Iğdır Üniversitesi Fen Bilimleri Enstitüsü Dergisi, 13(3): 1625-1632, 2023
Journal of the Institute of Science and Technology, 13(3): 1625-1632, 2023

ISSN: 2146-0574, eISSN: 2536-4618

Biology **DOI: 10.21597/jist.1228129**

Research Article

Received: 02.01.2023 **Accepted: 06.05.2023**

To Cite: Albayrak Turgut, B. and Bezirganoglu, İ. (2023). Callus Induction and Bioactive Compounds Production from Various Cultivars of *Medicago sativa* L. (*alfalfa*). *Journal of the Institute of Science and Technology*, 13(3): 1625-1632.

Medicago sativa Çeşitlerinde Kallus İndüklenmesi ve Biyoaktif Bileşiklerin Üretimi

Büşra ALBAYRAK TURGUT*, İsmail BEZİRGANOĞLU

Öne Çıkanlar:

- *Medicago sativa* Çeşitleri
- Genetik Çeşitlilik
- Sekonder Metabolitler

Anahtar Kelimeler:

- *Medicago sativa*
- Kallus İndüklenmesi
- Fenolikler
- Flavonoidler
- Tanenler

ÖZET:

Yonca (*Medicago sativa* L.), Türkiye'de yaygın olarak yetiştirilen baklagiller familyasına aittir. Fenolik bileşenler, flavonoid, temel amino asitler (valin, lösin, treonin ve lizin), tanenler, vitaminler (A, B1, B2, B6, B12, C ve E) veya β-karoten gibi biyoaktif bileşikler açısından zengindir. Bu çalışmada, explant sekonder metabolit içeriğinin kallus biyokütlesine etkilerinin araştırılması amaçlanmıştır. Bu amaçla kotiledon explantları steril koşullarda elde edilip kallus oluşumu 1 mg/L 2,4-D (Diklorofenoksi Asetik Asit) ve 0.0125 mg/L kinetin içeren standart MS ortamına aktarılmıştır. Explantların fenolik, flavonoid ve tanen içerikleri de belirlenerek biyokütleleri ile ilişkisi belirlenmiştir. Kallus biyokütlesi belirlenmesi için 74 *M. sativa* L. çeşidinin kotiledon eksplantları kullanılmıştır. Test edilen 74 yonca çeşidi, kallus gelişimi ve kallus biyokütle oluşumu bakımından farklılık göstermiştir. Van-22, Konya-Ereğli, Alsancak, Gözlü-1 ve İside çeşitlerinde daha yüksek kallus biyokütlesi gözlenirken, doku kültüründe Van-Gevaş, Bitlis-Hizan ve Van-Çaldıran daha düşük kallus biyokütlesi ile yanıt vermiştir. Doku kültürü koşullarında kotiledon eksplantları açısından yüksek kallus biyokütlesi olan bir yonca çeşidinin, düşük kallus biyokütlesi çeşidine göre daha yüksek toplam fenolik, flavonoid ve tanen içeriğine sahip olduğu gösterilmiştir. Toplam fenolik içeriği, düşük kallus biyokütlesine kıyasla daha yüksek kallus biyokütlesine sahip kotiledon eksplantlarında önemli ölçüde yüksektir. Yaprak taneni ve flavonoid birikiminin kallus biyokütlesi ile güçlü bir şekilde bağlantılı olduğu belirlenmiştir. Kallusun artan biyokütlesi ile doğru orantılı olarak kotiledon fenolik ve flavonoid içeriği artan bir eğilim göstermiştir.

Callus Induction and Bioactive Compounds Production from Various Cultivars of *Medicago sativa* L. (*alfalfa*)

Highlights:

- Medicago sativa Cultivars
- Genetic Diversity
- Sekonder Metabolites

Keywords:

- *Medicago sativa*
- Callus Induction
- Phenolics
- Flavonoids
- Tannins

ABSTRACT:

Alfalfa (*Medicago sativa* L.) belongs to fabacaea family widely grown in Turkey. It is rich in bioactive compounds such as phenolic compounds, flavonoid, essential amino acids (threonine, leucine, lysine, and valine) and tannins, vitamins (A, B1, B2, B6, B12, C and E) or β-carotene. In this study, it was aimed to investigate the impact of secondary metabolite content of explants on callus biomass. For this purpose, cotyledon explants were obtained under sterile conditions, and transferred to standard MS medium containing 1 mg/L 2,4-D (Dichlorophenoxy Acetic Acid) and 0.0125 mg/L kinetin to induce callus formation. The phenolic, flavonoid and tannin contents of the explants were also determined. Leaves and cotyledons explants of 74 *M. sativa* L. cultivars have been used for callus biomass. The 74 tested *alfalfa* cultivars varied in their callus growth and callus biomass formation. Van-22, Konya-Ereğli, Alsancak, Gözlü-1 and Iside cultivars were observed with higher callus biomass: Conversely, Van Gevaş, Bitlis Hizan and Van-Çaldıran responded with lower callus biomass in tissue culture. A high-callus biomass cultivar of *alfalfa* has been shown to have higher total phenolic, flavonoid and tannin content activity than the lower-callus biomass cultivar in terms of leaf explants under tissue culture conditions. Total phenolic content activity was significantly increased in cotyledon explants with higher callus biomass as compared to lower callus biomass. The accumulation of leaf tannin and flavonoid was strongly linked to callus biomass. Cotyledon phenolic and flavonoid content exhibited an increasing trend in response to the increasing biomass of callus.

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INTRODUCTION

Alfalfa (*Medicago sativa* L.) is a forage crop species that originated in various region at the world. This plant species has been cultivated for a different of aims such as soil fertility, animal feed, medicinal uses and nitrogen benefits (Bora&Sharma, 2011; Bezirganoglu, 2021; Tussipkan&Manabeyeva, 2022). *Alfalfa* is used as a traditional medicine to treat many health conditions due to its high vitamin, coumarines, alkaloids, porphyrines, stachydrine, homostarchydrine and flavonoids (Steppler&Nair, 1987; Rafinska et al. 2017; Singh and Sharma 2020; Suwingyo et al. 2023). Various plant secondary metabolites are found in *alfalfa*. Several bioactive molecules have been found in plant tissue culture, especially callus culture such as flavonoids and stilbenes, sterols, cardenolides and phenolic acids (Maneechai et al., 2012; Szopa&Ekiert, 2014). On the other side, alfalfa has the most cultivated area around the World. According to data from TUIK, the cultivated area of alfalfa has increased by 154 percent from 2002 to 2020 in parallel with the World and continues to increase because of its high nutrient value.

Alfalfa secondary metabolites plays important roles in several stress conditions such as cold, drought and salt. To overcome this stress factors, plants produce secondary metabolites together with other bioactive moleculesv (Rafinska et al. 2017). In recent years, plants have treated with nanoparticles, hormones and combination of them to increase quality of the secondary metabolites or stress resistance (Sajid et al. 2021; Paramo et al. 2023; Wang et al. 2023; Zhang et al. 2023).

In vitro culture methods are the most effective ways for providing bioactive molecules with a characterized production system and limited culture conditions, which supplies a continuous produce of metabolites of interest. Also, cells are without diseases and are not subject to seasonal variations (Qiang et al., 2020; Tahiri et al., 2022). Moreover, it has the potential for the production of new molecules not normally found in the parent plant. Plant growth regulators are largely used to stimulate callus, as they boost cell growth by inducing cell division and elongation through synergistic, antagonistic, and additive interactions. 2,4-dichlorophenoxyacetic acid (2,4-D, an auxin) has been commonly used either alone or combined with cytokinins, specially 6-benzylaminopurine (BAP), to induce callus initiation and determine bioactive molecules in tissue culture (Junairiah et al., 2021). However, the dosage and combination of these plant growth regulators must be identified for each species (Loredo-Carrillo et al., 2013). Various studies have indicated that the advantageous effects and the biological properties of the essential oils and their major components including terpenes and terpenoids (principally sesquiterpenes and monoterpenes) (Stephane & Jules, 2020). Although various effects of secondary metabolites in *alfalfa* have been reported to date. Currently, there are no reported effects of secondary metabolite on flavonoid and tannin, for Turkish local *alfalfa* ecotype. In our study, callus induction of *alfalfa* was provided by using MS medium supplemented with 2,4-D and kinetin (a cytokinin) as plant growth regulators. The aim of this study was to determine the effect of 2,4-D and kinetin dosage on the induction of calluses and the secondary metabolite profile of *alfalfa* cotyledon and leaves.

MATERIALS AND METHODS

Plant Cultivars, Culture Conditions and Callus Biomass

In this study, cotyledons types of explants of 74 *M. sativa* cultivars and ecotypes were used as the material for the response to callus induction. Heirloom seeds used in this study was gathered from different area of Van, Bitlis, Erzurum, Bingöl, Konya, Uşak, Sivas and Muş. Also, the cultivars developed in Turkey was obtained from Agricultural Research Institutes such as Alsancak, Savaş, Sazova, İside, Nimet, BATEM, Bilensoy, Bilensoy 82, MA-225, Kalender, Gözlü, Kayseri, Elçi, Ömer

Bey and Plato. The mature seeds were disinfected with 1 % NaClO (sodium hypochlorite) 20 min, and then, washed three times with sterile dH_2O (distilled water). These seeds were placed in Petri dishes containing MS medium (Murashige and Skoog, 1962) for 15 days at 26 ± 1 ^{0}C and in 16/8 h light and dark photoperiod at 1500 lX illumination intensity. Cotyledons and leaf explants were removed aseptically using forceps and transferred on MS medium (Murashige and Skoog 1962) with 1 mg /L 2,4-D, 0.0125 mg /L kinetin.

Seconder metabolite extraction

Seconder metabolites were extracted by using previously described method by Sagharyan et al. (2020) with some modifications. Briefly, leaves or cotyledons (50 mg) were crushed to a fine powder with liquid nitrogen. 2 ml methanol was added to the powder and sonicated at 500 w, 40 ^{0}C for 22 minutes by using a bath sonicator (KUDOS), and subsequent shaken for 12 hours at room temperature. After that, the extracts centrifuged at 20000 g for 10 min and supernatants were transferred to new 2 ml tubes.

Total content assays

Total phenolic content

100 µl of the extract was added to 1 ml of Folin Ciocalteu reagent and 2 ml of dH_2O. After 3 minutes, 1 ml of 20 % (w/v) sodium carbonate (Na_2NO_3) was added and stirrid vigorously in a vortex mixer. The mixture was stored in the dark condition at room temperature for 45 min. The reaction mixtures were neasured by using spectrophotometer at 725 nm. Total phenolic contents were calculated by a standard curve prepared with Gallic Acid Equivalent (GAE, 40-1250 µg/ml). Total phenolic contents were expressed as mg per g fresh weight (FW).

Total flavonoid content

0.5 ml of the extracts were supplemented with 150 µl of 20 % (w/v) sodium nitrite ($NaNO_2$) and 2 ml of dH_2O). After 5 minutes, 150 µl of 10 % (w/v) aluminum chloride ($AlCl_3$) was added and followed by the addition of 1 ml of 1 M sodium hydroxide (NaOH) and 5 ml of dH_2O. Then, the mixtures were shaken and kept in the dark condition for 30 min. The absorbtion of the mixtures was measured using spectrophotometer at 510 nm and the concentrations were determined by using a standard curve prepared with Catechin Equivalent solution (CE, 2-64 mg/ml). The results were expressed as mg per g FW.

Total tannin content

250 µl of the extract was added to 750 µl pure methanol. Then, 3 ml of 1 % (w/v) methanol vanilin solution and 2.5 ml of 9 N methanol sulfuric acid were added. The mixtures were incubated in the water bath at 38 ^{0}C for 15 min. After that, the absorbtions was measured by using spectrophotometer at 500 nm. Total tannin content was calculated by a standard curve of CE solution (2-32 mg/ml). The results were expressed as mg per FW.

Statistical analysis

All experiments were carried out in three replicates in our study. Correlation analysis with Pearson's coefficients (R) were calculated to evaluate the correlations between callus biomasses and total secondary metabolite contents at α = 95 % ($P<0.05$) using IBM SPSS Statistics 22 software (SPSS Inc., Chicago, IL, United States).

RESULTS AND DISCUSSION

Plant tissue cultures are widely used techniques to produce valuable secondary metabolites of pharmaceutical, nutritional and industrial significance. The recent advances in plant tissue organ and cell culture have shown promising results to significantly improve biomass growth and productivity (Murthy et al. 2014). The most excellent initial material to be used for production of secondary metabolite was callus culture or suspension cell culture produced there from since it provides shortening of time, fast production, overcoming seed dormancy and seed viability. Secondary metabolite accumulation in plants can be influenced by several factors, including explant sources, genotype, developmental stage, and environmental conditions. The optimization of media components and culture environmental factors are key approaches that should be identified for each plant species at the initial stage of the culture process (Toivonen et al. 1992; Shohael et al. 2006). Total phenolic, flavonoid and tannin substance amounts of the samples obtained by extracting the callus of the alfalfa genotypes with methanol were evaluated as a result of the analysis. Obtained results are shown on graphs and the differences among callus biomass and explant sources used are compared. In the present study, all of 74 genotypes were produced callus in medium including 1 mg /L 2,4-D and 0.0125 mg /L kinetin, and from these samples the 5 samples with the highest bio-mass and the 3 samples with the lowest bio-mass were chosen to evaluate the total phenolic, flavonoid and tannin content of the callus (Table 1). In terms of leaf explants, results indicated that 2,4-D and kinetin had a significant effect on the content of biochemical compounds ($P \leq 0.05$). The phenolic content of Alsancak and Konya-Ereğli genotypes was found to increase remarkably in response to in vitro culture. Based on callus biomass, Alsancak, Van-22 and Konya-Ereğli exhibited a high level of biomass, and they were higher phenolic content that accumulated and present in in vitro culture. In contrast, the Gözlü 1 ecotype did not show the expected phenolic content in the cotyledons after inoculation with callus culture (Figure 2). Although the Van-Gevaş, Bitlis-Hizan and Van-Çaldıran ecotypes exhibited a low level of biomass, and they were sufficiently phenolic content that accumulated in in vitro culture. It was detected that the biomass of fresh callus in samples was highly linked to plant growth regulation under *in vitro* culture and the lowest fresh callus biomass was detected in Bitlis-Hizan ecotype. (Fig. 2). This confirms that genotype and plant growth regulation are directly related to the interactions of in cell culture medium. The results shown in Figure 2B demonstrate that the *alfalfa* Konya-Ereğli had the highest total flavonoid content in terms of its fresh callus biomass when compared to the other genotypes. Among the other *alfalfa* genotypes, Van-Gevaş, Alsancak and Iside had higher rates of total flavonoid content as compared with Bitlis-Hizan and Van-Çaldıran.

Table 1. Callus Biomasses of *M. Sativa* Cultivars Grown in 1 ppm 2,4-D and 0.0125 ppm Kinetin

Cultivars	Biomass (g)
Alsancak	0.521 ± 0.065
İside	0.428 ± 0.055
Van-22	0.630 ± 0.024
Gözlü-1	0.455 ± 0.059
Konya-Ereğli	0.524 ± 0.103
Van-Gevaş	0.026 ± 0.001
Bitlis-Hizan	0.025 ± 0.009
Van-Çaldıran	0.027 ± 0.005

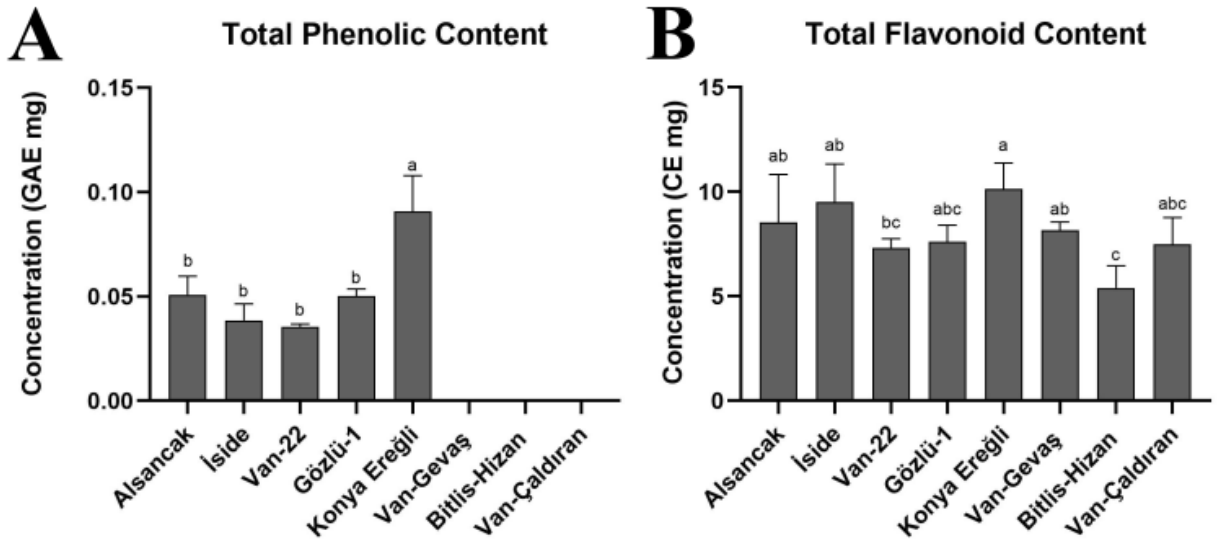

Figure 1. Total Phenolic (A) and Flavonoid (B) Content of Cothyledons From M. Sativa Cultivars (GAE: Gallic Acid Equivalent mg, CE: Catechin Equivalent mg)

Callus growth potential and total flavonoid content were greatly influenced by the genotype. Our results indicated that the percentage of total flavonoid content varied significantly depending on the genotype and fresh callus biomass. A wide variation was detected between the genotypes tested in response to total tannin content. In this study, it was observed that genotypes with high callus biomass produced higher amount of tannin, whereas genotypes with low callus biomass did not produce tannin. Interestingly, tannin formation was observed even though the Van-Çaldıran genotype had low callus biomass (Fig. 2C). The results indicated that total tannin content decreased significantly in low callus biomass. Our results indicated that fresh callus biomass rates ranged from 0.630 to 0.0251, suggesting significant genotypic variations in the total tannin content potential between the eight genotypes. The total tannin content was observed with a highs biomass but it is not observed in total tannin content with a low biomass. In our cases, total tannin content was at different levels in all tested genotypes under callus culture including 1 mg /L 2,4-D and 0.0125 mg /L kinetin. These findings are consistent with one of the first reports on callus induction and secondary metabolite profile obtained from *Elephantopus scaber* (Junairiah et al., 2021). A similar result of callus induction and production of phenolic compounds production under callus cultures of *Nepeta binaloudensis* Jamzad (*Lamiaceae*) was also reported (Sagharyan et al., 2020). Their results demonstrate that 1⁄2 MS supplemented with 2 µM reduced-glutathione and 1B, 2N increased the frequency of callus formation and biochemical compounds, including phenolics, flavonoids, and tannins content compared with the control medium. In terms of cotyledons explants, All genotypes with high callus biomass produced total phenolic content, but those with low callus biomass did not produce any total phenolic content. In terms of genotypes, the highest total phenolic content was found in Konya-Ereğli (Figure 1A). The lowest total phenolic content was found in Van-22. This result shows that it affects not only the callus biomass but also the total phenolic content in the regions to which the genotypes are grown. Mahood et al. (2022) showed that the type of explant and 2,4-D concentration affected the type and morphology of callus formation. These findings are in agreement with that reported by Castro et al. (2016) in the study on callus induction and phenolic component production from *Byrsonima verbascifolia*. Their results showed that callus produced remarkably amounts of phenolic compound and flavonoid regardless of the presence or absence type and dosage of hormone, light. However, the combination of different dosage of affected a direct relationship among callus induction and callus biomass. Figure 1b shows

the content of flavonoid compounds in in vitro cultures of *alfalfa*. Konya-Ereğli, Iside and Alsancak were most abundant flavonoid content, followed by Van-Gevaş and Gözlü-1. Van-22 and Van-Çaldıran presented intermediate amounts while Bitlis-Hizan had the lowest contents. Although the production of flavonoid content relies on several factors such as explant type, culture medium and genotype, their synthesis during the culture medium seems to promote callus structure induction. The high levels of callus biomass in tissue culture is associated with high level of presence of flavonoid, while low levels of callus biomass are hypothetical indicators of the induction of embryogenic structures rather than flavonoid content production in *alfalfa*. Our findings showed that the accumulation of total phenolic, flavonoid, tannin content and callus growth potential varied significantly depending on the genotype, similar to some of earlier reports. For instance, the concentration of quinoline alkaloid camptothecin alters among various genotypes including *Ervatamia heyneana, Camptotheca lowreyana, Camptotheca acuminata, Camptotheca yunnanensis, Ophiorrhiza pumila, Ophiorrhiza rugosa, Ophiorrhiza mungos, Nothapodytes nimmoniana, and Nothapodytes foetida* (0.03 – 0.4 % DW; Ramesha et al. 2008). Similarly, the amount of Bacoside A, a triterpenoid saponin, varies among different species of *Bacopa monnieri*, ranging from 3.53 to 18.36 mg g-1 DW (Naik et al. 2012). This is consistent with earlier reports showing that secondary metabolite accumulation is greatly linked to genotype. Therefore, the main factor is the choice of precious genotypes and specific organs for the induction of *in vitro* cells, organs, or callus.

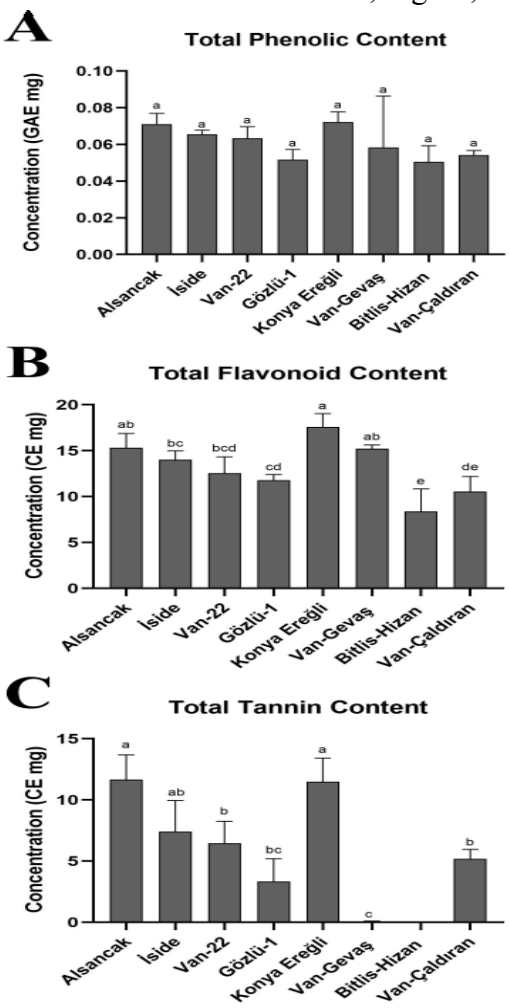

Figure 2. Total Phenolic (A), Flavonoid (B) and Tannin (C) Content of Leaves From *M. Sativa* Cultivars (GAE: Gallic Acid Equivalent mg, CE: Catechin Equivalent mg)

The present study is the first report to evaluate bioactive compounds production for alfalfa through fresh callus biomass with cotyledon in combination with 2,4-D and kinetin. It was found that the leaves of *alfalfa* contained the considerable amounts of bioactive compounds, such as phenolic, flavonoid and tannin. On the other hand, phenolic compound, flavonoid content were found in in vitro-grown cotyledon extract. Generally, concentrations of bioactive compounds in leaves were found at least two times higher than in cotyledon explants.

CONCLUSION

The present study is the first report to evaluate bioactive compounds production for *alfalfa* through fresh callus biomass with cotyledon in combination with 2,4-D and kinetin. It was found that the leaves of *alfalfa* contained the considerable amounts of bioactive compounds, such as phenolic, flavonoid and tannin. On the other hand, phenolic compound, flavonoid content were found in in vitro-grown cotyledon extract. Generally, concentrations of bioactive compounds in leaves were found at least two times higher than in cotyledon explants.

Conflict of Interest

The article authors declare that there is no conflict of interest between them**.**

Author's Contributions

All methods given in this article and the evaluation and interpretation of the results obtained from the applied methods were carried out by Büşra ALBAYRAK TURGUT. This article was written by İsmail BEZİRGANOGLU.

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