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# Study of the effect of plant growth regulators, size, and cultivar of the grape inflorescence explant on production of phenolic compounds in an *in vitro* condition

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**ABSTRACT**

**Introduction:** Phenolic compounds are a large number of secondary metabolites that have useful and desirable effects in the field of agriculture, medicine, and food. This research was aimed to achieve methods of in vitro propagation of grapevine in order to apply biotechnologies for correction, growth, and optimization of products and compounds of the cultivated plant in relation to phenol ratio.

**Methods:** In this interventional study, the effects of cultivar and size of the inflorescence explant and the gibberellin hormone in two levels, benzylaminopurine, and auxin hormones in three levels with three replicates per treatment were evaluated in relation to phenol ratio, in order to evaluate the effect of plant growth regulators, the type and size of the grape inflorescence explant on the phenol production.

**Results:** The type of plant growth regulators affected phenolic substances production. The production of phenolic substances decreased in a medium with the highest concentration of growth regulators, 4 and 2.5 µM concentration of benzylaminopurine, and 4.9 µM of auxin. Production of phenolic substances increased in the free-plant hormone medium. In smaller samples tendency to turn brown was more regarding high amount of the sugar.

**Conclusion:** The plant sample and the cultivar as important factors in producing phenol environment are induced by environmental stimuli like sugar, light, temperature, stress, ozone, and wound and can be actually applied to increase phenol production.

*Implication for health policy/practice/research/medical education:*
Phenolic compounds have useful and desirable effects in the field of agriculture, medicine, and food. The results of this study showed that plant sample and the cultivar as important factors in producing phenol environment are induced by environmental stimuli like sugar, light, temperature, stress, ozone, and wound. Hence they can be applied to increase phenol production.

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## Introduction

Phenolic compounds are a large group of secondary metabolites with low molecular weight and there are about 8000 different compounds in this group. Given their aromatic nature, phenolic compounds are easily oxidized. Several enzymes like monooxygenase and peroxides catalyze enzymatic oxidation. Their oxidation leads to browning and production of toxic compounds (1). These compounds have significant effects in the field of food, chemistry, pharmacy, and medicine. Also they have favorable biological effects including antioxidant, antibacterial, anti-inflammation, antiradical, and vasodilator properties (2) and play an important role in the maintenance of food products and preservation of human health (3). Phenolic compounds as secondary metabolites are introduced in most of the plant species (4,5). External

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and internal factors often affect the type and amount of phenolic compounds (6). Nutritional conditions, stressful conditions, plant damages, pathogens, and plant pests affect on concentration of phenols produced in plants (7,8). Plant regulators or PGR also affect the production and activity of the phenol (9). Distribution and type of carbohydrates affect phenol production so that high concentration of glucoses has role in the phenol production. But their deficiency forces the plant to resist stress condition and destroy the plant by producing toxic compounds (10). Metabolism of phenols has close relationship with the metabolism of hydrocarbonic compounds that provide phenols synthesis from the Shikimic acid pathway. Searches have reported that the bromine (Br) deficiency causes condensation of sugars and phenols in several herbaceous species while its intermediate amount cannot affect woody plants. This shows that there is very close relationship between phenols and sugars but if deficiency of Br increases, phenolic compounds as antioxidants prevent accumulation of the sugar (11). Phenols constitute about 30-40% of dry matter in plants, which represents the importance of composition of phenols from sugar (12). Plant growth regulators play a crucial role in the growth and production of secondary metabolites in the plant tissue and cells culture. For example, type and concentration of the auxin or cytokinin and also auxin-cytokinin ratio have significant effects on both the growth and accumulation of secondary metabolite in plants (13). Grapes are among the fruits containing the highest content of phenolic substances and synthesis of these compounds in the plant is genetic. There is significant difference between types of grape in terms of quantity of phenolic and antioxidant compounds presented in different parts of the plant. Content of phenolic compounds is different in stages of the flower and fruit development in a way that it reaches to the highest amount 50 days after flowering and is decreased by the grain maturation (14). In the fluorescence medium, the number and the place of flowers, sampling time, hormones concentration, and amount of carbohydrates are important in the flower growth. One of the limitations in the fluorescence growth is phenol concentration, so this study was aimed to investigate the effect of plant growth regulators, cultivar and size of the grape fluorescence explant on the phenol production ratio, to determine a criterion for survival and growth of cultivated plant samples in view of phenol ratio in the *in vitro* propagation condition, to make plant pathogen-free, and to optimize plant products and compounds.

## Materials and Methods

To perform the in vitro culture in the first experiment the basic medium and fluorescence samples with concentrations different from other growth regulators were used in compliance with the Table 1. Explants were disinfected for 15 minutes using 1% sodium hypochlorite and then cultured on prepared mediums. Jars containing cultured samples were maintained in the growth chamber with the temperature of 25±2 °C and light intensity about 70 µm/m$^2$ and the phenol concentration was studied in two types in 18 mediums.

In the second experiment mediums containing the lowest and the highest concentration of the phenol in the first experiment, that is the medium 15 and 17, and the control medium were used. Concentration of the hormone used in these mediums included control with zero concentration, cytokinin hormone in 2 levels (benzylaminopurine in concentrations of 2.5 and 4 µm) and the auxin hormone in three levels (indole-3-butyric acid in concentration of 4.9 µm) and the type of the fluorescence (big and small florets) and the cultivar (ruby seedless and white seedless).

In the experiments 2 and 3, the experiment was performed as factorial in the form of completely randomized design. The medium (18 mediums) and type of the variety factors were considered in the experiment 1 and factors of the inflorescence type, variety, and medium [control, 15(benzylaminopurine in concentrations of 2.5 and indole-3-butyric acid in concentration of 4.9 µm], 17(benzylaminopurine in concentrations of 4 µm and indole-3-butyric acid in concentration of 4.9 µm) were considered in the experiment 2. The phenol production was conducted in the medium or flowers using the indicator the Folin-Ciocalteu. If amount of phenol was decreased in the medium, sample was collected from flowers. Data were analyzed in the SAS software and means were compared using Duncan's test.

**Table 1.** Hormone compounds used for inflorescence growth and estimation of the phenol amount in the medium

| | **Levels of auxin(µm)** | | |
|---|---|---|---|
| | Level 3 of auxin (A3=4.9) | Level 2 of auxin (A2=2.5) | Level 1 of auxin(A1=0) |
| **Culture Medium** | 13$^{th}$ Medium, A3C1G1 | 7$^{th}$ medium, A2C1G1 | First medium,A1C1G1 |
| | 14$^{th}$ medium, A3C1G2 | 8$^{th}$ medium, A2C1G2 | 2$^{nd}$ medium ,A1C1G2 |
| | 15$^{th}$ Medium, A3C2G1 | 9$^{th}$ medium, A2C2G1 | 3$^{rd}$ medium, A1C2G1 |
| | 16$^{th}$ Medium, A3C2G2 | 10$^{th}$ Medium, A2C2G2 | 4$^{th}$ Medium, A1C2G2 |
| | 17$^{th}$ Medium, A3C3G1 | 11$^{th}$ Medium, A2C3G1 | 5$^{th}$ Medium , A1C3G1 |
| | 18$^{th}$ Medium, A3C3G2 | 12$^{th}$ Medium, A2C3C2 | 6$^{th}$ Medium, A1C3G2 |

A1, A2 and A3: Amount of auxin with concentrations of 2.5,.4.9, respectively; C1,C2, and C3: amount of the cytokine with concentrations of 2.5 and 4, respectively; G1 and G2: amount of gibberellin with concentrations of 0 and 2.89.

## Results

It was seen in the first experiment that the highest amount of the phenol was measured in the free-hormone medium and the lowest amount was seen in the medium 15 and 17 (Figure 1). Since inflorescences were cultured randomly in the replicates of different treatments and were seen as necrotic, the ruby seedless cultivar had produced more phenol compared to the seedless cultivar (it should be noted that all inflorescences had the same size in the first experiment, the second experiment was planned and the following results were obtained. Regarding the type of the flower, results showed that young flowers showed browning despite the high sugar content especially when their number was high in the inflorescence cluster Because high density and the hormonal and sugar imbalance caused browning (Figure 1a). Larger flowers receive sugar substances from smaller samples due to passing the sensitive stage and go through evolution stages until complete opening (Figure 1b and c). Since the sepals and cupules undergo the most amount of photosynthesis and production of sugar substances and despite these tissues become brown, the ovary may receive less phenolic substances (Figure 1d). According to observations some flowers in the cluster were brown and some were green. Especially in the early stages, due to evolution of reproductive organs more sugar substances are required and competition between flowers happens (Figure 1e). Among white seedless and ruby seedless cultivars smaller samples showed more browning compared to large flowers. These cultivars in the medium containing hormone became less necrotic than the free-hormone medium and seedless cultivar showed less browning compared to the ruby seedless (Table 2). Type of the medium showed that production of phenol substances decreased alongside increase in the concentration and type of growth regulators especially auxin (Figure 2). Some browned samples became again green after a while after culture or the green fruit grew between browned flowers of the sample. This greening can be due to the effect of antioxidant activity of sugars or the activity of enzymes related to the sugar and hormones on decrease of the phenol amount so that flowers remain brown in flowers with control treatment and in the control medium high phenolic compounds cause oxidation of the medium (Figure 1e and d). The amounts of various sugar concentrations (sucrose, hexose, starch, or fructose) among different cultivars are different and type of genes expression in different stages of inflorescence growth and type of the cultivar show their ability in phenol produced. The ruby seedless cultivar is very sensitive compared to the white seedless cultivar and produces more phenol. It seems that since phenolic compounds and sugars have the same pathway and flowers need more sugar than other plant organs, hormone compounds play role in production of antioxidant compounds except phenols and receive more sugar and compounds by flowers in a way that in some hormone samples fruits or green flowers grow after a while, representing sugars consumption and balance in increase of other compounds. In experimental observations, brown samples were seen with phenol production in the hormone-free medium. Samples without the brown medium and only the brown inflorescence or medium with low concentration of phenols were related to size of florets and benefiting from foods, sugars, and carbohydrates. But in the control medium, despite high concentration of phenol, oxidant compounds increased and the medium was seen brown

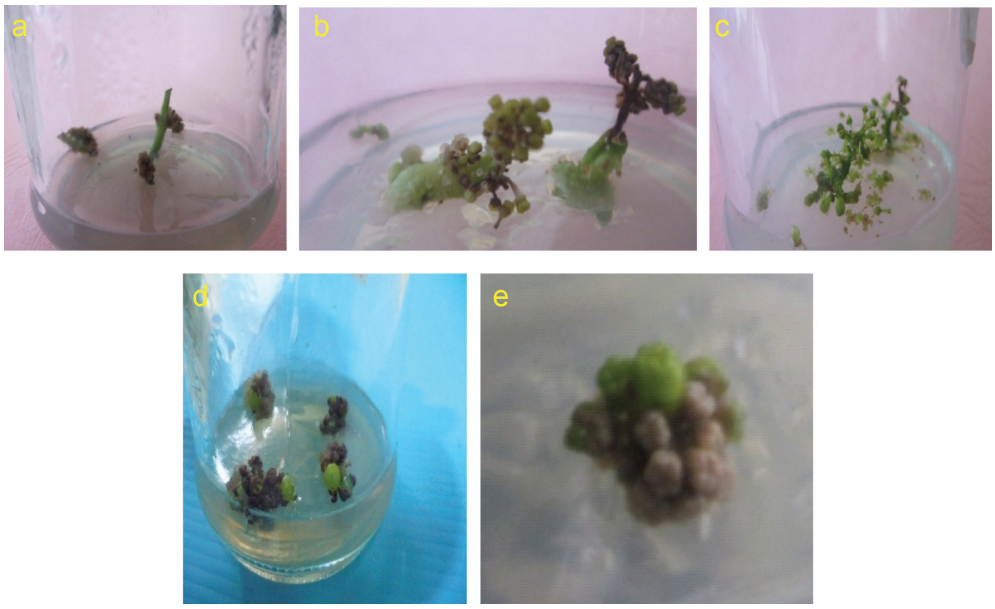


**Figure 1.** Brown flowers in the control treatment (a) small brown flowers and the large green flower in the free-phenol medium with the hormonal treatment; (b) green flowers with the hormonal treatment with large florets (c). The figure (e,d) production of green fruit between brown florets with the hormonal treatment and turning the brown flower into green.

**Table 2.** The effect of hormonal compound, type of the inflorescence and cultivar on the phenol amount.

| Parameter | Phenol amount ( mg/l) |
|---|---|
| Without hormone, small seedless floret | 1/75[b] |
| Without hormone, large seedless floret | 1/20[d] |
| Without hormone, small Ruby floret | 1/94[a] |
| Without hormone, large Ruby floret | 1/55[c] |
| A3C2G1, 15th medium, small floret white seedless | 0/55[f] |
| A3C2G1, 15th medium, large floret white seedless | 0/00[g] |
| A3C2G1, 15th medium, small floret seedless Ruby | 0/62[e] |
| A3C2G1, 15th medium, large floret seedless Ruby | 0/00[g] |
| A3C3G1, 17th medium, small floret white seedless | 0/52[f] |
| A3C3G1, 17th medium, large floret white seedless | 0/00[g] |
| A3C3G1 17th medium, small floret seedless Ruby | 0/69[e] |
| A3C3G1 17th medium, large floret seedless Ruby | 0/00[g] |

$A_3$ amount of auxin with concentration of 4.9. and $C_3$ amount of the cytokine with concentrations of 2.5 and 4. G1 amount of gibberellin with concentration. (C= Cytokinin, G= gibberellin, A= auxin).

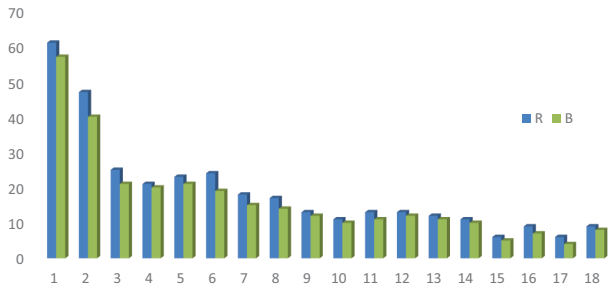


**Figure 2. The effect of hormone compounds of LAA, BAP, gibberellin (GA), and indole acid acetic on the percent of browning of the grape inflorescence in two cultivars of R ruby and B white seedless.** Percent of browning of the grape inflorescence in two cultivars of R (ruby) and B (white seedless) in mediums 2, 3, 6, 7, 8, 11, 12, 16 and 17 compared to each other is significant. Also this percent in the R (ruby) cultivar is higher than that in the B (white seedless cultivar. This percent is maximum in the medium 1 and is minimum in the medium 17.

(Figure 1a and b).

Obtained results in this research are not consistent with with the results obtained by North *et al.* (11). They stated that the medium with high concentration of the hormone produced more phenolic compounds. Perhaps, in the studied species of the explant production of the ethylene has been more important and less antioxidant properties or sugar existed in samples. Balance of sugars and hormone substances is important in the inflorescence growth and preventing toxic compounds with regard to the type of the explant.

## Discussion

Results obtained on the effect of growth regulators on the amount of medium phenol showed amount of phenolic compounds increase when the medium is free of hormones. These growth-inhibiting compounds provide major problem in the medium. So, plant sample and the cultivar as important factors are important in the phenol production. Collecting the explants in the special time of the year, when amount of phenolic compounds of the desired species is minimum, decreases the amount of the browning and becomes stimulated by environmental stimuli like sugar, ozone, temperature, stress and wound (15-17). The explant tissue and the medium become brown due to released negative compounds and cells disintegration (18). These reactions are catalyzed through polyphenol oxidase and tyrosinase enzymes (19). The meiosis stage is also a key stage in the evolution of reproductive organs and the sugar stress dries flowers, turns them brown, and causes them to fall (20). The grape inflorescence contains chlorophyll from the opening time to the time the fruit is matured. The amount of the chlorophyll of the inflorescence decreases in the growth period regularly. The highest amount of the chlorophyll is when the bud has opened recently, when its amount is comparable to that in leaves (21). In the next stages toward the fruit production, its amount reaches to 50% of the leaves photosynthesis level that is the highest amount in sepal and cupule (22). Stems of the inflorescence and ovaries also photosynthesize, the ovary may grows to the fruit despite these tissues turn brown (Figure 1e). Amount of the photosynthesis should be estimated in the inflorescence considering the respiration rate in them. When the inflorescence grows and the photosynthesis decreases the respiration rate increases indicating high metabolism activity and decrease in the sugar and phenol (23). Therefore, collecting the inflorescence explant in the special time of the year when amount of the chlorophyll and phenolic compounds of the desired species is high is important. These processes are stimulated by environmental stimuli like sugar, light, temperature, wound, etc. This research complies with the research performed previously on the *Strelitzia reginae* plant and indicates effects of antioxidants, growth regulators of the plant tissue on the phenols production and their concentration in production of phenolic compounds (24). It was determined in another study, conducted on the sugar beet culture, that explants with 0% concentration of sucrose showed the minimum phenol and nigrescence. But the best growth was obtained in the medium containing 1% sucrose, 2% mg/l indole acetic acid and 1 mg/l benzylaminopurine (BAP). When concentration of the sucrose was increased in the medium, the phenol concentration and necrotic tissues were increased and

the shoot regeneration was decreased (25). Substrates that are rapidly oxidized by the polyphenol oxidase in the grape fruit include catechin, epicatechin, caffeic acid, and catechol. The tissue browning is frequently observed in species containing tannin or other hydroxy-phenols, which are as substrate for *peroxidases* (POD) and *polyphenol oxidase*s (PPO) and cause oxidation of phenolic compounds (26). Achieving methods of *in vitro* propagation of grapevines has prevented the oxidation process. Adding catechin to sport energy drinks plays two main roles: first, supplying daily need of adults' body for antioxidants for preventing harmful oxidation reactions in the human's brain an second, preventing oxidation of perfumes and added flavors to drinks (27). Also, epicatechin is added to cosmetics and, in some cases, to food products like oils to prevent oxidation of fatty acids, to livestock feed in order to maintain the livestock healthy and improve quality of livestock products and also is used as an antibacterial compound in some foods and finally as an active compound for human health in foods (28).

## Conclusion

*In vitro* condition of the inflorescence to study physiologic process of the flowering and formation of the fruit and also determining stressful condition on this process that is not directly possible in the *in vitro* condition can solve many physiologic processes. Browning and death of plant tissues is one of the important problems in the *in vitro* propagation condition that depends on amount of phenolic compounds secreted into the medium. Determining the phenol amount can determine and improve the factors affecting production and decrease of the phenol production for establishment and growth of compounds.

## Authors' contributions

All the authors wrote the manuscript equally.

## Conflict of interests

The authors declared no competing interests.

## Ethical considerations

Ethical issues (including plagiarism, misconduct, data fabrication, falsification, double publication or submission, redundancy) have been completely observed by the authors.

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