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SECONDARY METABOLITES ACCUMULATION IN LONG-TERM CALLUS CULTURES OF *VITIS VINIFERA* CV. NEGRU VÂRTOS UNDER DIFFERENT STRESS CONDITIONS

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Abstract

Negru Vârtos is a variety of Vitis vinifera cultivated since the Dacian period. It is one of the most appreciated varieties in Romania, giving it a balanced and intensely colored wine. Our research aimed to increase the production of secondary metabolites in a long-term V. vinifera cv. Negru Vârtos callus culture obtained many years ago. Seven different abiotic factors represented by different media composition, salt addition, and UV or low-temperature exposure were tested to achieve this goal. The phenolic, flavonoid, anthocyanidin, and proanthocyanidin content was determined. Also, antioxidant activity was estimated by the α-diphenyl-α-picrylhydrazyl free radical-scavenging method (DPPH). Although all the tested variants showed better results concerning the callus morphometrical parameters as callus survival percentage, callus growth index, or callus moisture, only the variants treated with a low temperature of 4°C for 24 h revealed an increase of flavonoids, proanthocyanidins, and anthocyanidins content. Also, the production of anthocyanin was slightly stimulated in the R2 variant.

Key words*: abiotic stress, long-term callus, metabolites with biological activity, phenolic content, Vitis.*

INTRODUCTION

Vitis vinifera, the common grape vine, is an important crop plant that produces a variety of secondary metabolites with beneficial properties, such as resveratrol, anthocyanins, and flavonoids, compounds with recognized antioxidant, anti-inflammatory, and anticancer activities (Ali et al., 2024; Radulescu et al., 2020).

Negru vârtos (synonyms Gordan Negru, Corb, Neagră Vârtoasă, Negru Bătut, Negru Tare, Corb Negru) named because of the black colour of the grape skin, is one of the *V. vinifera* varieties of cultivated in Romania before the invasion of *Phylloxera* (Constantinesu, 1958); characterized by a long growing season with high production potential, with late ripening (https://grapevinevariety.com/details/show/4892), with unknown ancestry, used as a crossing partner for the Negru de Drăgășani and Novac varieties. No stocks have been reported since 2016 (https://glossary.wein.plus/negru-virtos).

Plant tissue culture offers alternatives for investigating metabolic pathways and factors influencing the biosynthesis of natural products of interest, in a controlled environment, independent of seasonal and geographical conditions. One of the possibilities for the production of valuable metabolites *in vitro* is based on callus culture (Ozyigit et al., 2023; Grąbkowska et al., 2016). Callus culture (an undifferentiated plant cell mass) is a valuable tool for studying and producing valuable compounds, including secondary metabolites (Ozyigit et al., 2023). Secondary metabolites represent compounds produced by plants, non-essential for growth, development, or reproduction, but with great benefits in adapting to environmental conditions (Seker & Erdogan, 2023). Different stress conditions can trigger the production of secondary metabolites in plants (Ramakrishna et al., 2011).

Over time, the long-term callus cultures become increasingly homogeneous (Fehér, 2019). In this context, the long-term callus cultures of *V. vinifera* represent a valuable source of natural antioxidants with potential applications in various industries (Sharafan et al., 2023).

Proanthocyanidins are a type of polyphenol, found in high concentrations in grapes (seeds and skin) with antioxidant properties with potential health benefits. Anthocyanins, a very attractive class of natural compounds, have different applications as colorants (food industry), nutraceuticals and therapeutic compounds (pharmacy), UV protectors, antioxidants, and anti-cancer compounds (cosmetics industry). Its biosynthesis is strongly induced by various stress factors (Hatier & Gould, 2009).

Our study aimed to evaluate the accumulation of the secondary metabolite (polyphenols, proanthocyanins, anthocyanidins, and flavonoids) in long-term callus cultures of *V. vinifera* cv. Negru Vârtos under different stress conditions.

MATERIALS AND METHODS

Plant material. The plant material used for our experiments was represented by *Vitis vinifera* long-term callus culture maintained for more than 40 years *in vitro* conditions. This long-term callus culture of *V. vinifera* cv Negru Vârtos was initiated in 1980 from leaf fragments from *in vitro* plantlets cultivated on a basal Gamborg (1968) medium supplemented with 0.1 mg/l NAA (α- naphthalene acetic acid), 0.2 mg/l kinetin, 2 g/l casein hydrolysate, 30 g/l sucrose, 8 g/l Plant Agar (Duchefa Biochemie, The Netherlands) (Brezeanu et al., 1980). The callusculture was subcultivated, over the years, on the MS (Murashige & Skoog, 1962) (Duchefa Biochemie, The Netherlands) media variant supplemented with the same additives previously mentioned (MSC) and maintained in the *in vitro* culture collection of the Institute of Biology Bucharest, Romanian Academy.

Experimental conditions. The initial explants, represented by 0.5g fragments of pale red callus from *V. vinifera* cv. Negru Vârtos long-term callus culture were spread on 0.5 cm surface of MSC medium variant (MS with NAA and kinetin) in each the Petri dish (6 cm diameter).

Different stress conditions used in the experiment are represented in Table 1. The stress conditions were induced by adding different chemicals to the MSC medium or by applying different physical factors at various exposure times.

Table 1. Stress conditions used for secondary metabolites induction in *V. vinifera* cv. Negru Vârtos long-term callus

	Growth conditions/Time	Variants
Control	MS/ 3 weeks	**A**
Chemical	MSC+CaCl2 · $2H_2O$ (440 mg/L) / 3 weeks	**B**
	MSC+NaCl (50 mM) / 3 weeks	**R1**
	MSC+NaCl (100 mM) / 3 weeks	**R2**
Control	MSC/ 3 weeks	**M**
Physical	MSC + 4°C / 24 h	**T1**
	MSC + 4°C / 48 h	**T2**
	MSC + UV exposure / 10 minutes	**U1**
	MSC + UV exposure / 15 minutes	**U2**

After exposure to different stress conditions, the callus was subcultivated on the MSC medium variant and maintained under *in vitro* conditions at 24 ± 2°C of temperature and 16/8 h photoperiod. After 4 weeks of subcultivation, the following morphometrical parameters were registered:

- callus survival percentage expressed after the formula: % survival = (number of developed calli / total number of inocula) × 100 %;
- callus fragment growth (cm) was quantified by measuring the diameter of the developed callus;
- callus growth index (CGI) was calculated as $CGI = (W_f - W_i)/W_i \times 100$ (Sahraroo et al., 2014) where W_i and W_f are the fresh weights registered at the beginning and the end of the growth period (4 weeks)
- fresh weight (FW, g) of the callus was recorded at 4 weeks, and dry weight (DW, g) was determined by incubation of a fresh callus sample in an oven at 55°C until the constant weight was reached;
- moisture content was calculated using the formula: Moisture content % = [(FW - DW) / FW] × 100.
- callus color intensity using a visual scale of yellow, red pale, and intense red.

Biochemical analysis

Extract Preparation. For extract preparation, 1 gram of *V. vinifera* callus (fresh weight) was crushed using a mortar and pestle, then 5 ml of methanol was added, and the samples were vigorously vortexed for 1 minute at room temperature. Homogenized samples were placed on an orbital shaker (Heidolph Instruments GmbH, Germany) and extracted for 2 days under continuous shaking, in the dark, at room temperature (RT). After this, samples were centrifuged (15 min, 12000 rpm, 4°C) using an Eppendorf centrifuge (Germany). The supernatant was transferred into a clean tube and stored at 4 °C.

Evaluation of Total Phenolic Content (PC). Total phenolic content was determined using the Folin-Ciocalteu method (Stankovic et al., 2011). Briefly, 2.5 ml Folin Ciocalteu solution and 2 ml Na_2CO_3 7.5% were added to 0.5 ml of diluted extract. The samples were incubated (30 min, RT) and the absorbance was measured at 765 nm and compared to the blank prepared with methanol. Results were expressed as mg of gallic acid equivalents (GAE)/g of DW, based on a calibration curve (10-100 µg/mL, $R^2 = 0.9968$).

Evaluation of Total Flavonoid Content (FC). Total flavonoid content was measured using a protocol adapted from Cai et al. (2012). In brief, 0.5 ml of dried extract was mixed with 2 ml distilled water and 0.15 ml 5% $NaNO_2$. The mixture was incubated (5 min, RT), then a 10% $AlCl_3$ solution was added. After 6 min at RT, 2 ml of 4% NaOH (m/v) and 1.2 ml distilled water were added to reach a final volume of 5 ml. The samples were stirred (15 min, RT) and the absorbance for each sample was read at 510 nm and compared to the blank. Results were expressed as mg of rutin equivalents (RE)/g of DW, based on a calibration curve (100-1000 µg/ml, $R^2 = 0.9996$).

Evaluation of Proanthocyanidin Content (PAC). The content of proanthocyanidins was determined according to Sun et al. (1998). Briefly, 400 µl of diluted extract was mixed with 3 ml of 4% vanillin solution in methanol (m/v) and 1.5 µl of hydrochloric acid. After 15 min incubation, 5 ml of glacial acetic acid was added, and the absorbance was read at 500 nm and compared to blank. Results were expressed as milligrams of catechin equivalents (CE)/g of DW, based on a calibration curve (20-200 µg/ml, $R^2 = 0.9924$).

Evaluation of Total Anthocyanins Content (TAC). Total anthocyanin content was determined using the standard method (Pasqualone et al., 2015). The dilution samples were prepared in methanol - 2N HCl solution (85:15 v/v). The absorbance of each sample was measured at 520 nm against a blank prepared with methanol - HCl. The absorbance of each sample was calculated according to the following formula:

Anthocyanin conc. (mg/g DW) = (A × MW × DF × 1000)/(ε × 1)

where: MW = molecular weight (449.2 g/mol C-3-G); DF = dilution factor; 1 = optical path (1 cm); ε = molar absorptivity (26,900 L × mol^{-1} × cm^{-1} for C-3-G).

Results were expressed as mg cyanidin-3-glucoside equivalents (C-3-GE)/g DW.

Evaluation of Antioxidant Activity Through DPPH Assays. The DPPH (2,2-Diphenyl-1-picrylhydrazyl) assay was applied according to Marxen et al. (2007). In brief, 2.25 ml methanol was mixed with 0.1 ml of diluted extract and 0.150 ml DPPH solution (1.27 mM). The reference and blank samples were prepared by mixing 2.35 ml methanol with 0.15 ml DPPH and 2.4 ml methanol with 0.1 ml diluted extract, respectively. Samples were incubated (30 minutes, RT) in the dark, and the absorbance was measured at 515 nm and compared to the reference sample. Results were expressed as mM of Trolox equivalents (TE)/g of DW, according to a standard curve (50-150 µg/ml, $R^2 = 0.9873$.

Statistical analysis. All the experiments consisted of 15 samples/treatments and the experiment was 3 times repeated. The results are reported as the means ± SD.

Before statistical analysis, the Shapiro-Wilk test was used to determine the normality of the dataset. The significant result ($p < 0.05$) of the test indicated the necessity to apply non-parametric techniques.

The Kruskal-Wallis test is a non-parametric method used to determine if there are statistically significant differences between two or more independent groups. If the test indicated significant differences between groups, pairwise comparisons were applied

using Dunn's post hoc test. To estimate the test distribution, the Monte Carlo method with 10,000 simulations was used. The technique is an alternative to the one-way ANOVA analysis applied when the normality of data conditions is not met (Ostertagova et al., 2014).

To analyze the relationship between bioactive compounds and antioxidant activity, the Partial Least Squares (PLS) regression method was used. This method was chosen due to its efficiency in managing data sets, allowing the identification of relationships between the independent variables (bioactive compounds) and the dependent variable (DPPH antioxidant activity). PLS models are particularly recommended for data sets with correlated variables, as they generate a set of components that best explain the dependent variable. The following coefficients were selected from the analysis*: Coefficients of determination* (R^2Y and R^2X) - R^2Y indicates the proportion of the variation in the dependent variable (Y) explained by the model. An R^2Y close to 1 suggests a good model fit. R^2X measures how well the PLS components explain the independent variables (X). A high R^2X suggests a good representation of the explanatory variables by the model components, *Predictive power* (cumulative Q^2), Q^2 (how) is a parameter that indicates the ability of the model to make predictions for new data. A Q^2(cum) close to 1 suggests high predictive power and *Variable Importance in Projection (*VIP) is an indicator of the contribution of each independent variable to the model. Variables with VIP > 1 are considered essential for prediction, and those with VIP < 0.8 can be eliminated to simplify the model (Tenenhaus et al., 2005).

Statistical analyses were performed with the XLSTAT software (2013).

RESULTS AND DISCUSSIONS

In the context of increasing demands for obtaining plant biomass with high secondary metabolite content, which can be exploited in various industries, the use of *in vitro* cultures for *V. vinifera* cv. Negru Vârtos was fully justified.

Previously, various studies were conducted regarding the isolation of *V. vinifera* callus lines with high proliferative capacities and secondary metabolites production (Brezeanu et al., 1993a; Brezeanu et al., 1999b; Lupşea & Brezeanu, 1999; Cogălniceanu at al., 2000a; Matienco et al., 2004; Mihai et al., 2009; Mihai et al., 2010; Mihai et al., 2013). Different factors like light intensity, KH_2PO_4 or NH_4NO_3 depletion (Karaaslan et al., 2013) and different elicitors such as salicylic acid, abscisic acid, jasmonic acid or mannitol (Mihai et al., 2010) were tested for increased the anthocyanin accumulation, the phenolic content and antioxidant activity in the grape callus lines.

In this study, two new chemicals ($CaCl_2 \cdot 2H_2O$ excess and NaCl) and two physical factors (low temperature and UV) to induced stress conditions were tested for elicitation of secondary metabolites as polyphenols, proanthocyanins, anthocyanidins, and flavonoids accumulation in long-term callus cultures of *V. vinifera* cv. Negru Vârtos.

The morphometrical parameters of the long-term callus cultures of *V. vinifera* cv. Negru Vârtos exposed to these stress conditions are illustrated in Table 2.

The various types of abiotic stress did not affect the callus survival, except for the UV treatments which determined a decrease in the survival rates to 84.13% (for U2 variant). The Kruskal-Wallis test (χ^2; = 29.7335; df = 7, p = 0.0002) indicated a significant difference between the analyzed groups in terms of survival rates. Dunn's post hoc pairwise comparison analysis reveals several significant differences. Thus, samples exposed to ultraviolet radiation for 10 and 15 minutes – the U variants) were clearly distinguished from the other groups, differing significantly (p = 0.0082 and p = 0.0004) from all other variants tested.

The callus growth index (CGI) and growth area were used to quantify the 3-D callus growth in surface and weight. The callus growth index was more affected by chemical treatments, unlike short treatments with physical stressors. All variants treated with $CaCl_2 \cdot 2H_2O$ in excess or NaCl registered a decrease in callus growth indexes than control, with the lowest value of 53.27% for the R1 variant treated with 50mM NaCl (Table 2). In the case of physical stressors the T1 and U2 variants showed increased values for callus growth index than control, indicating that these treatments induced callus growth.

Table 2. Morphometrical parameters of the long-term callus cultures of *V. vinifera* cv. Negru Vârtos exposed to different stress conditions

Stress variants	Survival percent (%)	Callus growth index (%)	Callus growth area (cm)	Moisture (%)	Callus color intensity
A	100	69.87	2.02 ± 2.76	98.53	Red
B	100	66.88	1.37 ± 0.51	98.48	Pale red
R1	100	53.27	2.56 ± 3.17	98.23	Intense red
R2	100	60.62	1.75 ± 0.41	98.24	Intense red
M	100	80.31	2.02 ± 2.76	98.36	red
T1	100	81.41	2.68 ± 1.61	98.54	Pale red
T2	100	75.12	3.3 ± 1.31	98.26	Pale red
U1	94	74.48	2.26 ± 1.17	98.48	Pale red
U2	84.13	82.01	2.29 ± 1.15	98.36	Pale red

The callus growth index was not associated with callus diameter growth (cm), which varied between 1.37 ± 0.51 cm for the B variant (excess of $CaCl_2 \cdot 2H_2O$) to 3.3 ± 1.31 cm for the T1 variant (4°C for 24 hours). These results suggested that the callus exposed to low temperature was more stressed, with callus cells being conditioned to spread on a medium surface to be in contact with the nutrients from the substrate.

Fresh and dry weight are two very important measurable variables that provide precise measurements of produced biomass. Based on the Kruskal-Wallis test and Dunn's post hoc pairwise comparisons, no statistically significant differences were found between the tested treatments analyzed in terms of fresh weight. Maintaining adequate moisture is crucial for the proper functioning of enzymes and the transport of nutrients within callus cells (Saurabh, 2015). In our case, the high moisture content was associated with the high survival rate of the callus, the stress conditions tested did not cause water accumulation in the cells. In their study, Mihai et al. (2013) underlined that high rates of biomass did not affect the phenolics and flavonoids production.

After 4 weeks, the calli maintained on the control variant (A) were almost red (100%), the calluses exposed to excess $CaCl_2 \cdot 2H_2O$ (B variant) were pale red, the calli subjected to excess salt (R variants) were intense red, while the calli subjected to low temperature (T variants) and UV (variants U) showed a pale red color. Under the red callus, in contact with the medium, islands of green callus were observed, especially in T variants (Figures 1 and 2).

Figure 1. *V. vinifera* cv. Negru Vârtos long-term callus initial explant

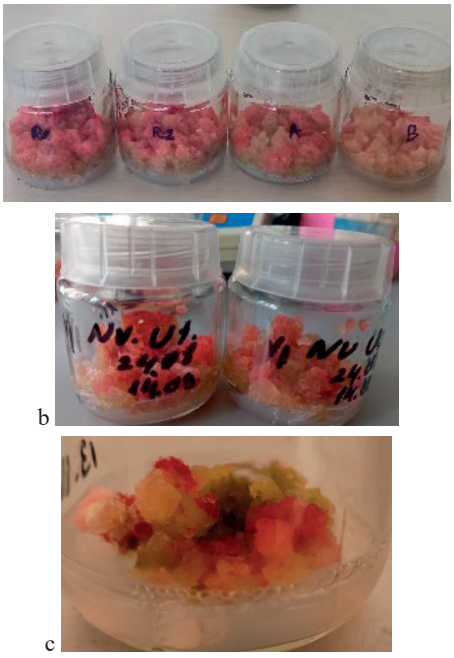

Figure 2. *V. vinifera* cv. Negru Vârtos long-term callus after stress experiment (a - R1, R2, A, B variants; b - UV variants; c - T1 variant)

Different abiotic stress was tested to induce secondary metabolite accumulation in long-term callus cultures of *V. vinifera* cv. Negru Vârtos.
Comparing different stress conditions, the Kruskal-Wallis test revealed significant differences between the variants analyzed for each of the studied compounds ($p < 0.0001$).
The highest accumulation of polyphenols was registered in unstressed variant M (control) and varied between stress conditions, from the highest content in the callus exposed to 4°C for 24 hours (T1 variant) (36.90 mg GAE/g DW) and the lowest content (11,757 mg GAE/g DW) in the callus exposed to an excess of $CaCl_2 \cdot 2H_2O$ (Figure 3a). For polyphenols ($\chi^2 = 31.49$), df = 7; $p < 0.0001$), the callus sample exposed to 4°C for 24 hours (T1 variant) was significantly different from the B variant ($CaCl_2 \cdot 2H_2O$ in excess) ($p < 0.0001$) but also beside the other variants exposure to the stress conditions. These results suggested that the T1 variant was the least affected option.
Concerning proanthocyanidins accumulation, callus exposure to 4°C for 24 hours (T1 variant) showed a slight increase in control (Figure 3b). Also, this variant had significant differences from B (excess $CaCl_2 \cdot 2H_2O$) ($p < 0.0001$) and R1 (50 mM NaCl) ($p = 0.0007$) variants.
In the case of anthocyanins, significant differences were recorded between variants ($\chi^2 = 28.25$; df = 7; $p < 0.0001$), the most notable being between the M variant (Control) and T1 variant ($p = 0.0006$), this treatment with low temperature stimulated synthesis of the anthocyanins (Figure 3c).
The flavonoid content varied between 16.39 (R1 variant) and 91.98 mg RE/g DW (T1 variant), the most significant differences were observed between T1 and B ($p = 0.00$), R1 ($p < 0.0001$), R2 ($p < 0.0001$) variants. Again, T1 was the variant treatment with the most differences from other variants and control, indicating a significant influence of low temperature on flavonoid content (Figure 3d). Our results are superior to Cetin's (2014) where a maximum total flavonol content (7.12 mg) was obtained in Öküzgözü callus exposed to UV for 5 min.

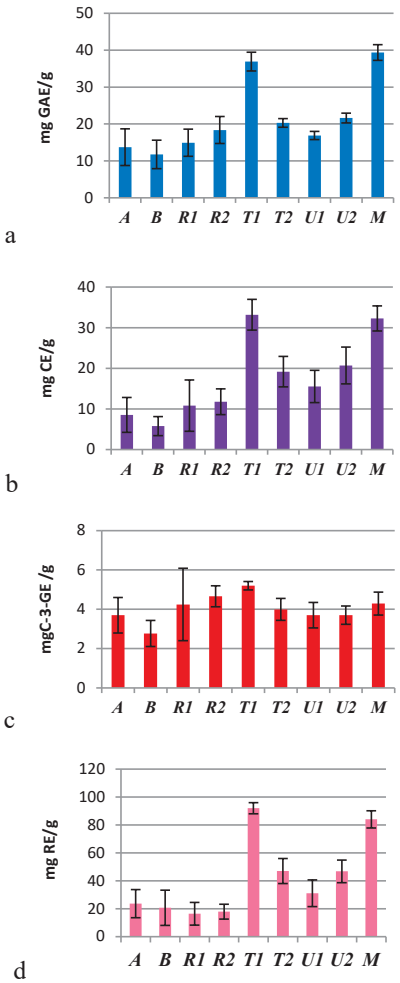

Figure 3. Secondary metabolites content in *V. vinifera* cv. Negru Vârtos long-term callus after exposure to different stress factors, polyphenols (a), proanthocyanidins (b), anthocyanidins (c), and flavonoids (d)

The antioxidant activity varied between 32.77-137.82 mM TE/g DW, but none of the stressed variants outperformed the control variants. The T1 variant (exposure for 24 hours at 4°C) showed significant differences from the B ($p < 0.0001$) and R1 ($p = 0.0001$) variants. Also, differences were observed between U2 and B ($p < 0.0001$), and B and R2 ($p = 0.001$) variants (Figure 4).

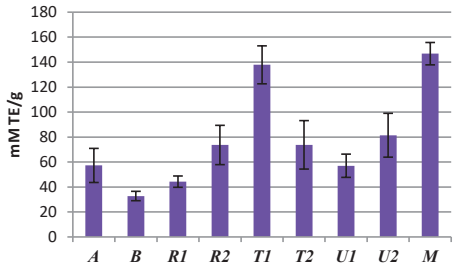

Figure 4. Antioxidant activity in *V. vinifera* cv. Negru Vârtos long-term callus after stress experiment

Compared with the control for both stress conditions (chemical - A and physical - M) no significant differences were observed, only a slight increase in the case of anthocyanidins, and flavonoids in the case of callus from the T1 variant.

The relationships between antioxidant activity and secondary metabolites tested analyzed through Partial Least Squares Regression (PLS Regression), highlighted their role in determining the antioxidant activity, with important variations between the types of samples, depending on the influence of each class of bioactive compounds. Analysis of PLS regression equations reveals how each bioactive compound influences DPPH antioxidant activity. In most samples, proanthocyanidins and polyphenols had either a positive or negative influence on DPPH, suggesting a variable impact on antioxidant activity. Flavonoids showed both positive and negative effects, but in general, their contribution was less compared to the other compounds. Anthocyanins had a different impact depending on the variants tested, in some cases having the strongest positive effect on DPPH activity and in others contributing negatively. Overall, polyphenols and proanthocyanidins are the most important factors in determining DPPH activity, while flavonoids and anthocyanins contribute to a variable extent (Table 3).

Table 3. PLS Regression Results: Q^2, R^2, and VIP Values for Evaluating Model Performance and Variable Importance

Variants	Q^2	R^2Y	R^2X_c	VIP			
				Proanthocyanidins	Polyphenols	Flavonoids	Anthocyanidins
A	0.78	0.82	0.98	0.86	1.07	1.04	1.02
B	0.58	0.72	0.71	1.35	1.25	0.35	0.71
R1	0.54	0.61	0.99	1.01	1.02	0.95	1.02
R2	0.67	0.74	0.97	1.12	0.87	1.08	0.88
T1	0.90	0.93	0.74	1.17	1.10	0.42	1.11
T2	0.98	0.99	0.73	1.15	0.84	1.17	0.77
U1	0.99	0.99	0.92	1.04	1.01	1.04	0.90
U2	0.93	0.94	0.90	0.77	1.02	1.09	1.09

Legend: Q^2 - Predictive power; R^2Y and R^2X - Coefficients of determination; VIP - Variable Importance in Projection

To identify the composition of the callus growth medium variant, it must be taken into account that in the process of growth and development of the callus, there are 2 types of cells (those at the base, colorless, which help the growth, and those at the surface, colored, characterized by the accumulation of secondary metabolites (Deroles, 2009). Balancing the nutrient composition to obtain both growth and secondary metabolites is the desired task. Knowing that calcium chloride ($CaCl_2 \cdot 2H_2O$) is often used in viticulture to improve fruit quality, we tested an excess concentration. Calcium, one of the essential nutrients, plays crucial roles in cell wall structure, enzyme activation, and signal transduction (White et al., 2003). An excessive application of $CaCl_2 \cdot 2H_2O$ can have a significant impact on the secondary metabolite profile of *V. vinifera* (Martins et al., 2021), fact was also proven in our case (2 times $CaCl_2 \cdot 2H_2O$ concentration - B variant, compared to the control variant), where all the secondary metabolites analyzed in the callus were in reduced quantity compared to the control, but also to the other stress variants (Figure 3).

Salt stress can induce the accumulation of phenolic compounds, such as flavonoids (anthocyanins, flavonols), with antioxidant effects protecting cells from damage caused by salt stress (Almagro et al., 2022). Salt stress can also alter the relative proportions of different secondary metabolites, affecting the ratio of anthocyanins to flavonols and influencing color. In our case, the callus subjected to 50-100 mM NaCl (R variants) was characterized by a color intensification of the cells (higher concentration, higher intensification), which proves a higher anthocyanin load compared to the control (Figure 3).

A 24-hour exposure of long-term callus cultures of *V. vinifera* cv. Negru Vârtos at 4°C (T1 variant) assures the highest secondary metabolite accumulation among all the tested variants. Kumar et al., 2023 showed that grapevines can trigger the production of certain secondary metabolites as a defense mechanism in response to cold stress. Prolonged cold exposure may have detrimental effects (Ramakrishna & Ravishankar, 2011), in our case, the 48-hour exposure (T2 variant) reduces almost half the secondary metabolites accumulation.

UV radiation has been shown to have the most significant impact on secondary metabolite production in grapevines, triggering various physiological responses, leading to the accumulation of beneficial compounds (Del-Castillo-Alonso et al., 2021); enhancing the phenolic compounds (e.g., anthocyanins, flavonols) biosynthesis, compounds with contribution to the color, astringency, and antioxidant properties of grapes and wine (Cozzolino, 2025).

The presentations of secondary metabolites analyzed in both fresh and dry-weight will be helpful, in future research, to normalize the data, determine water-soluble vs. water-insoluble compounds, understand the metabolite accumulation, track the metabolite accumulation, optimize extraction methods, etc. (Oancea et al., 2025)**.**

CONCLUSIONS

The results of these studies could have important implications for the production of valuable secondary metabolites from *V. vinifera*. By understanding the factors that influence the accumulation of secondary metabolites in callus cultures, it may be possible to develop more efficient methods for producing these compounds for use in the pharmaceutical, food, and cosmetic industries.

Between treatments, the cold storage at 4°C for 24 hours (T1 variant) ensures not only the biomass growth but also triggers the accumulation of secondary metabolites of interest in a higher quantity, as anthocyanidins and flavonoids.

In perspective, the combined testing of abiotic factors and different protocols for stress induction will be considered.

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REFERENCES

Ali, K., Maltese, F., Choi, Y.H., Verpoorte, R. (2010). Metabolic constituents of grapevine and grape-derived products. *Phytochemical Review, 9*(3). 357-378.

Almagro, L., Calderón, A.A., Pedreño, M.A., Ferrer, M.A. (2022). Differential Response of Phenol Metabolism Associated with Antioxidative Network in Elicited Grapevine Suspension Cultured Cells under Saline Conditions. *Antioxidants (Basel), 11(*2). 388.

Brezeanu, A., Iordan, M., Roşu, A. (1980). The micropropagation of *Vitis vinifera* L. I. The induction and characterization of callus from tissues of somatic origin, *Review Roumaine Biology -Biology.Vegetale*, *25*(2). 135-142

Brezeanu, A., Matienco, B., Cogălniceanu, G., Călin, A., Cârciumărescu, D. (1993a). Iniţierea şi caracterizarea calusului din fruct şi rahis la *Vitis vinifera* L., In I. Anghel, A. Brezeanu, D. Cachiţă-Cosma, (Eds), *Lucrarile celui de al V-lea Simpozion Naţional de Culturi de Ţesuturi si Celule Vegetale* (pp. 181-191). Bucureşti, Romania, Editura Universităţii Bucureşti.

Brezeanu, A., Matienco, B., Cogălniceanu, G., Maximova, E., Lupşea, S. (1999b). Hypothetical model of intracellular anthocyanins trafficking pathways in grapevine long term callus culture, In M. Falcă (Ed.), *Proceedings of the Institute of Biology* (pp. 263-272), vol. II, Bucureşti, Romania, Alcris.

Cai, S., Wang, O., Wu, W., Zhu, S., Zhou, F., Ji, B., Gao, F., Zhang, D., Liu, J., Cheng, Q. (2012). Comparative Study of the Effects of Solid-State Fermentation with Three Filamentous Fungi on the

Total Phenolics Content (TPC), Flavonoids, and Antioxidant Activities of Subfractions from Oats (*Avena sativa* L.). *Journal of Agricultural and Food Chemistry*, *60* (1), 507-513. DOI: 10.1021/jf204163a

Cetin, E.S. (2014). Induction of secondary metabolite production by UV-C radiation in Vitis vinifera L. Öküzgözü callus cultures. Biological Research, 47, 1-7. https://dx.doi.org/10.1186/0717-6287-47-37

Cogălniceanu, G., Brezeanu, A., Matienco, B., Lupşea, S. (2000a). Factori implicaţi în stimularea proliferării celulare şi a metabolismului secundar (biosinteza de antociani) în cultura de calus long - term la *V. vinifera*. *Acta Horti Botanici Bucurestiensis,* 28, 282-291.

Constantinesu, G. (1958). Ampelografia. Editura Agro-Silvica de stat Bucuresti, Romania.

Cozzolino, D. (2025). Phenolics and spectroscopy: challenges and successful stories in the grape and wine industry. *Journal of the Science of Food and Agriculture,* 105(3), 1408-1412.

Del-Castillo-Alonso, M.Á., Monforte, L., Tomás-Las-Heras, R., Ranieri, A., Castagna, A., Martínez-Abaigar, J., Núñez-Olivera, E. (2021). Secondary metabolites and related genes in *Vitis vinifera* L. cv. Tempranillo grapes as influenced by ultraviolet radiation and berry development. *Physiologia Plantarum, 173*(3).709-724.

Deroles, S. (2009). Anthocyanin biosynthesis in plant cell cultures: A potential source of natural colorants. In C. Winefield, K. Davies, K. Gould (Eds.) *The Anthocyanins*. (pp108-167), Springer New York.

Fehér, A. (2019). Callus, Dedifferentiation, Totipotency, Somatic Embryogenesis: What These Terms Mean in the Era of Molecular Plant Biology? *Frontiers in Plant Science, 10*. 536.

Gamborg, O.L., Miller, R.A., Ojima, K. (1968). Nutrient Requirements of Suspension Cultures of Soybean Root Cells. *Experimental Cell Research*, *50*. 151-158.

Grąbkowska, R., Matkowski, A., Grzegorczyk-Karolak, I., Wysokińska, H. (2016). Callus cultures of *Harpagophytum procumbens* (Burch.) DC. ex Meisn.; production of secondary metabolites and antioxidant activity. *South African Journal of Botany*, *103*. 41-48.

Hatier, J.-H.B. & Gould, K.S. (2009). Anthocyanin functions in vegetative organs. In C. Winefield, K. Davies, K. Gould (Eds.), *The Anthocyanins* (pp1-19). Springer New York.

https://glossary.wein.plus/negru-virtos (accesed on 15.01.2025)https://grapevinevariety.com/details/show/4892 (accesed on 15.01.2025)

Karaaslan, M., Ozden, M., Vardin, H., Yilmaz, F.M. (2013). Optimisation of phenolic compound biosynthesis in grape (Bogazkere Cv.) callus culture. *African Journal of Biotechnology, 12*(25), 3922-3933.

Kumar, K., Debnath, P., Singh, S., Kumar, N (2023). An Overview of Plant Phenolics and Their Involvement in Abiotic Stress Tolerance. *Stresses*, *3*. 570-585

Lupşea, S., Brezeanu, A. (1999). Reactivitatea *in vitro* a sp. *Vitis vinifera* L. în funcţie de genotip, natura explantului şi mediul de cultură, *Acta Horti Botanici Bucurestiensis,* 27. 87-92.

Martins, V., Unlubayir, M., Teixeira, A., Lanoue, A. Gerós, H. (2021). Exogenous Calcium Delays Grape Berry Maturation in the White cv. Loureiro While Increasing Fruit Firmness and Flavonol Content. *Frontiers in Plant Science, 12*, 742887.

Marxen, K., Vanselow, K.H., Lippemeier, S., Hintze, R., Ruser, A., Hansen, U.P. (2007). Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. Sensors, 7, 2080-2095.

Matienco, B., Brezeanu, A., Maximova, E., Marinescu, M., Cogălniceanu, G. (2004). Carpoculture *in vitro*. Nonmorphogenetic pathway, *Î.E.P. Ştiinţa*, Chişinău, pp 136.

Mihai, R., Brezeanu, A., Cogălniceanu, G. (2009). Aspects of some elicitors influence on non-morphogenetic callus of *Vitis vinifera* L. var. Isabelle, *Romanian Biotechnological Letters*, *14*(4). 4511-4518.

Mihai, R., Mitoi, M., Brezeanu, A., Cogălniceanu, G. (2010). Two-stage system, a possible strategy for the enhancement of anthocyanin biosynthesis in a long-term grape callus cultures, *Romanian Biotechnological Letters, 15*(1). 5025-5033.

Mihai, R., Mitoi, M., Manole, A., Brezeanu, A. (2013). A preliminary study on the dynamics of total phenol and anthocyanin accumulation in *Vitis vinifera* L. cv. Negru Vârtos callus culture, *Journal of Academy Science of Moldova (Life Science),* 3(321). 68-72.

Murashige, T. & Skoog, F. (1962). A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. *Plant Physiology, 15*, 473-497.

Oancea, E., Tula, I.A., Stanciu, G., Ştefan-van Staden, R.-I., van Staden, J.F., Mititelu, M. (2025). Advanced Amperometric Microsensors for the Electrochemical Quantification of Quercetin in *Ginkgo biloba* Essential Oil from Regenerative Farming Practices. *Metabolites*, *15*, 6.

Ostertagova, E., Ostertag, O., Kováč, J. (2014). Methodology and application of the Kruskal-Wallis test. *Applied mechanics and materials*, *6* (11), 115-120.

Ozyigit, I.I., Dogan, I., Hocaoglu-Ozyigit, A., Yalcin, B., Erdogan, A., Yalcin, I.E., Cabi, E., Kaya, Y. (2023). Production of secondary metabolites using tissue culture-based biotechnological applications. *Frontiers in Plant Science, 14*, 1132555.

Pasqualone, A., Bianco, A.M., Paradiso, V.M., Summo, C., Gambacorta, G.,Caponio, F., Blanco, A. (2015). Production and characterization of functional biscuits obtained from purple wheat. *Food Chem. 180*, 64–70.

Radulescu, C., Buruleanu, L.C., Nicolescu, C.M.,Olteanu, R.L., Bumbac, M., Holban, G.C., Simal-Gandara, J. (2020). Phytochemical Profiles, Antioxidant and Antibacterial Activities of Grape (*Vitis vinifera* L.) Seeds and Skin from Organic and Conventional Vineyards. *Plants, 9.* 1470.

Ramakrishna, A., Ravishankar, G.A. (2011). Influence of abiotic stress signals on secondary metabolites in plants. *Plant Signaling & Behavior*, *6*(11). 1720-31.

Seker, M.E., Erdogan, A. (2023). Phenolic and carotenoid composition of *Rhododendron luteum* sweet and *Ferula communis* L. subsp. *communis* flowers. *Frontiers in Life Science and Related Technologies 4*(1). 37–42.

Saurabh, B. (2015). Chapter 2 - Plant Tissue Culture. Editor(s): Saurabh Bhatia, Kiran Sharma, Randhir Dahiya, Tanmoy Bera, Modern Applications of Plant Biotechnology in Pharmaceutical Sciences, Academic Press, Pages 31-107.

Sharafan, M., Malinowska, M.A., Ekiert, H., Kwaśniak, B., Sikora, E., Szopa, A. (2023). *Vitis vinifera* (Vine Grape) as a Valuable Cosmetic Raw Material. *Pharmaceutics, 15*(5), 1372.

Sahraroo, A., Babalar, M., Mirjalili, M.H., Fattahi Moghaddam, M.R., Nejad Ebrahimi, S. (2014). *In-vitro* Callus Induction and Rosmarinic Acid Quantification in Callus Culture of *Satureja khuzistanica* Jamzad (Lamiaceae). *Iranian Journal of Pharmaceutical Resources, 13*(4). 1447-56.

Sun, B., Ricardo-Da-Silva, J.M., Spranger, I. (1998). Critical factors of vanillin assay for catechins and proanthocyanidins. *Journal of Agricultural Food Chemistry, 46*:4267–4274. doi: 10.1021/jf980366j

Stankovic, M. (2011). Total phenolic content, flavonoid concentration and antioxidant activity of *Marrubium peregrinum* L. Extracts. *Kragujevac Journal of Science*, *33*, 63-72

Tenenhaus, M., Pagès, J., Ambroisine, L., Guinot, C. (2005). PLS methodology to study relationships between hedonic judgments and product characteristics. *Food Quality and Preference*, *16(*4), 315-325

White, P.J., Broadley, M.R. (2003). Calcium in plants. *Annals of Botany, 92*(4), 487-511.

XLSTAT pro. (2013). *Data analysis and statistical solution for Microsoft Excel*. Addinsoft.