next 5 min, and kept constant at 20% methanol in water for an additional 5 min. Isoflavones were analyzed at a wavelength of 254 nm (Wang et al. 2000). Chemical standards [daidzein, genistein, formononetin, biochanin A, (Sigma-Aldrich, Mississauga, ON, Canada)] were used to identify isoflavones on chromatograms and to calculate their concentration.

4.3.5 Statistical analyses

The experiment was set up in a randomized complete block design with split-split plot restriction and three replicates, with harvest time (2 and 8 days after elicitor application) as the main plot, cultivar ('Azur' and 'Start') as the split-plot, and elicitor treatment (untreated control, 1, 2, 3, and 4 g L⁻¹ yeast extract, 125, 250, 500, and 1000 mg L⁻¹ chitosan, and 10, 100, 250, and 500 mM acetic acid) as the split-split plot. Statistical analyses were performed using the GLM procedure in SAS (SAS 1989). *A priori* contrasts with a single degree of freedom were used to compare treatment means. Effects and differences between treatments were declared significant at $P < 0.1$. This P-value was selected to minimize Type II errors.

4.4 Results and discussion

Daidzein and genistein represented only a very small proportion (i.e., 1%) of the total isoflavone concentration and remained essentially unaffected by cultivars, sampling

date and elicitor treatments. Differences were, however, observed between the two cultivars in formononetin, biochanin A, and total isoflavone concentrations ($P < 0.05$), 'Azur' containing an average of 7742, 9218, and 17148 $\mu\text{g g}^{-1}$ DM of formononetin, biochanin A, and total isoflavones, respectively, over all elicitor treatments and sampling dates, compared to 5656, 6764, and 12601 $\mu\text{g g}^{-1}$ DM for 'Start'. A time by cultivar interaction was also observed ($P < 0.1$) for total isoflavones reflecting a 20% increase in concentration between day 2 and day 8 with 'Azur'; in contrast total isoflavone concentration of 'Start' did not change between sampling dates (**Figure 4.4**). Total isoflavone concentration in 'Azur' was greater than 'Start' at both sampling dates. There was no interaction ($P < 0.1$) between either cultivar or sampling date and elicitor treatments.

Effects of elicitors were limited (**Table 4.4**). Contrasts indicated that overall, across cultivars and sampling dates, elicitors increased total isoflavone concentration ($P < 0.05$) by an average of 13% when compared to untreated control plants. Specifically, this increase was of 12, 14, and 15% with yeast extract ($P < 0.1$), chitosan ($P < 0.05$), and acetic acid ($P < 0.05$), respectively. There was however no differences in total isoflavone concentration between the various elicitors and between concentrations of each elicitor. Elicitors also had few effects on the concentration of specific isoflavones, acetic acid increasing biochanin A concentration by 17% ($P < 0.1$), and elicitors overall, as well as chitosan, increasing formononetin concentration by 14 and 16%, respectively ($P < 0.1$). Again there were few differences for specific isoflavones between elicitor types or concentrations. Finally, no differences were observed between chitosan and the 500 mM acetic acid treatment, in which chitosan was dissolved, suggesting that differences

observed between chitosan-treated plants and untreated ones may be due to the acetic acid present in the chitosan solutions as opposed to the chitosan itself.

The lack of differences observed between the various concentrations of specific elicitors may reflect that concentrations evaluated may not have maximized plant response, others might have been more effective. Kneer et al. (1999) and Al-Tawaha et al. (2005) indeed both reported that response to various elicitor compounds of *Lupinus luteus* roots and soybean plants is highly concentration dependant, with very specific concentrations maximizing plant response. The limited response to elicitors we observed could reflect that their effects may be transient and only occur more closely to the time of elicitor application. The time between elicitor application and harvest (i.e., 2 and 8 days) in the present study may be on the tail end of any plant response. Tebayashi et al. (2001), found that elicitation of isoflavones in treated red clover roots was highest 24 hrs after elicitor application. Conversely, it is possible that there may have not been enough time for effects to be seen. Elicitors were also applied during the late vegetative stage, just before flowering. Elicitor response may be more pronounced if application occurred earlier, perhaps not directly before plants are putting most resources into their reproductive cycle. Finally, we only applied elicitors once, it may be more effective to have multiple applications to reinforce any defense responses that are initiated or the stimuli may need to be repeated or maintained for a time to fully stimulate any changes in the isoflavone synthesis pathway. This is the case in cell culture studies or any studies where elicitors are applied to the roots through the growth medium (e.g., Raskin and Poulev 2002).

4.5 Conclusion

This experiment suggests that foliar applications of elicitor compounds hold promise as a means of increasing isoflavone concentrations in red clover foliage. However, further research is necessary to elucidate which elicitors are most effective, how they specifically should be used, as well as how they produce such a response.

Table 4.1. Isoflavone concentrations in the foliage of red clover plants treated with foliar application of acetic acid, chitosan, or yeast extract at different concentrations. Results are averaged over two cultivars ('Azur' and 'Start') and two sampling times after application (two and eight days) (n=12).

Treatment	Daidzein	Genistein	Formononetin	Biochanin A	Total
	----- ug g ⁻¹ DM -----				
Untreated water control	49	139	6590	6457	13235
Acetic acid 10 mM	38	108	7767	7095	15008
Acetic acid 100 mM	50	118	7704	7581	15453
Acetic acid 250 mM	45	141	7314	7330	14830
Acetic acid 500 mM	57	125	7106	8087	15375
Chitosan 125 mg L ⁻¹	56	123	7163	7250	14592
Chitosan 250 mg L ⁻¹	43	95	7252	7162	14552
Chitosan 500 mg L ⁻¹	44	103	8626	7520	16293
Chitosan 1000 mg L ⁻¹	72	187	7460	6985	14704
Yeast Extract 1 g L ⁻¹	74	140	7744	7574	15532
Yeast Extract 2 g L ⁻¹	55	139	7702	6525	14421
Yeast Extract 3 g L ⁻¹	48	131	6893	7605	14677
Yeast Extract 4 g L ⁻¹	84	147	7353	7114	14698
SEM	12	24	500	529	808

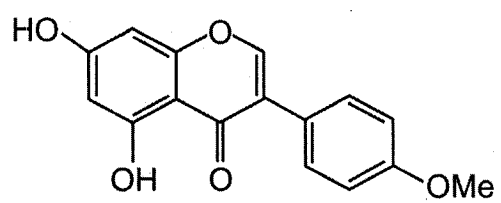
Contrasts

Elicitors vs. control	NS	NS	*	NS	**
Acetic acid vs. control	NS	NS	NS	*	**
Chitosan vs. control	NS	NS	*	NS	**
Yeast extract vs. control	NS	NS	NS	NS	*
Acetic acid vs. chitosan	NS	NS	NS	NS	NS
Chitosan vs. yeast extract	NS	NS	NS	NS	NS
Acetic acid vs. yeast extract	**	NS	NS	NS	NS
Chitosan vs. 500 mM acetic acid	NS	NS	NS	NS	NS

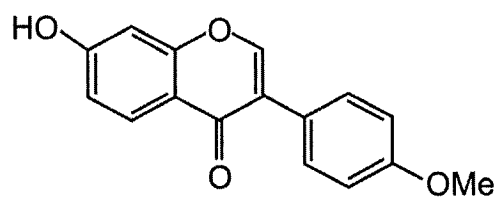
*, Significant at P < 0.1; **, Significant at P < 0.05; NS, Not significant (P > 0.1).

Figure 4.1. Structures of a) biochanin A, b) formononetin, c) genistein, and d) daidzein.

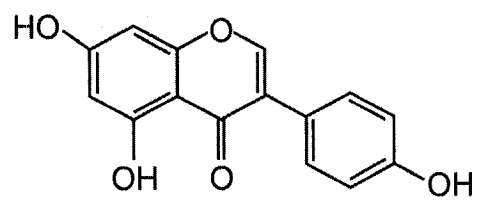
(a)



(b)



(c)



(d)

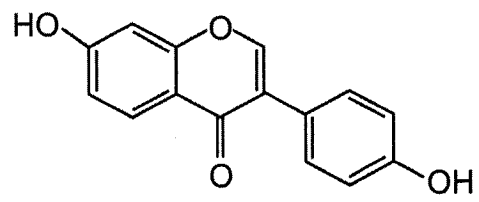
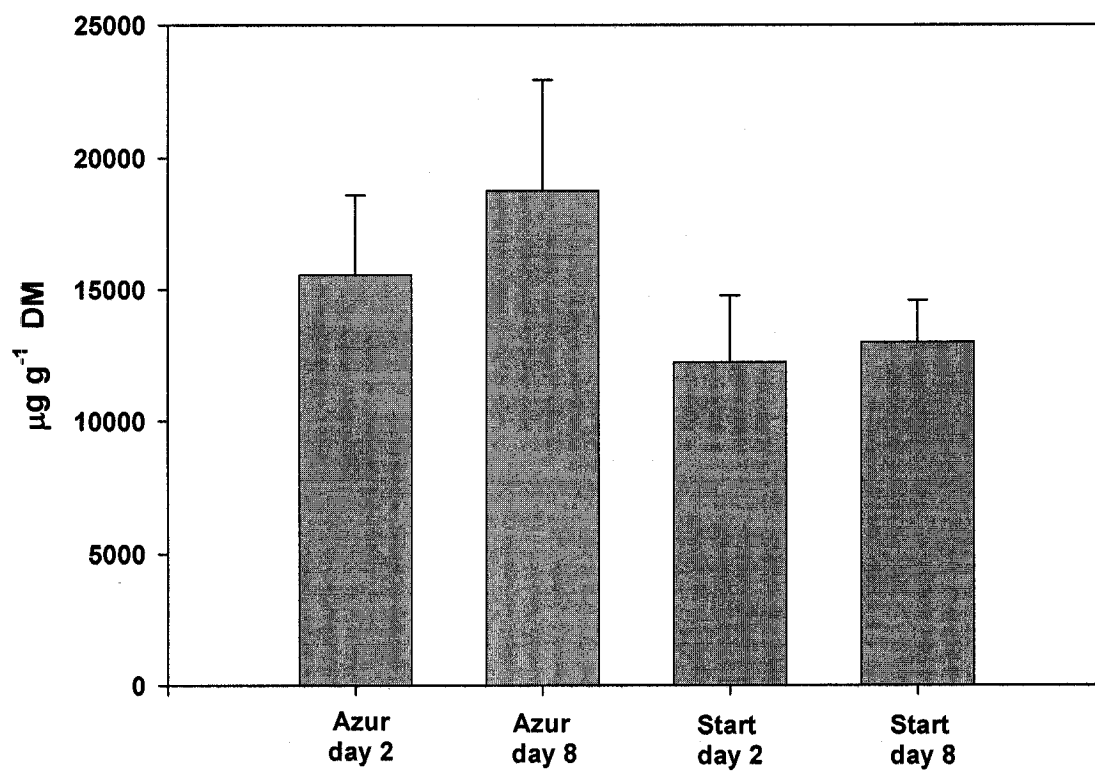


Figure 4.2. Total isoflavone concentration in the foliage of two red clover cultivars ('Azur' and 'Start'), 2 or 8 days after foliar application of elicitors (acetic acid at 10, 100, 250, and 500 mM, yeast extract at concentrations of 1, 2, 3, and 4 g L⁻¹, and chitosan at concentrations of 125, 250, 500, and 1000 mg L⁻¹) or water for untreated control plants. Results are averaged over 13 elicitor treatments and 3 replicates and represent the cultivar by sampling date interaction (n=39). Vertical lines represent \pm SD.



5.0 General Discussion and Conclusion

This project was conducted to gain a more complete understanding of the factors which affect isoflavone concentration in red clover, and therefore provide producers with the ability to better control isoflavone content in red clover destined for a number of end uses. Screening of currently recommended cultivars showed differences in both individual and total isoflavone concentrations. The cultivar 'Start' distinguished itself from other cultivars containing low concentrations of both formononetin and biochanin A; differences between other cultivars were less consistent. Site main effects and interactions of site with other factors suggest that environmental factors can have a dramatic effect on isoflavone content in red clover; these observations are in agreement with results from studies on other species (Smith et al. 1986, Seguin et al. 2004, Seguin and Zheng 2005).

Isoflavone concentration was strongly dependent upon maturity stage and plant part, as well as the interaction between the two. Averaged across maturity stages and cultivars, leaves had the highest content ($11970 \mu\text{g g}^{-1} \text{DM}$), followed by stems ($4896 \mu\text{g g}^{-1} \text{DM}$) and flowers ($3297 \mu\text{g g}^{-1} \text{DM}$). Leaves and stems had similar formononetin and total isoflavone concentrations prior to the onset of flowering. Concentrations of formononetin and total isoflavones decreased in both leaves and stems as plants neared the onset of flowering. Isoflavone concentrations remained fairly constant during flowering in leaves, while decreasing slightly in stems. The result was leaves having much higher isoflavone concentrations than stems during flowering. Biochanin A was higher in leaves than stems at all stages tested and not affected by maturity stage. Inflorescences had nearly identical formononetin and biochanin A concentrations as

leaves upon first appearance (stage 4), much higher than stems. By the next stage, however, isoflavone concentrations in inflorescence had fallen to approximately that of stems, and by stage 6 (at least one open flower on main stem) had lower formononetin and total isoflavone concentrations than did stems. This experiment illustrates that isoflavone concentrations do indeed change as red clover matures, but changes vary according to plant part. These results are generally in agreement with the results of a greenhouse experiment reported by McMurray et al. (1986). This experiment suggests that in order to maximize isoflavone concentrations in red clover herbage, it may be advisable to harvest during the vegetative stages, prior to the onset of flowering. Alternatively, if low isoflavone concentrations are sought, as when destined for feed for ruminants and reproductive problems are of concern, harvesting during flowering may be advantageous. This supports the current recommendations of harvesting red clover at early flowering for ruminant feed. Continuing this practice would minimize animal exposure to phytoestrogens present in red clover feed.

Storage and preservation methods were found to affect formononetin and total isoflavone concentrations in red clover herbage. Total isoflavone concentration was 22% higher in fresh herbage ($14464 \mu\text{g g}^{-1} \text{DM}$) than either silage or hay (12200 and $11604 \mu\text{g g}^{-1} \text{DM}$, respectively). Formononetin concentration was highest in fresh herbage, followed by silage, while hay had the lowest value (9021 , 7220 , and $6468 \mu\text{g g}^{-1} \text{DM}$, respectively). The effect of storage and preservation techniques on isoflavone content in red clover is important for producers looking to control phytoestrogen exposure in sheep and cattle. Feeding hay, or alternatively silage, rather than fresh herbage could minimize risks associated with red clover feeding.

The foliar application of the elicitor compounds yeast extract, chitosan, and acetic acid resulted in modest increases in total isoflavone concentration versus control plants (13% increase averaged across elicitors, dose, and sampling times). There was no difference observed between chitosan treatments and the acetic acid control, suggesting that increases observed in chitosan treatments may be due to the acetic acid present in the solution, and not to the chitosan itself. Results did not reveal differences between individual elicitors tested or between concentrations of each elicitor. This study suggests that while elicitor application has potential to increase isoflavone concentration in red clover, more research is needed to elucidate the most effective elicitors and their optimum concentrations, application times, and methods.

Isoflavone concentrations in red clover are influenced by both genetic and environmental factors. Manipulation of isoflavone concentrations in red clover is thus possible to achieve by genetic means through breeding or biotechnology and through agronomic practices. More research is needed in order to more fully understand ways to control isoflavone concentrations in red clover.

6.0 Recommendations for Future Research

The results of this project suggest several areas that warrant further research related to isoflavone production in red clover. Future research could include:

1. Breeding programs to increase isoflavone concentration in red clover.
2. More comprehensive studies on elicitor-induced production of isoflavones in red clover including testing different elicitors, application methods, response times, and compound concentration.
3. Exploring if specific ensiling conditions (eg. pH, temperature, etc.) can affect isoflavone concentration of ensiled herbage.

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