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Research Article

Content of Fatty Acids in Callus Cultures of Endemic A*juga vestita* BOISS.

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ABSTRACT

Ajuga L., a member of the Lamiaceae family, is one of the most popular species due to its medicinal, decorative, and pharmacological properties. One of them is *Ajuga vestita* BOISS. which is endangered and placed in the "Endangered (EN)" category. In the present study, using *in vitro* tissue culture techniques, *Ajuga vestita* BOISS. seeds were germinated in hormone-free 1/4 MS medium, and callus were regenerated from leaf explants of germinated seeds in 1/1 MS medium containing BAP (benzylaminopurine) and Kin (kinetin). It was aimed at comparatively examining the fatty acid content of *Ajuga vestita* BOISS. callus. When the fatty acid content of the callus obtained from the control (hormone-free), BAP, and Kin media were examined, major saturated fatty acid amounts were palmitic acid in all three extracts (59.137%, 38.836%, and 24.295%) for control, BAP, and Kin respectively. The major unsaturated fatty acid amounts were octadecanoic acid (21.097%, 32.283%) for control and BAP, respectively, and linoleic acid (21.209%) for Kin considering that high amounts of unsaturated fatty acids make the plants more reliable in terms of health, the higher amount of unsaturated fatty acid content determined in the Kin extract in our study compared to the control will shed light on future studies in this sense that can be done with tissue culture techniques.

1. Introduction

Rare or locally endemic species are sensitive to changes in environmental conditions because they have less genetic diversity than common species. When their environmental conditions change, they are more in danger of extinction. According to the IUCN 2001 criteria, approximately 600 of our endemic species are in the "Very Endangered CR" category, and 700 of them are in the "Endangered EN" category [1, 2]. For this reason, studies on conservation and reproduction methods gain more importance. It is at this point that *in vitro* plant culture has emerged for the conservation and mass clonal propagation of rare plants.

The ajuga genus belonging to the Lamiaceae family contains more than 300 annual or perennial species, and the demand for them has increased sharply due to their medicinal, decorative, and pharmacological properties. These wide-ranging plants are in danger of rapid extinction due to over-collection for ornamental and medicinal purposes, as well as habitat destruction and deforestation. *Ajuga boninsimae*, *Ajuga bracteosa*, *Ajuga ciliate*, *Ajuga genevensis*, *Ajuga incisa*, *Ajuga makinoi*, *Ajuga multiflora*, *Ajuga pyramidalis*, *Ajuga shikotanensis*, *Ajuga reptans* and *Ajuga vestita* are categorized as endangered and protected plants. One of these species is *Ajuga vestita* BOISS. which is medically important, endemic, and in the “EN-Endangered” category.

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Phytochemical studies have revealed that ajuga species contain many bioactive compounds, such as anthocyanidin-glycosides, essential oils, iridoid glycosides, flavonoids, phytoecdysteroids, sterols, and terpenoids. The studies conducted with other Ajuga species have observed that these species have phenolic compounds, the components of their essential oils are determined, and they have many pharmacological activities such as antioxidant, cytotoxic, and antimicrobial activity [3-7].

In recent years, there has been an increasing interest in the production of components with pharmacological effects (secondary metabolites, fatty acid components, etc.) by tissue culture methods. These components can also be obtained by conventional methods, but these methods have disadvantages, especially for rare endemic plants. *In vitro* culture methods provide different alternatives to overcome the problems encountered in obtaining these metabolites naturally. With these studies, it is aimed at obtaining a large number of plants to be produced and then to producing them on an industrial scale and at a low cost for some applications [8].

In this study, it was aimed at comparatively determining the fatty acid content (control, BAP, Kin) in callus regenerated from leaf explants of seedlings obtained from *in vitro* shoots of *Ajuga vestita* BOISS. The fact that there is no study in the literature in which the fatty acid compositions of this plant were presented comparatively before increases the importance of this study. These data will shed light on any possible future study with this and similar endemic plants.

2. Material and Method

2.1. Plant material and cultivation

Ajuga vestita BOISS. seeds, which were used as starting material in the study, were collected from the Savur district of Mardin and identified by Prof. A. Selçuk ERTEKIN (*Ajuga vestita*-HM00000282) [9].

Mature seeds of *Ajuga vestita* were washed and then pre-sterilized by soaking them in 70% alcohol for 30 seconds. The seeds were soaked in a 5% NaOCl solution for 15 minutes and sterilized. They were then rinsed in sterile distilled water five times for five minutes each to remove NaOCl. The seeds were cultured in ¼ MS medium supplemented with 30 g/L sucrose with a pH adjusted to 5.8 to support germination and growth [10]. Sterile seeds, along with cracked testa, were cultured and left to germinate in the growth chamber (25±2 ^{O}C temperature, 16 hours of light, 8 hours of dark light period; 3000-5000 lux).

After a two-week culture period, the shoot tips of the germinated seeds were cultured in medium supplemented with Kin for propagation, and *in vitro* shoots were obtained for use as starting material in callus formation studies. Following 4-weeks development period, leaf explants from *in vitro* shoots were used in callus formation studies with different concentrations of BAP and Kin (0.125-4.0 mg/L). Callus samples grown in 1/1 MS medium containing BAP and Kin, as well as those without hormones (control), were dried in a cool and dry environment for use in fatty acid content studies.

2.2. Determination of fatty acid methyl esters (FAME)

Each explant, which was powdered, then extracted for 4 hours by the Soxhlet continuous extraction method using hexane. The extracted product was evaporated in reflux, and the obtained extract was completed with 10 mL of hexane. 1 mL of 2 N methanolic KOH solution was added to the hexane extract and shaken vigorously. The sample was kept in the dark for 1-2 hours until the phase separation took place and the upper phase became clear, then the supernatant was taken into vials and the fatty acid methyl ester analysis was performed with a gas chromatography flame ionization detector (GC-FID).

Analysis was performed on the Shimadzu GC-2030 instrument combined with the AOC-20 i Plus auto injector. The Restek RT-2560 column (0.20 µm film thickness, 0.25 mm inner diameter, 100 m length, cat# no, 13198) was used. The column temperature program began 100 °C hold for 4 minutes, followed by an increase at a rate of 3 °C/min until reaching a final temperature of 240 °C, where it was held for

20 minutes. The sample injection volume was set at 1.0 µl in a split mode ratio of 1:20, and helium (flow rate of 1 ml/min) was used as the carrier gas. The hydrogen flow rate of 32.0 ml/min and the air flow rate of 200.0 ml/min. The injection and detector temperatures were both set at 250 °C. The total time of the analysis was 70 minutes. The "Food Industry FAME Mix Standard, Restek (cat# no, 35077, 37 components)" was used as a standard. FAME's are identified according to the retention times of this standard.

3. Results and Discussion

Ajuga vestita BOISS. mature seeds were subjected to sterilization by first soaking them in 70% ethanol for 30 seconds, followed by immersion in 5% NaOCI for 15 minutes. The seeds were then rinsed with sterile distilled water to remove any residual NaOCI, completing the sterilization process. After sterilization, the seeds were placed in a hormone-free 1/4 MS medium and allowed to grow in a plant growth chamber.

Following a development period of 3 weeks, leaf explants from the germinated seedlings were used for callus initiation studies. These leaf explants were cultured in a 1/1 MS medium supplemented with hormone-free (control), BAP and Kin. The fatty acid contents of the callus extracts obtained from leaf explants cultured in the medium containing BAP, and Kin were compared with those of the control plants and presented in Table 1.

The researchers conducted a study on the fatty acid contents of four plants from the Lamiaceae family, including *Salvia verticillata*, using the GC/MS method [7]. They identified tricosanoic acid and lignoceric acid as the major saturated fatty acids, while nervonic acid, eicosadienoic acid, and docosadienoic acid were identified as the major unsaturated ones in each plant. The researchers emphasized the positive impact of high unsaturated fatty acid content on plant health and suggested that their findings would inform future studies involving these plants. In our study, while the total saturated fatty acid ratios were 63.661%, 45.373%, and 53.342%, the total unsaturated fatty acid ratios were 36.339%, 54.626%, and 46.659% for control, BAP, and Kin, respectively.

In another study, researchers conducted a comparison of the carotenoid, fatty acid, and tocopherol content of leaves from *A. multiflora* plants that were either transferred to soil or obtained from shoots cultured *in vitro* [11]. They found that the highest levels of carotenoids, fatty acids, and tocopherols were obtained from the leaves of shoots cultured in MS medium. This study, which compared these characteristics between plants from natural environments and those from *in vitro* cultures, underscores the significance of studies conducted using tissue culture techniques. It suggests that extracts from *in vitro* cultures yield superior results compared to those obtained from plants grown in natural environments. This finding highlights the potential advantages and importance of utilizing tissue culture techniques in plant research.

The leaves of *Ajuga iva* were analyzed for fatty acids, essential oils, and phenolic compounds in a study cited as [12]. The analysis revealed that linolenic and linoleic acids were the primary components of the fatty acid profile. Additionally, another study [13] conducted a comparative examination of neutral lipids, including their fatty acid composition, in callus cultures and leaves of *Ajuga genevensis* and *Ajuga chia* plants. The study highlighted the significant influence of species, the origin of the callus, and the age of the culture (number of subcultures) on both the composition and content of lipids and fatty acids. Among unsaturated fatty acids, linoleic acid has been reported as a predominant component in callus tissues, whereas palmitic acid is dominant in saturated fatty acids. Palmitic acid is a fatty acid that can be converted into stearic acid and oleic acid in all organisms [14].

The researchers concluded that the high palmitic acid content in all samples studied can be attributed to this situation. In the present study, palmitic acid was found to be the predominant component among saturated fatty acids in all treatments. Additionally, a similar study investigating the fatty acid compositions of *Ajuga reptans* L. plants yielded results consistent with our findings. According to the results of this study, palmitic acid exhibited the highest proportion among saturated fatty acids, while

linolenic and linoleic acids showed the highest proportions among unsaturated fatty acids [15].

Table 1. Fatty acid contents of callus extracts of *A. vestita* BOISS. (%m/m)

Fatty Acids	**Extracts**		
	Control	**BAP**	**KIN**
4:0 Butyric acid	ND	ND	1.324
6:0 Caproic acid	1.840	3.915	6.898
10:0 Decanoic acid	ND	ND	3.604
11:0 Undecanoic acid	ND	ND	2.827
12:0 Dodecanoic acid	0.884	1.159	0.864
14:0 Myristic acid	1.800	1.463	1.576
14:1 (*cis*-9) Myristoleic acid	ND	ND	0.909
15:1 (*cis*-10) Pentadecanoic acid	ND	ND	1.092
16:0 Palmitic acid	59.137	38.836	24.295
17:1 (*cis*-10) Heptadecanoic acid	8.284	7.524	0.985
18:0 Steraic acid	ND	ND	6.631
18:1 (*trans*-9) Octadecanoic acid	21.097	32.283	13.498
18:1 (*cis*-9*)* Oleic acid	ND	ND	0.508
18:2 (*cis*-9,12) Linolelaidic acid	2.578	12.033	21.209
18:3 (*cis*-9,12,15) Linolenic acid	ND	2.087	5.873
20:0 Arachidic acid	ND	ND	2.328
22:0 Behanic acid	ND	ND	1.026
22:1 (*cis*-13) Erucic acid	ND	ND	2.585
22:6 (*cis*-4,7,10,13,16,19) Docosahexaenoic acid (DHA)	1.281	0.143	ND
23:0 Tricosanoic acid	ND	ND	0.438
24:0 Lignoceric acid	ND	ND	1.531
24:1 (*cis*-15) Nervonic acid	3.099	0.556	ND
Saturated fatty acids	63.661	45.373	53.342
Monounsaturated faty acids	32.48	40.363	19.577
Polyunsaturated faty acids	3.859	14.263	27.082
Total	100.000	99.999	100.001

BAP: benzylaminopurine, KIN: kinetin, ND: not dedected

In our study, arachidic acid was detected only in the Kin extract, albeit in low amounts, while linolenic acid was present in extracts other than the control. It is known that linoleic acid is converted to jasmonic acid, which plays a significant role in plant metabolism, particularly in defense mechanisms, through 12-oxophytodienoic acid [16-19]. The level of linoleic acid was found to be the lowest in the control group and the highest in the medium supplemented with Kin in the present study.

The major fatty acids were determined by reviewing literature studies that investigated the fatty acid composition of the plants. Plant contents may vary due to numerous factors, including the geographical location where the plant was collected, the time of collection, the specific plant part used, the solvent utilized for extract preparation, and the conditions of analysis. This situation renders it impossible to obtain exactly the same results as the studies previously recorded in the literature. A study comparing the amount and composition of neutral lipids and their fatty acids in the leaves and callus cultures of *Ajuga genevensis* and *Ajuga chia* plants in their natural environment found that lipid and fatty acid content varied

depending on factors such as species, the origin of the callus, and the number of subcultures. [12]. Topdemir et al. applied different hormone concentrations and combinations (BAP and NAA) to the cotyledon explants of kiwi plants (*Actinidia deliciosa*). They observed a decrease in the ratios of palmitoleic, stearic, and linoleic acids in groups with different hormone combinations, while an increase in the ratio of linolenic acid was detected [20].

Aly et al. investigated the effect of 2,4-D and benzyladenine on the stimulation of fatty acid synthesis *in vitro*. They found a decrease in saturated fatty acids, such as palmitic and stearic acid levels, in groups where plant growth regulators were added compared to the control group [21]. In the current study, hormone application led to a decrease in palmitoleic acid content and an increase in linoleic acid and stearic acid levels compared to the control group. The differences in the results of these studies may be attributed to the use of different plant species as materials and the variation in the hormones applied.

4. Conclusion

Unsaturated fatty acids, due to the double bonds they contain, exhibit higher reactivity compared to saturated fatty acids. This reactivity increases proportionally with the number of double bonds in the fatty acid chain. Unsaturated fatty acids play constructive and reparative roles in the biochemical and physiological activities of the body, as well as in maintaining healthy tissue development and balanced organ function [22]. They are known to play a crucial role in preventing various diseases such as cardiovascular diseases, depression, migraines, rheumatism, diabetes, high cholesterol, hypertension, allergies, and cancer [23].

Therefore, the high content of unsaturated fatty acids found in the callus extracts of *Ajuga vestita* BOISS., particularly those containing BAP and Kin obtained from leaf explants under *in vitro* conditions, holds significant importance for the aforementioned reasons.

The fact that a limited study comparing the fatty acid compositions of this plant has been found in the literature further emphasizes the importance of this study. The data obtained from this study will provide valuable insights for future research involving these plants. It will serve as a reference for any potential studies conducted with this plant in the future.

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The Declaration of Research and Publication Ethics
The authors of the paper declare that in the writing process of this study, international scientific, ethical and citation rules were followed, and no falsification was made on the collected data. Sakarya University Institute of Science Journal and editorial board bear no responsibility for all ethical violations that may be encountered. All responsibility belongs to the corresponding author and this study has not been evaluated in any academic publications other than Sakarya University Journal of Science.

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