In this research, it was examined the changes in total phenolic contents and seven major phenolic compounds (gallic acid, (+)-catechin, catechol, chlorogenic acid, o-coumaric acid, rutin, and quercetin) of two grape cultivar (Cardinal and Uslu) shoot tips collected in different months, in order to determine their effects on the explant browning during establishment stage of shoot tip culture. The concentrations of phenolic compounds varied depending on the cultivars and the months. Phenolic compounds showed various correlation coefficients with the explant browning. Total phenolic content and some individual phenolic compounds including (+)-catechin, catechol, gallic acid, chlorogenic acid and rutin quantified in this study showed significant positive correlations with the explant browning, while o-coumaric acid and quercetin did not exhibit any significant one. According to our results, explant browning is affected by the phenolic compounds at different ranges. In both grape cultivars, shoot tips collected in May exhibited the lowest explant browning during the establishment stage as compared to those collected in the other months. So it may be possible to increase the success of shoot tip culture with the selection of the most suitable terms of explant collection.

Introduction
During the establishment stage of in vitro culture, oxidative browning of explant is one of the main drawbacks. Browning is mainly due to the presence of phenolics, released from the cut surfaces of the explants (9, 13). It may be a natural defence mechanism in plants against invasion by pathogens through the wound. Releasing phenolics from cut surface to the culture medium show the phytotoxic effect. The oxidized products not only may contribute to failure of the explant development, but also may lead to the death of explants (2, 9). Like other woody species, grapevine tissues also exhibit high levels of polyphenols and tannins (8) and lipid peroxidation products (1).

Some factors including type of explant, light exposure, age and vigour of the donor plants, source of explant, genotype, size of explant affect the controlling of explant browning (15, 16, 18, 19, 20). Time of the year at explant collection is also another important factor influences the explant browning. It is possible to alleviate the browning of explant with the selection of season which will present the minimum phenolic compounds (5, 6, 19).

The aim of the present work was to investigate the fluctuation and distribution of some phenolic compounds in two different grape cultivar (Uslu and Cardinal) shoot tips collected at different months in order to determine their possible effects on the explant browning in the establishment stage of shoot tip culture.
Materials and Methods

Plant materials
1-2 cm long shoot tips were collected from vines of Cardinal and Uslu grape cultivars growing at Eğirdir Horticultural Research Institute, Isparta, Turkey. Vines were fourteen-year-old and guyot trained. Shoot tips were taken with a 15 day intervals from May to August (Two samples per month except August. Because enough shoot tips were not found for analyses in the second part of August). While a part of shoot tips collected at each month was used in tissue culture studies, immediately; the other part of them was stored at -20 °C until phenolic analysis.

Shoot tip culture
30 shoot tips for each experiment were sterilized for 10 min sodium hypochlorite (1 % activated chlorine) containing a few drops of Tween 20 and rinsed three times in sterile-distilled water. Shoot tips approximately 1-2 mm in length were excised under aseptic condition and placed on medium which consisted of MS basal medium (12) supplemented with 2.5 mg/l BA (6-benzyladenine), 0.5 mg/l GA3, 30 g/l sucrose solidified with 7 g/l agar. The pH was adjusted to 5.7-5.8. After inoculation, the cultures were incubated at 25±1 ºC under cool-white fluorescent illumination with a 16 h day-length. After three weeks, the percentages of browning explant were determined. The dead explants because of the browning were discarded, while surviving explants were sub cultured to the fresh media.

Determination of total phenolic content
For total phenolic extraction, shoot tips were homogenized with 50% ethanol in water and left for 2 h in the dark. After filtration, the concentration of total phenolics in the shoot tips was determined by the Folin-Ciocalteu colorimetric method (17). Estimations were carried out in triplicate and calculated from a calibration curve obtained with gallic acid. Total phenolic contents were expressed as gallic acid equivalents (mg GAE/g).

HPLC separation of phenolic compounds
Phenolic compounds were extracted from shoot tips described by Karkacier (10). Briefly, 2.5 grams of shoot tips were mixed with 5 ml of HPLC-water, presence of ascorbic acid to prevent the oxidation. The mixture was homogenised by using ultratorax at 24.000 rpm and centrifuged at 6.000 rpm for 30 min at ambient temperature. Then, 2.5 ml of mixture was diluted with 2.5 ml of HPLC water. After centrifuging at 6.000 rpm, the final mixture was filtered through 0.45 μm membrane filter before 20-μl injections. High Pressure Liquid Chromatography (HPLC) analysis of phenolics was performed by HPLC on a Varian Model 9010 equipped with a UV-visible (Varian 9050 model) detector and an auto sampler (Marathon). Gradient elution was performed with %5 formic acid (Merck, eluent A) and methanol (Merck, eluent B), and gradient program followed as 0-8 min %70 A-%30 B, 8-8.5 min %65 A-%35 B, 8.5-20 min %60 A-%40 B, 20-30 min %54 A-%46 B, 30-30.5 min %40 A-%60 B, 30.5-34.5 min %15 A-%85 B and 34.5-35 min %70 A-%30 B. The UV detection was performed at 280 nm. The elution was conducted at room temperature using a Supelco C18 column (15 x 4.6 I.D.), and at a flow rate of 1 ml/min.

Initial identity assignment of phenolic was based on comparison retention data obtained with UV detector for standard compounds and sample components. Quantisation was achieved by using peak areas from external calibration with standard [gallic acid, catechol, quercetin, rutin, o-coumaric acid, (Sigma), chlorogenic acid and + (-) catechin (Fluka)] solutions. All determinations were done three times by using three different samples and data are expressed μg/g fresh weight.

Statistical analysis
Data for explant browning were subjected to angular transformation for normalizing the frequency distribution, prior to statisti-
TABLE 1
Changes in the explant browning and the phenolic compounds at different months of explant collection

<table>
<thead>
<tr>
<th>Cultivars</th>
<th>Months</th>
<th>Explant Browning (%)</th>
<th>Total phenolics (mg/g)</th>
<th>Gallic acid (μg/g)</th>
<th>(+) catechin (μg/g)</th>
<th>Catechol (μg/g)</th>
<th>Chlorogenic acid (μg/g)</th>
<th>Rutin (μg/g)</th>
<th>o-coumaric acid (μg/g)</th>
<th>Quercetin (μg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cardinal</td>
<td>May</td>
<td>55.83 {cd}</td>
<td>13.85 {de}</td>
<td>96.98 {d}</td>
<td>62.06 {c}</td>
<td>33.93 {d}</td>
<td>30.51 {b}</td>
<td>4.51 {bc}</td>
<td>1.51 {b}</td>
<td>1.15 {b}</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>67.50 {bc}</td>
<td>15.97 {cd}</td>
<td>211.72 {b}</td>
<td>146.87 {b}</td>
<td>34.18 {b}</td>
<td>4.70 {b}</td>
<td>5.41 {b}</td>
<td>2.18 {a}</td>
<td>2.25 {a}</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>75.00 {ab}</td>
<td>22.52 {a}</td>
<td>280.49 {a}</td>
<td>246.93 {a}</td>
<td>181.02 {a}</td>
<td>5.20 {b}</td>
<td>5.40 {b}</td>
<td>1.62 {b}</td>
<td>1.10 {b}</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>83.33 {a}</td>
<td>28.95 {a}</td>
<td>360.27 {a}</td>
<td>264.27 {a}</td>
<td>179.81 {a}</td>
<td>8.93 {a}</td>
<td>8.93 {a}</td>
<td>1.68 {b}</td>
<td>1.15 {b}</td>
</tr>
<tr>
<td>Uslu</td>
<td>May</td>
<td>29.17 {e}</td>
<td>8.14 {f}</td>
<td>77.12 {d}</td>
<td>41.77 {c}</td>
<td>37.51 {d}</td>
<td>12.80 {c}</td>
<td>3.43 {c}</td>
<td>1.07 {b}</td>
<td>0.58 {b}</td>
</tr>
<tr>
<td></td>
<td>June</td>
<td>45.00 {d}</td>
<td>11.97 {c}</td>
<td>178.91 {c}</td>
<td>73.73 {c}</td>
<td>105.66 {c}</td>
<td>16.66 {c}</td>
<td>4.08 {b}</td>
<td>1.48 {b}</td>
<td>1.11 {b}</td>
</tr>
<tr>
<td></td>
<td>July</td>
<td>53.33 {cd}</td>
<td>14.32 {d}</td>
<td>205.76 {bc}</td>
<td>145.84 {b}</td>
<td>116.64 {bc}</td>
<td>16.48 {c}</td>
<td>6.24 {b}</td>
<td>1.37 {b}</td>
<td>1.04 {b}</td>
</tr>
<tr>
<td></td>
<td>August</td>
<td>73.33 {ab}</td>
<td>17.43 {bc}</td>
<td>255.47 {a}</td>
<td>236.27 {a}</td>
<td>159.02 {a}</td>
<td>18.53 {c}</td>
<td>10.29 {a}</td>
<td>1.46 {b}</td>
<td>1.41 ab</td>
</tr>
</tbody>
</table>

* Within each column, means with the same letter are not significantly different (P≤0.05).

**Results and Discussion**

Changes in the browning percentage of grapevine explant

Some of the explants cultured in vitro remained viable and grew during the establishment stage of the in vitro culture, while some of them turned dark brown. A major browning phenomenon was observed after about 12-14 days of incubation. The data recorded after three weeks for the explant browning and the statistical separation of the means are given in Table 1. The percentages of browning explants changed significantly according to cultivars and the months of the explant collection (P≤0.05). In both cultivars, the lowest percentages of explant browning were detected in the shoot tips collected in May and explant browning gradually increased during the season, with high values in Cardinal.

Changes in the total phenolic content of grapevine explant

Total phenolic contents of shoot tips collected in the various months are presented in Table 1. Genotype and months of explant collection had significant effects on the total phenolic contents in shoot tips (P≤0.05). Total phenolic content in the Uslu shoot tips exhibited a low initial value in May, and then increased gradually, reaching a maximum level in August. In Cardinal, total phenolic content increased from May to August and the maximum total phenolic content was detected in shoot tips collected in August. Although Cardinal showed a similar pattern for total phenolic content with Uslu, this grape cultivar exhibited higher total phenolic contents than Uslu in all months.

Changes in the phenolic compounds of grapevine explant

The amounts and variations of phenolic compounds in the shoot tips collected in the different months are presented in Table 1. The genotype as well as the month of explant collection seemed to the major factors influencing the relative concentrations of the various phenolic compounds in the shoot tips. In both cultivars, the various phenolic compounds quantified in this study showed significantly differences in shoot tips collected in different months (P≤0.05).

Gallic acid and (+) catechin were the most abundant phenolic compounds in the shoot tips of both cultivars, with higher concentrations in Cardinal. In both culti-
vars, the lowest concentrations for gallic acid were found in the shoot tips collected in May. But gallic acid concentration increased at different rates up to August. Concentrations of +(-) catechin in Cardinal shoot tips significantly increased from May to July but there was no any significant difference for +(-) catechin between July and August. In Uslu, +(-) catechin also showed a gradual increase during the season and the highest value was detected in the shoot tips collected in August. The amounts of the second most abundant phenolic compounds in shoot tips were catechol and chlorogenic acid. In Cardinal, catechol concentration was relatively low in the shoot tips collected in May. But it subsequently exhibited a strong increase in the explants collected in June. After that this increase, but more slightly, continued up to August. Uslu also exhibited almost similar pattern with Cardinal for +(-) catechin. Concentrations of chlorogenic acid in the shoot tips were higher in Cardinal than Uslu, in all months. While concentration of chlorogenic acid in Cardinal did not significantly vary in the shoot tips collected in May, June and August, it showed a significantly increase in August. Although a lower chlorogenic acid concentration in Uslu was found in the shoot tips collected in August compared the shoot tips collected in the previous months, the values did not differ significantly. The amounts of rutin in Cardinal remained almost stable from May to July, but it showed a strong increase in the shoot tips collected in August. On the other hand in Uslu, concentration of rutin gradually increased during summer, and reached to maximum value in August. Concentration of o-coumaric acid in shoot tips of Uslu gradually increased during summer as it showed a sharp decrease from May to August in Cardinal. The highest concentration of quercetin in Cardinal was found in the shoot tips collected in June, and there was no any significant difference among the other months. Quercetin content of Uslu had a different pattern to that of Cardinal. Although it differed slightly from May to August, these differences were not significant.

**Correlation of the phenolic compounds with the browning percentage of grapevine explant**

Correlation coefficients between the explant browning and the phenolic compounds are given in Table 2.

The most remarkable positive correlation was found between explant browning and the total phenolic content. Gallic acid, +(-) catechin, catechol and chlorogenic acid in the explants exhibited a significantly strong positive correlation with the explant browning. But explant browning showed relatively weak positive relationship with rutin. Neither the concentration of o-coumaric acid, nor that of quercetin showed a significant relationship with the explant browning.

Browning and subsequent death of explants is a common problem in cultures of woody plants and has generally been attributed to the oxidation of phenolic compounds in explant tissues (9). Browning is of considerable importance, because it invariably leads to the death of the explants. In this study, it was determined that the browning percentages of explants varied significantly depending on the cultivars. Shoot tips of Cardinal exhibited higher ex-

<table>
<thead>
<tr>
<th>Endogenous compounds</th>
<th>Explant browning</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total phenolic content</td>
<td>0.839**</td>
</tr>
<tr>
<td>Gallic acid</td>
<td>0.665 **</td>
</tr>
<tr>
<td>+(-) catechin</td>
<td>0.713**</td>
</tr>
<tr>
<td>Catechol</td>
<td>0.692**</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>0.507**</td>
</tr>
<tr>
<td>Rutin</td>
<td>0.378*</td>
</tr>
<tr>
<td>o-coumaric acid</td>
<td>ns</td>
</tr>
<tr>
<td>Quercetin</td>
<td>ns</td>
</tr>
</tbody>
</table>

*P≤0.05, **P≤0.01, ns: not significant, n=42.
plant browning than those of Uslu when their percentages were compared in the same months. It has been observed that the explant browning is highly genotype dependent, as previously reported by Tango-lar et al. (18), Thomas and Ravidra (19) and Yu and Meredith (20).

Time of explant collection is in general a critical factor in explant browning (5, 6, 18, 19). In both cultivars, the lowest explant browning percentages were found in the shoot tips collected in May. Shoot tips collected in the other months showed more browning, turned brown sooner and showed no growth response. But Fanizza et al. (7) in grapevine; Roussos and Pontikis (15) and Roussos and Pontikis (16) in olive found that season of explant collection did not influence the explant browning.

Time of explant collection influences not only the percentage of explant browning but also the contents of total phenolics (5). In this study, it was shown a variation of explant browning and total phenols in relation to months of explant collection. The lowest browning percentages were achieved from the explants taken in May, with the highest browning percentages from those collected in August. Higher phenolic levels were associated with higher explant browning but lower survival. It was previously reported that season could an important role in the successful establishment of explants in in vitro culture (6) and explant survival was inversely related to phenolic content of explants (3, 4 5, 21). Similarly, higher phenolic content and explant browning were observed with Cardinal shoot tips while Uslu shoot tips showed lower phenolic contents and better survival.

Time of explant collection is one of the major factors influencing the relative concentration of the various phenolic compounds (16). In this study, phenolic compounds quantified by HPLC presented significant differences depending on the cultivars and the months. While some of the phenolic compounds including gallic acid, (+) catechin, catechol and chlorogenic acid showed highly positive correlations with explant browning, rutin exhibited more slightly correlation with the browning. On the other hand, there were not found any correlation between browning and some phenolics such as o- coumaric acid and quercetin. Different quinones produced by different phenolic compounds contribute to a different degree to the formation of the brown colour (14). In olive explants, Roussos and Pontikis (16) found significantly positive correlations between some phenolic compounds (luteolin-7-glucoside, quercetin and luteolin) and the browning while they did not found any correlation between browning and some phenolics including rutin, chlorogenic acid and oleuropein.

The concentrations of the different phenolic compounds change during the growing season, presenting the great effect of environmental conditions on the total phenol content of a tissue and especially on individual phenolic compounds (11). It is well known that the environmental properties, especially temperature and sunlight have important contribution in the formation of the phenolic compounds in plant tissues (18, 20).

There are a lot of studies conducted on determining the effects of some factors such as genotype, explant origin, season, vigour of vines and sunlight, on the total phenolic content and explant survival in different plants (5, 7, 15, 16, 18, 19, 20). But according to our knowledge, variabilities in the individual phenolic compounds of shoot tips and their effects on the success of shoot tip culture were not previously examined in grapevines. We have found that in shoot tip explants of grapevine there are important relationships between phenolic compounds and explant browning in the initial stage of cultures. And not only the variation of phenolic compounds but also the percentage of explant browning are related strongly to
months of explant collection. So, by the careful selection of months browning may be alleviated and thus it is possible to increase the success of shoot tip cultures of grapevine.

REFERENCES