

Phenolic Constituents of *Vaccinium* Species from Both Natural Resources and Micropropagated Plantlets

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Abstract: Fruits and leaves of *Vaccinium* species have rich bioactive phytochemicals. These bioactive phytochemicals make these plants particularly valuable for the medical and food industry. This novel approach was designed to determine the phenolic content of *Vaccinium* species obtained from both micropropagated and naturally growing leaves. An efficient micropropagation protocol was developed to produce tree *Vaccinium* species plantlets via direct organogenesis. Lateral buds containing one or two leaves were cultured in McCown woody plant medium (WPM), supplemented with zeatin/indole-3-butyric acid (IBA) (1.0/0.1 mg L⁻¹). In conclusion, Protocatechuic acid, Chlorogenic acid, Syringic acid and Routine phenolic compounds were determined in significant amounts. It has been determined that the phenolic compounds of leaves produced in tissue cultures is higher than the phenolic compounds obtained from naturally growing leaves.

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1. INTRODUCTION

Secondary metabolites are important compounds that human beings frequently use especially in the medical field. The development of new and effective production methods leading to these valuable compounds has gained momentum in recent years [1, 2]. *Vaccinium* species L., belonging the family Ericaceae, is an economically most important wild plant species growing in indigenous population of Turkey. The genus *Vaccinium* is represented by four species in the Turkish flora, namely *Vaccinium uliginosum* L., *Vaccinium arctostaphylos* L., *Vaccinium myrtillus* L., and *Vaccinium vitis-idaea* L., and their fruits are consumed by local people as dry or fresh fruit, marmalade, jam, compote, etc. The leaves of *Vaccinium* species

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have antioxidants and antibacterial properties as well as rich phenolic content. The leaves of these plant species have also been consumed as a herbal tea in the past [3, 4].

Because of these biological benefits, there is a growing interest in *Vaccinium* species, and many researchers study the commercial cultivation of these species. Due to the many advantages, including a mass effective clonal propagation, a fully controlled environment, a shortened growth cycle, an uninterrupted production process, and production of disease-free plants, plant tissue culture techniques have recently been preferred for the propagation of these species [5-8].

Collection of such valuable plants directly from natural population causes negative effects on natural habitats of plants and serious reductions in plant numbers; however, with plant tissue cultures, it is possible to prevent the use of natural plants for that purpose and keep nature's beauty intact [9, 10]. An also the micropropagation technique is useful for the production of active compounds during formation of secondary metabolites in medicinal and aromatic plants and in particular, to increase the levels of some important metabolites [11, 12]. The purpose of this novel study was to develop an efficient and rapid micropropagation protocol for producing *Vaccinium* plantlets and thereafter compared their phenolics contents with naturally growing plants.

2. MATERIALS and METHODS

2.1. Plant Material

Actively growing young *Vaccinium* nodal segments were collected from the natural populations of different regions of Trabzon in 2013. Lateral buds were washed with tap water for 1 h, and then surface sterilized with 70% (v/v) ethanol for 1 min, followed by 15 (10 min for *V. uliginosum* explants) min of incubation in 3% sodium hypochlorite (NaOCl). Finally, they were washed with sterile distilled deionized water 3 times for 15 min. Sterilized explants dried on sterile whatman filter paper and cultured on approximately 50 mL of nutrient media in 98.5 × 59 mm glass containers. Each treatment was performed in triplicates and 30 explants were used for each treatment.

2.2. Experimental

For shoot multiplication, McCown woody plant medium including vitamins (WPM) [13], supplemented with zeatin/ indole-3-butyric acid (IBA) (1.0/0.1 mg L⁻¹), was used to obtain in large quantities number of plants. WPM basal medium containing 2% sucrose and 0.8% agar. Zeatin and IBA used in the study were sterilized with 0.22-µm filters and added to the cooled media after autoclaving. The medium pH was adjusted to 5.0 before autoclaving. Cultures were incubated in a growth chamber maintained at 24 ± 2 °C under a 16/8-h day and light conditions. A subculturing protocol was performed every 8 weeks. The regeneration ability of cultures was then evaluated on the basis of mean number of shoots per explant, length of shoots emerged from each explant, mean number of nodes, fresh and dry weight. Each experiment was repeated in triplicate.

2.2. Extraction

In vitro grown biomass was extracted as reported elsewhere [14, 15]. Briefly, 200 mg of each sample was macerated in n-hexane (HE) and dichloromethane (DCM) (10 mL each) for 10 min, solvent was filtered out and residue was removed via methanol (MeOH) extraction for 30 min, and methanol was evaporated in vacuo. The obtained methanol extract was then dipped in pH 2.0 ± 0.1 water and shaken vigorously followed by extraction three times with 5 mL diethyl ether and ethyl acetate. Organic phases were combined, evaporated and made up in methanol (2.0 mL) for HPLC analysis after passing through 0.45 µm filter. Determination of

total phenolic contents were determined with previously reported method [16]. Briefly, 750 μL of Folin-Ciocalteu's/water mixture (1:14) were added to 50 μL sample and after 3 min, 200 μL of 20 % Na_2CO_3 was added. Then the reaction mixture was incubated in dark for 30 min. Absorbance was measured on an ultraviolet-visible (UV-Vis) spectrophotometer (Unicam UV2-100) at 760 nm and methanol was used as blank. Gallic acid was used as standard and total phenol contents in extracts were calculated as mg gallic acid equivalent total phenolic in mg Gallic Acid Equivalent/100 (GAEq mg/100 gm) gm dry weight of plant.

2.3. HPLC analysis of phenolic compounds

A previously developed and validated HPLC method [16] was used in the quantification of phenolics. The analysis was carried out on an ELITE LaChrome (Hitachi HPLC system), quaternary pumps (L-2130 model), auto injector (model L-2200) and variable wavelength PDA detector (photo diode array (L-2455 Model). A C-18 reverse phase column (250 mm \times 4.6 mm id, 5 μm particle size, Agilent (USA)) was used in the analysis which was fixed in column oven (Model-2300). Mobile phase was a mixture of solvent A (2 % acetic acid in water) and solvent B (70:30, acetonitrile/water). The injection volume was 20 μL and column was kept at 30 $^\circ\text{C}$. The flow rate was kept constant at 1 mL min^{-1} using gradient programming; starting the flow of mobile phase as B (5 %) to three min, gradual increase (up-to 15, 20, 25, 40 and 80 % at 8, 10, 18, 25 and 35 min, respectively) and drop back to 5 % at 40 min and left for 10 min to equilibrate in column. Eluent was continuously monitored through PDA by measuring at three different wavelengths, i.e., 280, 315 and 350 nm.

2.4. Statistical Analysis

Each treatment included 6 Magenta B-caps (each containing 5 explants) and each was carried out in triplicate for shoot multiplication. All data were analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). The data collected for mean shoot length, mean number of shoots, mean number of nodes for shoot multiplication, fresh and dry weight were analyzed using analysis of variance (ANOVA) with Pearson's correlation. Values are means \pm standard deviation.

3. RESULTS and DISCUSSION

3.1. Shoot Multiplication

Three different assessed *Vaccinium* species also improved in the WMP medium supplemented with zeatin/IBA. All *Vaccinium* species have higher shoot regeneration frequency (90%). The highest shoot multiplication was obtained in *V. arctostaphylos* with 5.08 shoots per explant. Although *V. myrtillus* and *V. uliginosum* gave the close shoot multiplication values, there was also a statistically significant difference between these species ($P \leq 0.05$, Table 1). In recent years, the production of secondary metabolites has been accelerated by tissue culture methods, and many researchers have reported many studies on the production of these valuable metabolites [1, 2, 17, 18]. In addition, although there are many studies to determine the secondary metabolite content of *Vaccinium* species fruits naturally growing in our country [19-22], there isn't any study to compare phenolic contents of *Vaccinium* species from both natural resources and micropropagated plantlets. Although researchers have found different results depending on the species studied with different media and plant growth regulators [23, 24] in terms of shoot number, the most effective results have been obtained from studies using zeatin [8, 25].

Our preliminary studies show us, multiplication depends not only on the presence or absence of the growth regulators but the basal medium and the plant species to be reproduced also has a great impact on micropropagation. The highest shoot length was again obtained from *V. arctostaphylos* with 44.60 mm. This value is higher than the shoot length obtained from *V.*

myrtillus and *V. uliginosum*, with 12.31 % and 21.57 %, respectively. Moreover, remarkable statistical differences were also established between these three *Vaccinium* species in terms of the shoot length ($P \leq 0.05$, Table 1). Unlike the shoot length, a different result was obtained between the three species in terms of the number of nodes. No significant difference was found between *V. arctostaphylos* and *V. myrtillus* in regard with the node number. The highest node number was obtained from *V. arctostaphylos* and *V. myrtillus* with 12.38 and 12.12 per shoot, respectively. This value was determined at 11.00 in *V. uliginosum* (Table 1). Well-developed plantlets production are needed in the production with micropropagation. Because of this, many researchers have applied different plant growth regulators on the micropropagation of depending on the plant species [26, 27]. Some of these researchers were reported that zeatin is more effective than other cytokinins in terms of the shoot length of *Vaccinium* species [6]. Also some other researchers were also reported that zeatin was more effective on shoot length than other cytokinins on *V. uliginosum* and *V. arctostaphylos* in terms of the shoot length [8, 28]. The findings obtained from previous reports support our findings.

In such biomass studies where the secondary compounds are sought to be obtained, fresh and dry weight findings have also great significance. In this context, the fresh and dry weights of the plant leaves obtained by natural and micropropagation were calculated. The higher fresh and dry weight parameters were achieved from collected natural plant leaves with 1.88 and 0.38, respectively. These findings were higher than *V. arctostaphylos* with 12.23% and 18.42%, *V. myrtillus* with 25% and 34.21%, and 33.51% and 50.00%, respectively. Above mentioned, natural collection methods are likely to impact negatively on the natural habitat of these plants and lead to a dramatic decrease in plant populations. Therefore, it is important to carry out studies to obtain valuable secondary products using the advantages of plant biotechnology. This study is the first report to investigate the phenolic content of *Vaccinium* leaves produced in tissue cultures. Naturally collected *Vaccinium* leaves were evaluated as control groups.

3.2. Determination of Phenolic Contents

Free phenolics were analyzed by HPLC and the findings were summarized in Table 2. Protocatechuic acid, Chlorogenic acid, Syringic acid and Rutin were calculated significant levels. Protocatechuic acid was highest in micropropagated *V. myrtillus* followed by *V. arctostaphylos* with 2.27 and 1.868. One of the dramatic results was showed from Protocatechuic acid content of natural *V. myrtillus* and *V. uliginosum* leaves and no statistically significant data could be obtained ($P \leq 0.05$, Table 2). In the Chlorogenic acid contents, a significant increase was observed in the leaves of *V. arctostaphylos* and *V. myrtillus* obtained by micropropagation. Micropropagated *V. arctostaphylos* leaves had 164.09% more Chlorogenic acid content than natural *V. arctostaphylos* leaves and there was a significant statistical difference between them ($P \leq 0.05$). A similar condition has also occurred for *V. myrtillus* and this ratio was calculated as 180.98% (Table 2). The lowest Chlorogenic acid content was achieved from micropropagated *V. uliginosum* with 1.62.

Table 1. Effect of zeatin in the presence of IBA (0.1 mg L⁻¹) on shoot multiplication of three *Vaccinium* species.

	PGRs Concentration (mg L ⁻¹)	Number of Shoots/ per Explant	Shoot Length (mm)	Node Numbers/ per shoot	Fresh Weight (mg)	Dry Weight (mg)
Natural	-	-	-	-	1.88 ± 0.21 a	0.38 ± 0.032 a
<i>V. arctostaphylos</i>	1.0/0.1	5.08 ± 0.48 a	44.60 ± 2.47 a	12.38 ± 0.91 a	1.65 ± 0.20 b	0.31 ± 0.039 b
<i>V. myrtillus</i>	1.0/0.1	3.86 ± 0.51 b	39.11 ± 1.62 b	12.12 ± 0.67 a	1.41 ± 0.07 c	0.25 ± 0.036 c
<i>V. uliginosum</i>	1.0/0.1	3.46 ± 0.54 c	34.98 ± 1.51 c	11.00 ± 0.99 b	1.25 ± 0.16 d	0.19 ± 0.011 d

Data were recorded 8 weeks after the culture with a total of 3 replicates of 20 plants per treatment for shoot regeneration. Values having the same letter(s) in the same column are not significantly different according to Duncan's multiple range test at P ≤ 0.05. PGRs: Plant Growth Regulators

Table 2. Phenolics identified of *Vaccinium* species from both natural resources and micropropagated plantlets though HPLC (mg/100 g)

	NATURAL			MICROPROPAGATED		
	<i>V. arctostaphylos</i>	<i>V. myrtillus</i>	<i>V. uliginosum</i>	<i>V. arctostaphylos</i>	<i>V. myrtillus</i>	<i>V. uliginosum</i>
Protocatechuic acid	1.752 ± 0.097 b	-	-	1.868 ± 0.017 b	2.27 ± 0.17 a	1.29 ± 0.0 c
Chlorogenic acid	7.52 ± 0.61 d	47.15 ± 1.70 b	133.85 ± 5.16 a	19.86 ± 0.45 c	132.48 ± 0.88 a	1.62 ± 0.18 e
Syringic acid	0.608 ± 0.077 d	0.63 ± 0.038 d	0.97 ± 0.091 c	1.72 ± 0.14 a	1.42 ± 0.08 b	0.66 ± 0.07 d
Rutin	2.998 ± 0.012 a	-	-	-	-	-

Data were recorded 8 with a total of 3 replicates. Values having the same letter(s) in the same line are not significantly different according to Duncan's multiple range test at P ≤ 0.05.

The importance of the study has emerged with the identified secondary compounds, which are predominantly high in micropropagated plants. Syringic acid content was also found to be higher in micropropagated plantlets than in natural ones except for again *V. uliginosum*. Rutin was calculated only natural sample of *V. artostaphylos* and amount of it was determined as 2.998. Although many researchers have mostly studied the biologically active components of *Vaccinium* fruits [29-31], very few researchers have studied on natural leaves of these valuable plants [32, 33]. In one of the these studies researchers performed such a study on *Vaccinium angustifolium* and reported that tissue culture techniques enhance the total phenolic contents of *V. angustifolium* [34]. The same researchers have reported that tissue culture technique used in the study can affect the phenolic content. Similarly, the other some researchers have reported that chlorogenic acid myricetin, syringic acid and rutin are main phenolic components of micropropagated blueberry seedlings [35]. These researchers have specified that micropropagated *Vaccinium* seedlings are a good source of antioxidant compounds in terms of the above mentioned phenolic compounds. These results also support our findings.

4. CONCLUSION

This study emphasized to determined efficient *in vitro* micropropagation method and to compare of phenolic compounds of micropropagated *Vaccinium* plantlets and natural ones. At this occasion, phenolic contents of natural *Vaccinium* leaves and micropropagated seedlings of three *Vaccinium* species growing in the Turkish flora compared for the first time. According to the results, there were significant increases in some phenolic compounds in micropropagated seedlings of some species. Some factors such as the type of plant species, the type of medium and the type and combination of plant growth regulator can affect these desired outcomes. The efficient results of these valuable plant species may be a pioneer of scientific reports and it may lead the way to enhance the further multidisciplinary experiments.

Conflict of Interest

The authors declare that there is no conflict of interests in this current study.

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