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Review paper

Analysis of the factors influencing red clover (*Trifolium pratense* L., Fabaceae) isoflavone content

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Summary. This paper summarizes published literature concerning different factors that could affect the content and composition of isoflavones in red clover (*Trifolium pratense* L., Fabaceae). Influence of genotype, plant part, geographic origin, ontogeny and storage conditions of raw material are discussed. Statistical analysis of available data on the profile of isoflavones in red clover suggest that, in general, samples are grouped according to country of origin, indicating the importance of climate conditions. An increase in biosynthesis of isoflavones as a response to different abiotic factors is also considered. Knowledge about these factors is of great practical importance, since red clover is a medicinal plant widely used for production of dietary supplements, and producers aim to breed cultivars with high isoflavone concentrations.

Keywords: ecological factors, genotype, geographic origin, isoflavone, ontogeny, plant part, red clover.

INTRODUCTION

Red clover (*Trifolium pratense* L., Fabaceae) is an important forage legume which is grown in many parts of the world. It is a highly valued plant due to its ability to fix atmospheric nitrogen in the soil. In recent decades, the presence of biologically active compounds has generated new increased interest in this plant. Namely, red clover has been recognized as an important source of phytoestrogens, secondary plant metabolites that can mimic or modulate the actions of endogenous estrogen (Setchell 1998). Three classes of phytoestrogens are found in red clover: isoflavones, coumestrols and lignans. Since the amount of coumestrols and lignans is small, they do not contribute in a large extent to the estrogenic effects of red clover (Booth et al. 2006a). Isoflavones are found in physiologically significant amounts in legumes, mainly soy and red clover (Messina 2010; Rietjens et al. 2017). Even though the most common source of isoflavones in human diet are soy and soy-based products, the concentration of isoflavones in red clover has been reported to be 2-10 times higher (Sivesind and Seguin 2005).

Isoflavones are a subclass of flavonoids (Wang et al. 2008). The key structural elements that are essential for the estrogenic effects of isoflavones are the phenolic ring and 2-4 hydroxyl groups in certain positions (Rosenberg-Zand et al. 2000; Smiley and Khalil 2009). According to Kolodziejczyk-Czepas (2016), sixteen isoflavonoid structures are present in red clover. Formononetin (7-hydroxy-4'-methoxyisoflavone) and biochanin A (5,7-dihydroxy-4'-methoxyisoflavone) have been listed as the most abundant isoflavones in *T. pratense* (Spanguolo et al. 2014). On the other hand, their demethylated forms, daidzein (7,4'-dihydroxyisoflavone) and genistein (5,7,4'-trihydroxyisoflavone), respectively, represent only a small portion of the total isoflavone concentration (Spanguolo et al. 2014) (Fig. 1). These compounds can be found in aglycone or in corresponding conjugated form (as acetyl, malonyl or β-glucoside) (Saviranta et al. 2010). Moreover, other minor aglycones, such are glycitein, irilone, orobol, pratensein, pseudobaptigenin and prunetin, as well as minor malonyl glycosides can be found (Spanguolo et al. 2014). Isoflavones are usually present in plants as glycosides and glycoside malonates: inactive forms that are suitable for storage of less soluble flavonoid aglycones (Beck et al. 2005; Xiao et al. 2017). It has also been reported that isoflavone glycosides are prone to hydrolysis by the enzyme β-glucosidase, which

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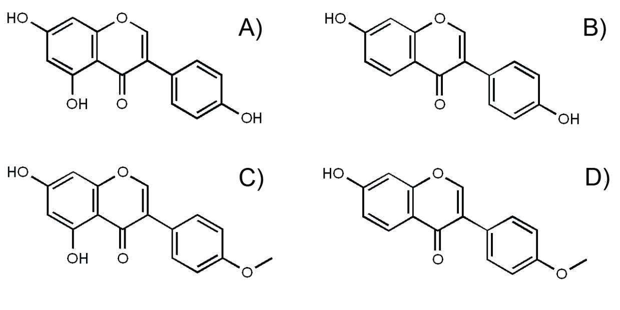

Fig. 1. Chemical structures of **A**) genistein, **B**) daidzein, **C**) biochanin A and **D**) formononetin.

can be activated upon mechanical damage of plant tissue (Xiao et al. 2017).

The estrogenic properties of red clovers are commonly listed as their main pharmacological activity, even though the efficacy of estrogenic action of plant-derived preparations is still widely discussed (Kolodziejczyk-Czepas 2016). Phytoestrogens can bind to ERα and ERβ and can modulate estrogen receptor function providing both agonist and antagonist effects (Sobenin et al. 2016; Rietjens et al. 2017).

Due to the increasing interest in natural alternatives to hormone replacement therapy, red clover extracts are becoming widely used for the treatment of menopausal disorders and for the amelioration of menopausal symptoms, such as hot flashes, night sweats, sleep disturbances, and vaginal dryness (Booth et al. 2006a; Sabudak and Guler 2009; Chen et al. 2015; Xiao et al. 2017). Phytoestrogens are also considered to provide beneficial effects on osteoporosis, cardiovascular diseases and in the prevention of breast cancer (Rassi et al. 2002; Taku et al. 2007; Ma et al. 2008; Patisaul and Jefferson 2010; Kolodziejczyk-Czepas 2012; Thorup et al. 2015; Sobenin et al. 2016; Shibu et al. 2017). In addition to their estrogenic activities, isoflavones can exert antioxidative effects (Umeno et al. 2016; Xiao et al. 2017). Furthermore, red clover has been traditionally used for the treatment of eczema, whooping cough, asthma and eye diseases. It is also considered as an antispasmodic, expectorant and sedative (Booth et al. 2006b; Saviranta et al. 2008; Raheja et al. 2018).

The distribution of isoflavones in red clover, however, is dependent on various factors (genotype, environment, climate, etc.) (Taujenis et al. 2015). Bearing in mind that red clover is a forage crop widely used as raw material for production of dietary supplements, there are two different aspects of cultivation depending on final use. If used as a feed, a lower amount of phytoestrogenic compounds may be desirable, in order to minimize possible effects on the reproductive health of farm animals. On the other hand, for the pharmaceutical industry it is important to select and breed cultivars with high isoflavone concentrations. Knowledge about the various factors which could influence isoflavone content is of a great practical importance. Therefore, in the present study we analyzed available data on isoflavone content (formononetin, biochanin A, genistein and daidzein) in *Trifolium pratense* cultivars and different factors influencing this trait.

MATERIALS AND METHODS

In order to investigate the factors influencing red clover isoflavone content, Science Direct, Pub Med and Google Scholar databases were searched with the following keywords: red clover, isoflavones, genotype, cultivation, climate and plant parts. Papers published over the last 25 years were taken into consideration. A total of 28 manuscripts dealing with the effects of genotype, plant parts, stage of ontogenetic development, geographical origin and cultivation conditions, as well as different stress factors on isoflavone content were selected and analyzed.

Furthermore, data from four studies describing the quantities of individual isoflavones in 25 red clover samples grown in Lithuania, Finland, Brazil and Serbia were processed by the use of multivariate statistics. Factor analysis, including extraction of principal components followed by rotation (varimax normalized) of the extracted factors, was used to describe the patterns of variability of selected compounds and consequential grouping of the examined samples. Canonical Discriminant Analysis (CDA) was performed to identify the compounds which mostly contribute to differences among the samples, while subsequent hierarchical cluster analysis performed on squared Mahalanobis distances evaluated similarities in grouping of samples based on the country of cultivation. All of the statistical analyses were performed in Statsoft Statistica, v 12.5 (Dell Inc. 2016).

RESULTS AND DISCUSSION

Effect of genotype on isoflavone content in red clover

Red clover varieties can typically display significant differences in isoflavone content. The reported content of total isoflavones is in the range from 1 to almost 34 mg/g (Vetter 1995; Ramos et al. 2008). It is considered that genetic factors and cultivation conditions have crucial effects on isoflavone content and composition in red clover. Numerous studies indicate that the red clover genotype has a major impact on the production of isoflavones and other secondary metabolites (Sivesind and Seguin 2005; Tsao et al. 2006; Saviranta et al. 2008; Butkutė et al. 2014; Mustonen et al. 2018). Oleszek et al. (2007) concluded that cultivar selection is essential for obtaining the highest yield of isoflavones. Additionally, it has been pointed out that ploidity of a cultivar had no statistically significant effect on total isoflavone content (Tsao et al. 2006; Lemežienė et al. 2015).

Isoflavone content in different plant parts

Isoflavones are not uniformly distributed throughout the plant and their concentrations in different red clover plant parts have been investigated in detail. Lemežienė et al. (2015) reported that the highest concentration of total isoflavones at the flowering stage was found in the leaves and the lowest in flowers (Fig. 2), in agreement with findings from several other researchers (Wu et al 2003; Sivesind and Seguin 2005; Tsao et al. 2006; Saviranta et al. 2008). Tsao et al. (2006) detected higher concentrations of isoflavones than the concentrations reported by other researchers in respective plant organs (up to 23.4 mg/g of total isoflavones in leaves). Booth et al. (2006b) also detected much higher concentrations of all four investigated isoflavones in the above-ground parts than in flower heads. However, these results are in contrast to those obtained by Vetter (1995), who detected the highest total isoflavone concentration in flowers when measured at a single sampling date. On the other hand, Bursać et al. (2011) found that the leaves are the richest in isoflavones, while the stems have the lowest amount of these compounds (Fig. 2). Another study showed that below-ground plant parts were the most abundant source of isoflavones. Moreover, that study also confirmed that stems had the highest total isoflavone concentrations among aerial plant parts (Butkutė et al. 2014) (Fig. 2).

The composition of isoflavones in the above ground parts of red clover plants have been investigated in detail and it has been noticed that two isoflavones, formononetin and biochanin A, are the most abundant (de Rijke 2004; Sivesind and Seguin 2005; Tsao 2006; Booth et al. 2006b; Oleszek et al. 2007; Ramos et al. 2008; Saviranta et al. 2008; Lemežienė et al. 2015). Formononetin, biochanin A and genistein were detected in every organ of red clover, while daidzein was found in every part except flowers (Saviranta et al. 2008). Saviranta et al. (2008) also reported that daidzein and genistein are predominantly accumulated in the petioles, while leaves contained the highest concentrations of formononetin and biochanin A. In addition, they found that leaves and roots contained the highest concentration of formononetin, while the largest amount of biochanin A was found in the leaves. On the other hand, few studies found that the concentrations of daidzein were higher than biochanin A in all plant parts (Vetter 1995; Bursać et al. 2011). It is clear that the results concerning isoflavone content and composition in different organs are contradictory, and it is difficult to establish some general pattern, but we can conclude that the majority of conducted studies indicate that the leaves are likely to be the best source of isoflavones.

Influence of the plant phenophase on isoflavone content

The same plant parts harvested at different growth periods show various concentrations of isoflavones, indicating that phenophase also affects isoflavone concentration in different organs of red clover (Tsao et al. 2006). Saviranta et al. (2008) reported that the highest formononetin and biochanin A concentrations were found in young leaves collected in June. The same was confirmed for formononetin and biochanin A concentrations in the stems and the four isoflavone concentrations in the roots. During the flowering period, the largest isoflavone content was detected in flower buds in June. The total concentration of isoflavones was also the greatest in young leaves collected in June, according to Saviranta et al. (2008). Sivesind and Seguin (2005) noted that as flowering progressed, formononetin and total isoflavone content differed in the three plant parts, being the highest in

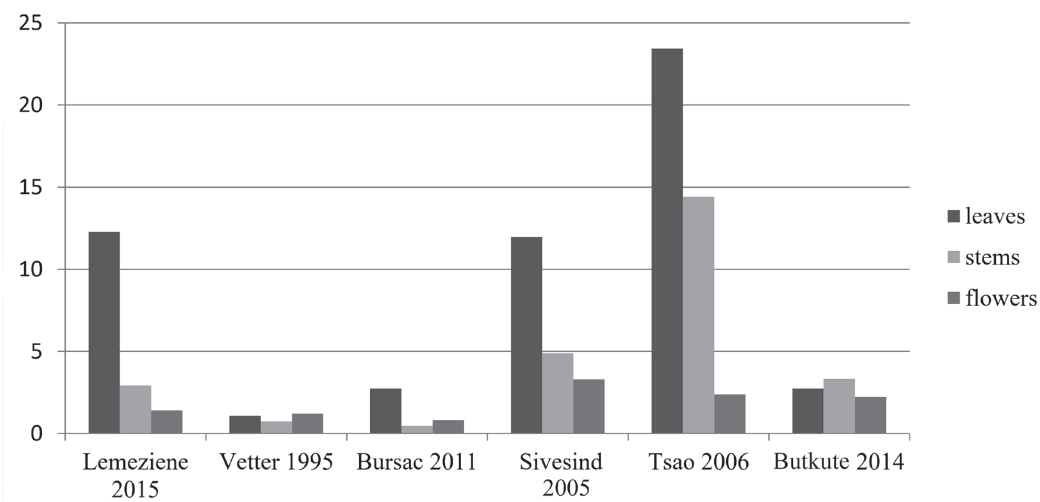

Fig. 2. Literature data on the average total isoflavone content (mg/g) in different red clover plant parts (leaves, stems, flowers).

leaves, intermediate in stems and lowest in inflorescences. Moreover, concentrations of all isoflavones decreased sharply in stems and flowers, while a smaller decrease, found only for formononetin, was observed in leaves. On the other hand, Sivesind and Seguin (2005) and Tsao et al. (2006) reported that in the leaves the average total isoflavone concentration significantly increased in late flowering stage, whereas in the stems and petioles the total isoflavone concentration decreased significantly as the plants matured. Booth et al. (2006b) also detected a peak in levels of formononetin and biochanin A in red clover aerial parts in early September, while levels of genistein and daidzein peaked around July. Furthermore, Mustonen et al. (2018) reported that concentration of total isoflavones were significantly higher at the later end of August/beginning of September, compared to earlier harvest times (July).

Geographic origin influencing isoflavone content

Published studies with detailed content and composition of the four dominant red clover isoflavones (formononetin, biochanin A, genistein and daidzein) were selected in order to elucidate the influence of geographic origin on isoflavone composition. Red clover samples from four different studies - eleven from Lithuania (Lemežienė et al. 2015), four from Finland (Mustonen et al. 2018), five from Brazil (Ramos et al. 2008) and five from Serbia (Bursać et al. 2011) were analyzed using multivariate statistics.

Results of chemical characterization of selected *T. pratense* samples reveal that the first two principal components describe more than 83% of the sample variability. The variability described by factor 1 mostly correlates with the detected amounts of formononetin and biochanin A, while in the case of factor 2, most of the variability is described by the quantities of genistein and daidzein (Fig. 3A). The position of the examined samples in the space defined by the first two factor axes shows grouping of samples originating from the same country based on their similar patterns of chemical profile variability. Namely, samples originating from Lithuania and Serbia are located in the negative part of the first principal component axis, as a consequence of low amounts of formononetin and biochanin A, and moderate amounts of genistein and daidzein. On the other hand, samples originating from Brazil and Finland are located in the positive part of the same axis. Furthermore, the notable position of samples originating from Finland is a result of high amounts of genistein and daidzein, followed by moderate quantities of formononetin and biochanin A (Fig. 3B). Previously conducted studies indicate that the biosynthesis of isoflavones increases with the exposure to UV radiation, as well as the amount of rainfall (Budryn et al. 2018; Reis et al. 2018). Furthermore, the assumption is that the biosynthesis of isoflavones is primarily directed toward the production of methylated metabolites, such as formononetin and biochanin A, while genistein and daidzein are formed by 4'-O-demethylation of the previously mentioned metabolites (Wu et al. 2003). This is in complete agreement with the results of our statistical analyses. Namely, considering the climate of the investigated countries, Brazil is characterized by the highest average monthly temperatures and precipitation, which positively correlates with its highest recorded amounts of formononetin and biochanin A.

The application of CDA reveals that only the amounts of genistein and daidzein significantly affect the discrimination of the examined samples, while hierarchical cluster analysis applied to the obtained Mahalanobis distances (Fig.

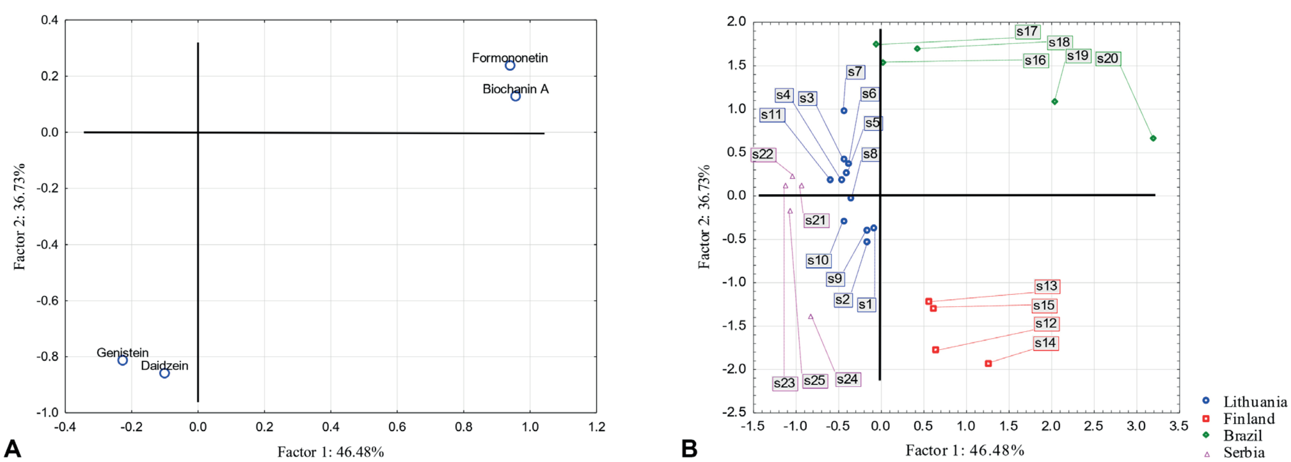

Fig. 3. A: Loadings of the first two factors after principal components extraction and Varimax normalized rotation; **B**: position of the examined *Trifolium pratense* samples (S1-S11 samples from Lithuania (Lemežienė et al. 2015), S12-S15 from Finland (Mustonen et al. 2018), S16-S20 from Brazil (Ramos et al. 2008), S21-S25 from Serbia (Bursać et al. 2011)) in the space defined by the first two principal components.

4) reveals the resemblance of chemical profiles of samples originating from Serbia and Finland with the samples originating from Lithuania. This is not surprising because similar climate conditions are present in these countries, especially in terms of precipitation and temperatures in the spring and the summer, when red clover is planted. On the other hand, samples originating from Brazil are a part of separate cluster as a result of low levels of genistein and daidzein.

Fluctuations in the chemical composition of red clover obviously depend on geographical origin, and this could be a consequence of different periods of sunlight, current weather conditions, soil characteristics and many other abiotic factors. Of course, it must be taken into account that there are certain differences in analytical procedures in the presented studies.

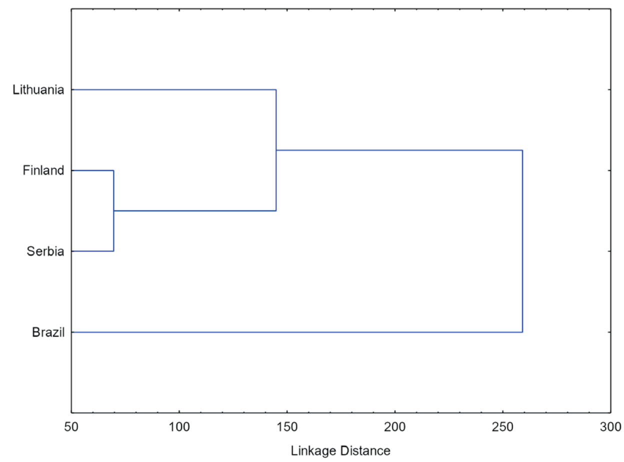

Fig. 4. Cluster analysis diagram of the examined *Trifolium pratense* samples based on their geographical origin (Ward's method, grouping variable is the country of geographical origin).

Possible approaches for maximization of isoflavones biosynthesis and production

Exposure to different biotic and abiotic factors can promote the synthesis of isoflavones. Temperature during plant growth appears to greatly affect the concentration of isoflavones. Budryn et al. (2018) evaluated the content of isoflavones in red clover sprouts cultivated under different conditions. Obtained results showed that the additional stress of high temperature (all-day access to white light in comparison to 12 hours of white light per day) induced more intensive biosynthesis of isoflavones. Even though the experimental cultivation was conducted using both UVA and UVB light considered as abiotic stress agents, the effects were both very positive. They also revealed that formononetin was the most abundant isoflavone, especially in the late stage of germination, and its glycoside in the early stage. Hence, the most favorable cultivation conditions in terms of bioavailability appeared to be red clover sprouts cultivated ten days under white light at 25 °C. The beneficial effects of continuous lighting on isoflavone biosynthesis were also observed by Swieca et al. (2012) and Gao et al. (2015). On the other hand, Mustonen et al. (2018) detected significantly higher total isoflavone concentrations in plants cultivated under challenging conditions with smaller effective accumulated temperature and greater precipitation.

An increase in the synthesis of isoflavones as a response to different abiotic factors was confirmed by Swinny and Ryan (2005). They reported that leaves from plants grown under enhanced UVB radiation contained markedly higher formononetin and biochanin A isoflavone levels than those exposed to ambient levels of UVB. The obtained results are in accordance with previously published data (Ryan et al. 1998, Ryan et al. 2002) and confirmed the hypothesis that abiotic stressors cause an upregulation of many of the genes in the phenylpropanoid pathway (Swinny and Ryan 2005). As ozone has a phytotoxic effect on plant tissues by generating reactive oxygen species, consequently, plants react against ozone-induced stress by rapid activation of several signaling pathways (Samuel et al. 2000; Kangasjarvi et al. 2005). Furthermore, increased transcription of genes involved in flavonoid biosynthesis were found in ozone resistant leguminous plants (Puckette et al. 2008). Even though there is constant synthesis of isoflavones, their concentrations are influenced by ozone exposure as an abiotic factor. Saviranta et al. (2010) found out that the concentrations of phenolics in the leaves were increased by ozone exposure. In older plants exposure to slightly elevated ozone still increased the content of flavonoids and other phenolics, but decreased the concentration of isoflavones. They assumed that an increased accumulation of isoflavones under elevated ozone conditions could be a consequence of an antioxidant defense response as isoflavones had been shown to possess antioxidative properties (Kroyer 2004; Occhiuto et al. 2009).

De Rijke et al. (2005) investigated the effects of disturbed root nodulation in response to waterlogging on the concentrations of the main isoflavones of red clover, since isoflavones are known to respond to environmental disturbances (Edwards et al. 1997). Concentrations of all isoflavones varied in a compound-specific way. It was noted that the concentrations of biochanin A, its glucoside and glucoside-malonate and genistein glucoside notably increased during the experimental period. Considering the obtained results, they concluded that isoflavones normally involved in the nodulation process accumulate in the leaves due to disturbed formation of root nodules in response to waterlogging. Saviranta et al. (2010) also reported that red clover plants partly damaged by hares contained increased concentration of aglycones. These results are in line with previous studies by Agrell et al. (2004). It was assumed that the increase in aglycones is due to hydrolysis of corresponding conjugated forms during stress (Tebayashi et al. 2001; Saviranta et al. 2010).

Postharvest drying and preservation methods may also have an effect on the isoflavone content in red clovers. Swinny and Ryan (2005) showed that the method of postharvest drying had a significant influence on the glucoside conjugate profile. They reported that freeze-drying method provides isoflavone compounds from red clover leaves in a condition closest to their natural state. On the other hand, vacuum drying resulted in almost complete conversion of glucosides to aglycones. In the air-dried harvest, a more abundant conversion of malonylated glucosides to their respective glucosides or aglycone equivalents in comparison with freeze-drying was noticed. In oven-dried material some β-glucoside hydrolysis occurred, but the percentage of aglycones was relatively low. This research demonstrated that the highest yield of bioactive isoflavone aglycones is obtained using vacuum-drying methods. Sivesind and Seguin (2005) reported that total isoflavone concentrations were higher in fresh material than either silage or hay. Conversely, Sarelli et al. (2003) found isoflavone content to be greater in ensiled red clover than in wilted herbage before ensiling. It was also observed that differences between studies could be due to variations in the ensiling process.

CONCLUSIONS

Isoflavone content in red clover is primarily a result of genetic factors, but is also influenced by many other complex environmental influences. Usually, these compounds are accumulated in red clover leaves and their content fluctuates during different phenophases. Statistical analysis of the available data on red clover isoflavone profiles suggest that, in general, samples are grouped according to country of origin, indicating that the ecological habitat characteristics, and in particular temperature and precipitation, are significant influencing factors. Concerning abiotic factors, isoflavone biosynthesis may be increased by exposure to continuous lightning (UVA and UVB radiation having the most important effect) and ozone. Also, other stress factors such as disturbed root nodulation in response to waterlogging and physical plant damage can increase their concentration. Additionally, the highest yield of isoflavones is obtained by vacuum-drying method, and isoflavone composition depends on the ensiling process. Since red clover is a forage crop which is recently becoming widely used as raw material for the production of dietary supplements, understanding these factors could enable production of final products with desirable characteristics.

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