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Comprehensive evaluation of phytoestrogen accumulation in plants and in vitro cultures of *Medicago sativa* L. 'Elçi' and natural tetraploid *Trifolium pratense* L.

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Abstract: The main goal of this study was to establish callus cell suspension cultures of *Medicago sativa* L. 'Elçi' and natural tetraploid *Trifolium pratense* L. and to compare the isoflavone production of the cultures to the original plants. The callus culture was transferred to liquid Murashige and Skoog media (MS3 and MS5) in order to establish the cell suspension cultures. The extracts were then analyzed by liquid chromatography–mass spectroscopy (LC-MS) for their isoflavones (phytoestrogen), mainly formononetin, biochanin A, daidzein, and genistein. The production of daidzein and formononetin was higher in cell suspension culture than in callus and herba of *M. sativa* L. 'Elçi,' while biochanin A and genistein content could not be detected. On the other hand, the production of phytoestrogens was more successful in the herba of *T. pratense* L. than in both of the cultures. It might be suggested that *T. pratense* L. can be grown in larger fields, whereas *M. sativa* L. can be utilized to establish in vitro cultures in order to produce isoflavone compounds for pharmaceutical purposes.

Key words: Callus, cell suspension culture, LC-MS, isoflavone, phytoestrogens, *M. sativa* L. 'Elçi', natural tetraploid *T. pratense* L. (Elçi red clover)

1. Introduction

Higher plants synthesize a wide variety of phenolic compounds during their regular growth and development. These phenolic compounds are products of the phenylpropanoid biosynthetic pathway and generally accumulate at relatively high concentrations in cell vacuoles, and they are usually chemically associated with sugars (Coronado et al., 1995). Flavonoids are found throughout the plant kingdom, whereas isoflavones are more restricted and particularly prevalent in the subfamily Papilionoideae of Fabaceae.

The Fabaceae taxonomic class includes a variety of plants with significant economic value, including soybean, alfalfa, clover, pea, and various beans. These leguminous plants accumulate a wide range of phenolic secondary compounds, including isoflavonoid conjugates (Kessmann et al., 1990b). An important group of legume natural products, isoflavones, have been better understood at the molecular genetic level (Dixon et al., 1995; Dixon 1999; Dixon et al., 2004). These compounds function as preformed or inducible antimicrobial, antiinsecticidal compounds, signaling molecules in symbiotic nodulation by *Rhizobium* bacteria, or as allelopathic agents. In vitro cultures, which are a key method for secondary metabolite production, have been established for some of the legumes. Cell suspension cultures of *Glycine max* contained higher amounts of total isoflavones than that of the callus cultures (Federici et al., 2003). Genistein, daidzein, formononetin, and biochanin A were observed in the cell suspension cultures of *Medicago truncatula* (barrel medic), which is the model legume in plant functional genomics (Suzuki et al., 2005; Farag et al., 2007). *T. pratense*, usually called red clover, has a variety of isoflavones, including biochanin A, genistein, daidzein, and formononetin, which were reported in high concentrations (Setchell et al., 2001; Khaosaad et al., 2008). Isoflavones may act as an estrogen agonist or antagonist, depending on the compound and content present in the organism (Dog, 2005). Thus, red clover has become popular as a food supplement for the amelioration of menopausal disorders (Dornstauder et al., 2001; Beck et al., 2003). Although red clover blossoms have been used in traditional herbal medicine for centuries, it is the semipurified isoflavone leaf extracts for relief of menopause-related symptoms that interest

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researchers. This positive effect on menopausal disorders has been confirmed by several clinical trials (Khaosaad et al., 2008). Wu et al. (2003) identified 31 isoflavones in the leaves and stems, 25 in the roots, and 26 in the flowers of red clover, with the highest concentrations of formononetin and biochanin in the leaves. Biochanin A and formononetin, the predominant isoflavones in alfalfa, chickpea, and red clover, are 4′-O-demethylation plant precursors to genistein and daidzein, respectively (Tham et al., 1998; Tolleson et al., 2002; Engelmann et al., 2009). Besides isoflavones, their heterosides daidzin, genistin, and ononin were also determined in *T. pratense*, *T. repens*, and *Genistella sagittalis* (Hanganu et al., 2010).

All 3 varieties of *T. pratense* L. (var. *pratense*, var. *sativum*, and var. *americanum*) grown in Turkey are diploids; however, *T. pratense* L. collected by Elci (1982) for the first time in the Tortum district of Erzurum was found to be a natural tetraploid with low seed settings and hard seed problems. Algan and Bakar (1997) reported that degenerations in the embryo sac might affect the rate of seed settings as well as fertility failure. Hard and soft seed structure, germination, and regeneration of the plant parts were also studied previously (Colgecen et al., 2008).

This is the first report of an efficient protocol of callus induction and production of isoflavones in *M. sativa* L. 'Elçi'. Additionally, analysis and comparison of herba specimens of *M. sativa* L. 'Elçi' and natural tetraploid *T. pratense* with their in vitro cultures are also reported using a liquid chromatography–mass spectrometry (LC-MS) system. The data from this study will facilitate the production of isoflavones on larger scales for pharmaceutics designed to ameliorate menopausal disorders.

2. Materials and methods

2.1. Plant material

In order to obtain the whole plant and the seeds for further usage, *M. sativa* L. 'Elçi' and natural tetraploid *T. pratense* L. (Elçi red clover) (E2-type 2n = 4x = 28 chromosomes) were grown in the experimentation gardens of the Department of Biology of Ankara University in 2010.

2.2. In vitro culture establishment

The seeds were surface sterilized twice, first in 96% ethanol for 1 min and then in a 10% sodium hypochlorite solution for 5 min. Later on, the seeds were rinsed 3 times with autoclaved distilled water. The seeds of *M. sativa* L. 'Elçi' and the scarified seeds of *T. pratense* L. (Elçi red clover) were germinated in glass jars (100 mm × 200 mm) containing hormone-free MS medium (Murashige and Skoog, 1962) supplemented with 20 g/L sucrose and 7 g/L plant agar. The culture medium was adjusted to pH 5.8 prior to autoclaving for 20 min at 121 °C. The seeds were maintained at 24 ± 2 °C with a 16-h photoperiod and a light intensity of 42 μmol m^{-2} s^{-1}.

2.3. Callus induction

Following the in vitro germination, 5 different types of explants [hypocotyl (0.5–1 cm), cotyledon, apical meristem (1 mm), epicotyl (0.5–1 cm), and young primary leaves] were excised from 20-day-old aseptic seedlings for both of the species. Whole explants were placed on MS media supplemented with a combination of kinetin (K), naphthalene acetic acid (NAA), and 2,4-dichlorophenoxyacetic acid (2,4-D) in sterile petri dishes (Table 1). The induced callus was friable with yellow color. Callus growth index (GI) was calculated by using the following formula (Sánchez-Sampedro et al., 2005):

GI = [final fresh weight (FW) – initial FW] / initial FW.

2.4. Establishment of cell suspension culture and growth determination

Calli (800 mg) derived from explants of *M. sativa* L. 'Elçi' and natural tetraploid *T. pratense* L. (Elçi red clover) were transferred to 40 mL of MS3 and MS5 media, the best for cell suspension culture, in a 125-mL Erlenmeyer flask for 20 days. Every 2 days, 3 flasks of each cell line were harvested, and the FW was determined by filtration through a cell dissociation sieve tissue grinder kit (Sigma, 200 mesh) from 30 to 60 s. The harvested cells were frozen and dried with a freeze-dryer (Lyolab, France) for 3 days, and the dry weights (DWs) of the cultures were determined before the extraction.

Table 1. Combinations of MS media.

MS (Murashige and Skoog, 1962)	Kinetin (mg/L)	NAA (mg/L)	2,4-D (mg/L)
MS1	0.5	0.5	0.3
MS2	1	1	0.5
MS3	1.5	1.5	0.7
MS4	2	2	0.9
MS5	2	-	0.3

2.5. Isoflavone extraction

Flowering and nonflowering herba of *M. sativa* L. and *T. pratense* L. were collected at the end of June 2011. Ten grams of fresh herba, root, stems, leaves, and flower samples were stored at –80 °C until the extraction. Freeze-dried samples were ground to powder by mortar and pestle. One gram of the powdered samples was extracted with 10 mL of 80% methanol by shaking at 180 rpm for 2 h. The samples were filtered and collected in a volumetric flask. Furthermore, 5 mL of 80% methanol was added to the samples and shaken overnight. All the extracts were combined and they were brought to a volume of 10 mL in volumetric flasks. Subcultures of both calli and cell suspension cultures of *M. sativa* L. and *T. pratense* L. were collected, their FWs were measured, and then they were freeze-dried to obtain their DWs. The same extraction procedure as above was applied to the 100 mg of powdered in vitro culture samples.

2.6. LC-MS analysis

Isoflavones were separated using an Agilent Eclipse Xdb-C18 column (5 µm, 4.6 × 150 mm) and analyzed using a 1200 HPLC series system (Agilent Technology Inc., USA) and Agilent MSD (LC/MSDVL, Model No: G1956A). Isoflavones were monitored at 254 nm using a photodiode-array UV-Vis detector. Daidzein, genistein, formononetin, and biochanin A (Sigma-Aldrich) were used as standards for isoflavone identification and quantification in the extracts. The wavelength absorption was monitored at 254 nm using a photodiode-array UV-Vis detector (PDA). Primary testing showed that major isoflavone compounds corresponded to peaks with retention times of 25.341 for daidzein, 27.255 for genistein, 28.527 for formononetin, and 31.212 for biochanin A. The injection volume was 10 µL for samples and standards with a flow rate of 0.8 mL/min. Solvent A was acetonitrile and solvent B was 40 mM formic acid. The mobile phase gradient system was 12%, 22%, 50%, 55%, and 12% of solvent B at 0, 20, 25, 30, and 32 min, respectively. To quantify the isoflavones in red clover dry extract, 3 concentrations of external standards were used. The standards were prepared in 100% methanol at the following concentrations: daidzein at 2.8, 7, 14, 28, and 280 µg/mL; glycitein at 2.2, 5.5, 11, 22, and 220 µg/mL; daidzein at 2.8, 7, 14, 28, and 280 µg/mL; glycitein and genistein at 3, 7.5, 15, 30, and 300 µg/mL; formononetin at 2.9, 7.25, 14.5, 29, and 290 µg/mL; and biochanin A at 2.6, 6.5, 13, 26, and 260 µg/mL.

3. Results

3.1. Establishment of callus and cell suspension cultures

Callus growth was observed within 3–7 days on cut surfaces of the hypocotyl, cotyledon, apical meristem, epicotyl, and young primary leaves of both of the species. The growth of calli index from *M. sativa* L. 'Elçi' demonstrated that MS3 medium was the best calli growth medium for the first subculture of explants, except young primary leaves. As for *T. pratense* L., MS4 medium was the best calli growth medium for the first subculture of explants, except cotyledon (Figure 1). MS2, MS3, and MS4 media were suitable for callus production in natural tetraploid *T. pratense* L.; MS3 medium for *M. sativa* L. 'Elçi' and natural tetraploid *T. pratense* L. exhibited similar and optimal growth curve in cell suspension culture. Because of that, MS3 was used for subculturing. The calli became uniform in appearance within 4 to 5 weeks and were used to initiate

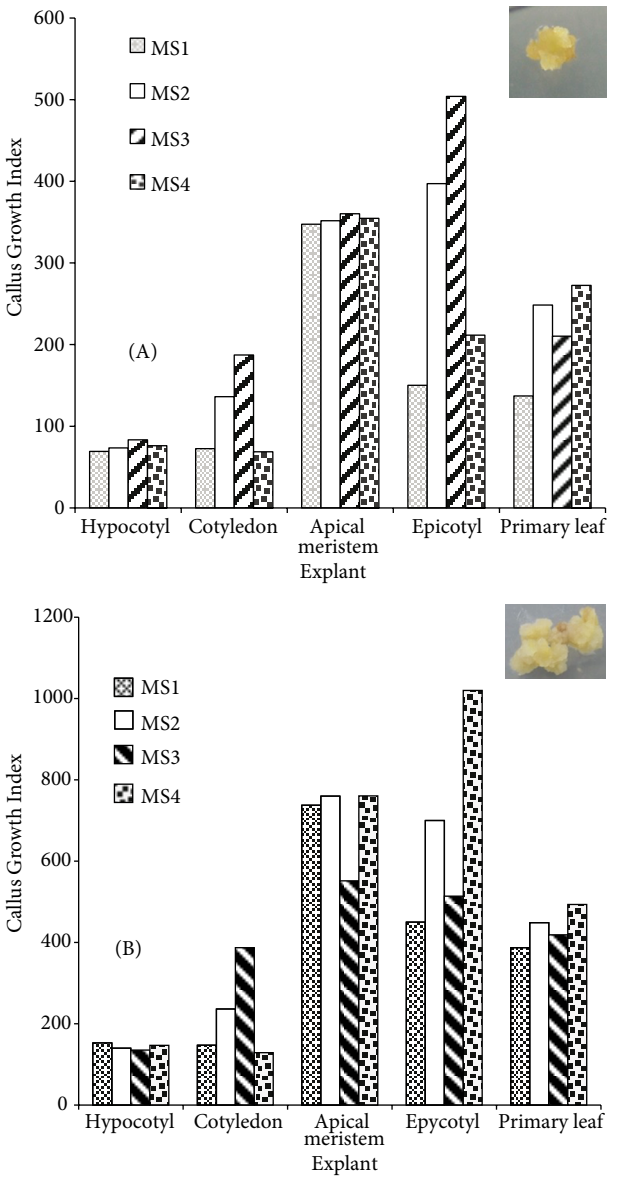

Figure 1. Yellow calli from apical meristem explants in MS3 from *M. sativa* L. 'Elçi' (A) and natural tetraploid *T. pratense* L. (B). Callus growth indexes of *M. sativa* L. 'Elçi' (A) and natural tetraploid *T. pratense* L. (B) from hypocotyl, cotyledon, apical meristem, epicotyls, and young primary leaves explants in MS.

suspension cultures, which were subcultured every 4 weeks in MS3 medium for both of them.

The growth characteristics of the cell suspension cultures were examined based on FWs during the 18 days of culturing. All cell lines of *M. sativa* L. and natural tetraploid *T. pratense* L. were in lag phase for 2–6 (MS3) and 2–10 (MS5) days, respectively. They then began to grow exponentially until reaching a stationary phase within 6–16 and 10–16 days, respectively. The highest fresh biomass of *M. sativa* L. and *T. pratense* L. was observed on day 15 in MS3 and day 13 in MS5, respectively.

3.2. Determination of isoflavonoid content in herba, callus, and cell suspension cultures

Herba, callus, and cell suspension cultures of the plants were analyzed by LC-MS. Daidzein, genistein, formononetin, and biochanin A were observed in herba and cultures of the plants.

3.2.1. Herba

Isoflavones were not detected in flowering and nonflowering roots, stems, and leaves of *M. sativa* L. 'Elçi' since they were under the detectable limit (Table 2). The highest isoflavone levels were determined as daidzein at 2.630 mg/g DW (stem), genistein at 0.276 mg/g DW (leaf), formononetin at 2.930 mg/g DW (leaf), and biochanin A at 6.958 mg/g DW (leaf) in nonflowering natural tetraploid *T. pratense* L. (Figure 2; Table 3). However, the highest levels of isoflavonoids were recorded as daidzein at 2.681 mg/g DW (root), genistein at 0.116 mg/g DW (root), formononetin at 1.934 mg/g DW (leaf), and biochanin A at 1.372 mg/g DW (leaf) in flowering natural tetraploid *T. pratense* L. (Table 3).

3.2.2. Callus and cell suspension cultures

All of the isoflavonoids in question were under the limit of detection in the herba of *M. sativa* L. 'Elçi', whereas daidzein was determined in the callus culture of *M. sativa* L. 'Elçi' when MS3 and MS4 media were applied. Besides daidzein, formononetin was also produced in cell suspension culture of *M. sativa* L. 'Elçi' with the utilization of MS3 and MS5 media. The total isoflavonoid contents increased from day 6 to day 14 of all cell lines of *M. sativa* L. 'Elçi' in MS3 and MS5 media. The highest levels of formononetin and daidzein production were 0.47 mg/100 mg DW and 0.23 mg/100 mg DW cell suspension culture in MS3 medium on day 14, respectively. Those results were similar in MS5 medium on day 14 (Table 2).

Daidzein, formononetin, and biochanin A were observed in the callus culture of natural tetraploid *T. pratense* L.; on the other hand, genistein was not determined. The major isoflavonoids that were found in every cell line were daidzein, genistein, formononetin, and biochanin A. The total isoflavonoid content increased from day 2 to 10 in all the cell lines of natural tetraploid *T. pratense* L. in the cell suspension culture. The best daidzein, genistein, formononetin, and biochanin A production of the cell suspension culture in MS3 medium was 0.074, 0.036, 0.125, and 0.0055 mg/100 mg DW, respectively. The best daidzein, genistein, formononetin, and biochanin A production was 0.018, 0.047, 0.096, and 0.01 mg/100 mg DW in cell suspension cultures in MS5 medium, respectively. Formononetin was the major isoflavonoid that accumulated in every cell line of MS3 and MS5 suspension cultures (Table 3).

4. Discussion

In our study MS medium was used, which is easily prepared in the laboratory or purchased premade at a low cost, to establish calli and cell suspension cultures for the 2 cultivars. On the other hand, different studies performed with *Medicago* species applied different medium contents. For instance, Kessman et al. (1990a, 1990c) and Jorrin and Dixon (1990) used only 10^{-7} M K. A cell suspension culture of *M. sativa* L. 'Du Puits' was established with 1 mg/L 2,4-D and 0.1 mg/L K in darkness (Schröder et al., 2001), which is different from our study.

Several flavonoids/isoflavonoids have been described in the mycorrhizal and nonmycorrhizal roots of *M. sativa* L. 'Sitel', such as coumestrol, medicarpin, ononin, formononetin (24 µg/g FW), daidzein (19 µg/g FW), and genistein (0.2 µg/g FW) (Larose et al., 2002). Moreover, 4 phytoestrogens (coumestrol, apigenin, luteolin, and quercetin) were found in field-grown alfalfa (*M. sativa* L.) in Montreal, Canada (Seguin et al., 2004). In this study, the samples were grown in an experimental garden, not in a field. Moreover, the differences in *M. sativa* L. varieties and/or growing conditions might have affected our results.

The most common isoflavonoids in the root of diploid *T. pratense* L. 'Kenland' were formononetin conjugates (4 µmol/g FW) (Tebayashi et al., 2001). Krenn et al. (2002) observed that the most common isoflavonoids in the herba of diploid *T. pratense* L. were daidzein (0.11 mg/g DW), genistein (0.10 mg/g DW), formononetin (2.89 mg/g DW), and biochanin A (2.04 mg/g DW), whereas Peng and Ye (2006) reported that the most common isoflavonoids in the herba of diploid *T. pratense* L. were daidzein (0.87 mg/g DW), genistein (0.49 mg/g DW), and biochanin A (2.11 mg/g DW). The most common isoflavonoids in the green parts of *T. pratense* L. from a natural habitat in Lublin, Poland, were daidzein (0.042 mg/g DW), genistein (0.278 mg/g DW), formononetin (1.541 µg/g DW), and biochanin A (1.627 mg/g DW) (Zgorka, 2009). In this study, much higher isoflavonoid contents were determined in the extracts of nonflowering and flowering natural tetraploid *T. pratense* L. than in any of the aforementioned studies.

In this study, we were also able to increase the amounts of daidzein and formononetin in callus and cell suspension

Table 2. Isoflavonoid content determined by LC-MS in herba, callus, and cell suspension culture in *M. sativa* L. 'Elçi'.

Plant	Nonflowering herba			Flowering herba					
mg/g DW	Root	Stem	Leaf	Root	Stem	Leaf	Flower		
Daidzein	nd	nd	nd	nd	nd	nd	nd		
Genistein	nd	nd	nd	nd	nd	nd	nd		
Formononetin	nd	nd	nd	nd	nd	nd	nd		
Biochanin A	nd	nd	nd	nd	nd	nd	nd		
Cultures	Callus culture								
mg/100 mg W	MS1	MS2	MS3	MS4					
Daidzein	0.018 ± 1.0	0.017 ± 1.5	0.022 ± 2.1	0.026 ± 0.9					
Genistein	nd	nd	nd	nd					
Formononetin	nd	nd	nd	nd					
Biochanin A	nd	nd	nd	nd					
	Cell suspension culture								
mg/100 mg DW	MS3								
Day	2	4	6	8	10	12	14	16	18
Daidzein	0.13 ± 1.1	0.14 ±2 .2	0.16 ± 1.5	0.13 ± 1.3	0.19 ± 2.3	0.22 ± 2.5	0.23 ± 1.5	0.21 ± 1.6	0.23 ± 1.2
Genistein	nd	nd	nd	nd	nd	nd	nd	nd	nd
Formononetin	0.14 ± 2.4	0.14 ± 1.8	0.15 ± 2.3	0.19 ± 1.1	0.24 ± 2.8	0.37 ± 1.9	0.47 ± 1.3	0.33 ± 2.7	0.32 ± 1.6
Biochanin A	nd	nd	nd	nd	nd	nd	nd	nd	nd
	Cell suspension culture								
mg/100 mg DW	MS5								
Day	2	4	6	8	10	12	14	16	18
Daidzein	0.04 ± 2.7	0.03 ± 1.2	0.03 ± 2.3	0.10 ± 2.1	0.11 ± 2.5	0.13 ± 2.1	0.17 ± 0.5	0.10 ± 1.6	0.10 ± 1.5
Genistein	nd	nd	nd	nd	nd	nd	nd	nd	nd
Formononetin	0.12 ± 2.1	0.20 ± 1.8	0.27 ± 2.2	0.38 ± 2.8	0.41 ± 2.9	0.46 ± 1.8	0.49 ± 1.1	0.29 ± 1.0	0.18 ± 2.5
Biochanin A	nd	nd	nd	nd	nd	nd	nd	nd	nd

Each value represents the mean ± SD of samples. DW: Dry weight, nd: not detected.

cultures of *M. sativa* L. As a result, the cell suspension culture was more successful than the callus culture and the herba with respect to isoflavonoids production.

To date, different researchers have analyzed varieties of *M. sativa* L. species for their isoflavonoid content. Medicarpin, afrormosin glucoside, and afrormosin glucoside malonate were determined in cell suspension cultures of *M. sativa* L. 'Calwest 475' (Kessmann et al., 1990c). Twenty-seven isoflavones, flavone glucosides, and/or glucoside malonates were determined in cell suspension cultures of *M. truncatula* L. (Farag et al., 2007). In roots grown under limited nitrogen supply, isoflavonoids were identified as formononetin 7-O-β-D-glycoside (ononin) and formononetin 7-O-β-D-glycoside-6′-malonate (malonylonoin) in *M. sativa* L. subsp. *varia* 'A2' and *M. sativa* L. subsp. *sativa* 'Nagyszenasi' (Coronado et al., 1995). In our study, the isoflavonoid contents of calli and cell suspension cultures of *M. sativa* L. 'Elçi' were much higher than in the intact plant. Several flavonoids/isoflavonoids have been described in mycorrhizal and nonmycorrhizal roots or cell suspension cultures of *M. sativa* L., such as coumestrol, medicarpin, ononin, formononetin, daidzein, genistein, biochanin A, 4′,7-dihydroxyflavanone, 4,4′-dihydroxy-2′-methoxychalcone, isoliquiritigenin, and 4′,7-dihydroxy-flavone (Dalkin et al. 1990; Kessmann et al. 1990a; Larose et al., 2002). Contrary to the other reports, all the reported isoflavonoids were under the detection limits in the analyzed plant samples. These differences are possibly due to the difference in *M. sativa* varieties and/or growing conditions.

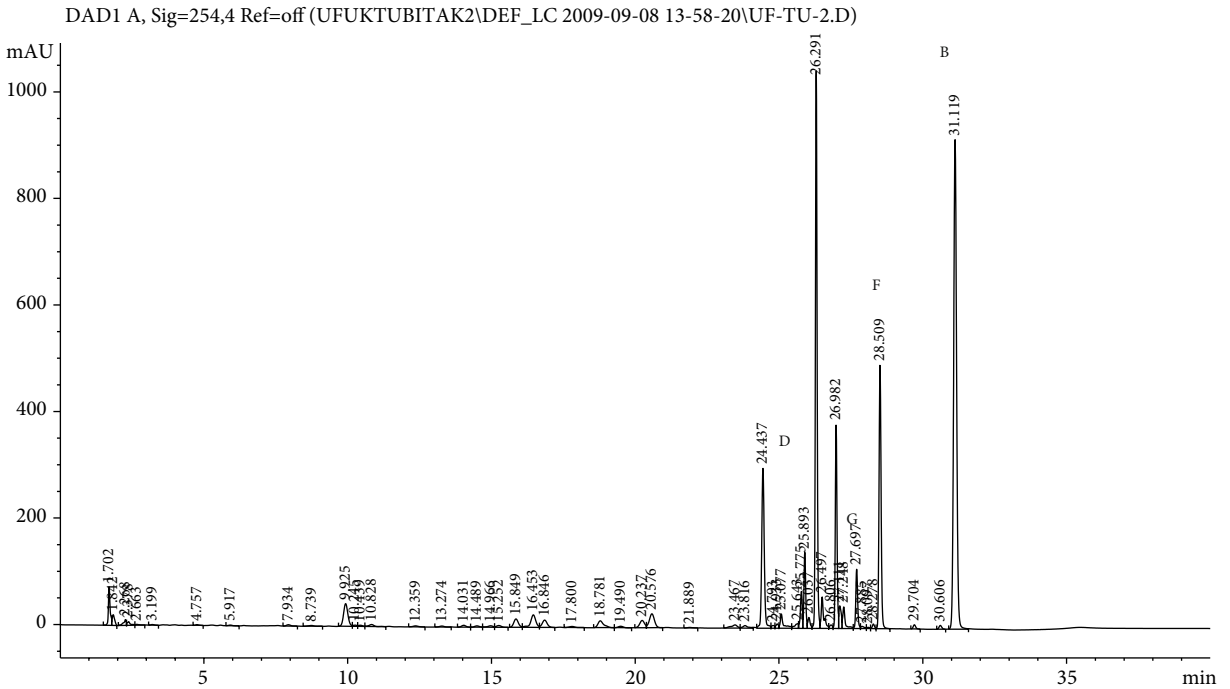

Figure 2. LC-MS chromatogram for daidzein (D), genistein (G), formononetin (F), and biochanin A (B) in leaves of nonflowering natural tetraploid *T. pratense* L.

In this study, the isoflavonoid accumulation patterns of *M. sativa* L. 'Elçi' cell suspension cultures were different from those of *M. sativa* L. 'Elçi' calli cultured on semisolid medium. This difference may be due to the factors that differ between suspension culture conditions and culturing of calli on semisolid media, including shear stress from agitation and limited oxygen supplied in liquid medium. These factors might affect growth and secondary metabolite accumulation (Zhong et al., 1995). According to our results, cell suspension cultures maintained in MS3 and MS5 of *M. sativa* 'Elçi' were able to produce higher amounts of nonconjugated isoflavonoids (daidzein and formononetin). Our results showed that the cell suspension cultures derived from *M. sativa* L. 'Elçi' could be a significant source for the production of daidzein and formononetin. Although there are numbers of studies of *Medicago* species and cultivars, mostly at the enzyme and/or gene levels (Dalkin et al., 1990; Kessmann et al., 1990a; Dixon, 2004), this is the first report on *M. sativa* L. 'Elçi' grown in Turkey. The results showed that the isoflavonoids daidzein and formononetin, which are pharmacologically important for women's health, can be successfully produced in suspension cultures. Previously, daidzein and formononetin were produced in root and cell suspension cultures of *M. truncatula*, which accords with our data. However, the researchers did not report the level of produced isoflavonoids (Farag et al., 2007).

Nonflowering herba and flowering herba of natural tetraploid *T. pratense* L. contained higher levels of isoflavonoids than the callus and cell suspension cultures. Most of the time, the plant herba contained the best amount of isoflavones (Babaoğlu et al., 2001). The best production of biochanin A was observed on day 8 in MS3 suspension culture medium. The best production of daidzein and genistein was observed on day 8 in MS5 suspension culture medium. Genistein was produced only in the cell suspension culture of *T. pratense* L. (Table 3). In this study, a lower amount of plant growth regulators and light flux were used than in the study by Engelmann et al. (2009) for the callus and the cell suspension cultures in natural tetraploid *T. pratense* L. In the leaf of the plant, the highest level of formononetin was 0.49 mg/g and biochanin A was 0.065 mg/g, whereas in calli that originated from the leaf, the formononetin level was 0.142 mg/g and biochanin A was 0.022 mg/g (Engelmann et al., 2009). There were more isoflavonoid compounds in the herba and cell suspension cultures of natural tetraploid *T. pratense* L. than in the diploid *T. pratense* L., whereas they were similar in the callus cultures of both tetraploid and diploid *T. pratense* L. (Engelmann et al., 2009). Kasparová et al. (2006) used Gamborg medium for callus and suspension cultures in 4 different diploid varieties of *T. pratense* L. As a result, TLC and HPLC only detected formononetin. Our results were in accord with the previous study (Ercetin et al., 2012) in terms of formononetin and genistein production in callus culture with Gamborg B5 medium.

Table 3. Isoflavonoid content determined by LC-MS in herba, callus, and cell suspension culture in natural tetraploid *T. pratense* L.

Plant	Nonflowering herba			Flowering herba					
mg/g DW	Root	Stem	Leaf	Root	Stem	Leaf	Flower		
Daidzein	1.656 ± 1.0	2.630 ± 0.7	0.490 ± 1.5	2.681 ± 1.0	1.291 ± 1.2	0.639 ± 1.7	nd		
Genistein	0.040 ± 0.5	0.199 ± 1.8	0.276 ± 0.8	0.116 ± 0.9	0.104 ± 0.7	0.110 ± 2.0	0.025 ± 0.4		
Formononetin	0.075 ± 1.7	0.319 ± 2.4	2.930 ± 1.1	0.054 ± 1.8	nd	1.934 ± 1.4	0.893 ± 1.3		
Biochanin A	0.011 ± 0.8	0.158 ± 2.0	6.958 ± 1.3	nd	nd	1.372 ± 0.9	0.192 ± 1.0		
Cultures	Callus culture								
mg/100 mg DW	MS1	MS2	MS3	MS4					
Daidzein	0.028 ± 1.6	0.030 ± 1.1	0.037 ± 0.8	0.028 ± 1.7					
Genistein	nd	nd	nd	nd					
Formononetin	nd	0.017 ± 0.9	0.012 ± 1.2	nd					
Biochanin A	0.002 ± 1.0	nd	nd	nd					
	Cell suspension culture								
mg/100 mg DW	MS3								
Day	2	4	6	8	10	12	14	16	18
Daidzein	0.074 ± 1.5	0.069 ± 2.4	0.067 ± 1.8	0.058 ± 0.9	0.056 ± 1.3	0.048 ± 0.8	0.028 ± 2.0	0.025 ± 2.2	0.022 ± 0.7
Genistein	0.016 ± 1.9	0.027 ± 2.1	0.036 ± 1.1	0.032 ± 2.1	0.029 ± 1.1	0.024 ± 0.5	0.009 ± 1.1	0.015 ± 1.9	0.011 ± 0.5
Formononetin	0.125 ± 1.0	0.105±1.9	0.083 ± 2.0	0.062 ± 1.8	0.045 ± 1.7	0.034 ± 1.0	0.025 ± 1.6	0.023 ± 2.0	0.016 ± 2.1
Biochanin A	0.0043 ± 2.0	0.0048 ± 1.5	0.0043 ± 1.6	0.0055 ± 1.2	0.0045 ± 1.2	0.0041 ± 1.5	0.0040 ± 1.9	0.0042 ± 1.7	0.0045 ± 1.9
	Cell suspension culture								
mg/100 mg DW	MS5								
Day	2	4	6	8	10	12	14	16	18
Daidzein	0.017 ± 1.4	0.018 ± 1.1	0.017 ± 1.5	0.018 ± 1.1	0.017 ± 1.8	0.016 ± 2.1	0.011 ± 2.0	0.015 ± 0.9	0.015 ± 2.3
Genistein	0.014 ± 1.9	0.015 ± 2.1	0.035 ± 1.2	0.047 ± 0.9	0.046 ± 1.4	0.025 ± 1.9	0.035 ± 1.7	0.011 ± 0.7	0.019 ± 2.0
Formononetin	0.096 ± 0.8	0.081 ± 1.7	0.076 ± 1.2	0.072 ± 1.0	0.068 ± 1.5	0.049 ± 1.6	0.044 ± 1.0	0.054 ± 1.2	0.048 ± 1.9
Biochanin A	0.010 ± 1.5	0.009 ± 1.0	0.009 ± 0.9	0.009 ± 1.6	0.008 ± 0.9	0.006 ± 2.0	0.001 ± 1.1	0.003 ± 1.2	0.004 ± 1.7

Each value represents the mean ± SD of samples. DW: Dry weight, nd: not detected.

In conclusion, a cell suspension culture method was developed for *M. sativa* L. 'Elçi', in which daidzein and formononetin were produced in as little as 18 days. Natural tetraploid *T. pratense* L. can grow in large fields as a medicinal plant. Better callus and cell suspension culture methods should be developed for the production of daidzein, genistein, formononetin, and biochanin A in the natural tetraploid *T. pratense* L. Large-scale production methods of daidzein, genistein, formononetin, and biochanin A by cell suspension culture or bioreactors can be investigated in further studies.

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