STUDY CONCERNING THE INVOLVEMENT OF GUAIACOL PEROXIDASE – PHENOLIC COMPOUNDS RELATIONSHIP ON ASSIMILATORY PIGMENT DEGRADATION IN *VITIS VINIFERA* L. LEAVES

STUDIU PRIVIND IMPLICAREA RELAȚIEI GUAIACOL PEROXIDAZA-COMPUŞI FENOLICI ÎN DEGRADAREA PIGMENȚILOR ASIMILATORI DIN FRUNZELE *VITIS VINIFERA* L.

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Abstract. *Previous research highlighted that chlorophyll and some carotenoids are bleached by the action of oxidative enzymes such as peroxidases in the presence of phenolic compounds. In the present investigation, leaves of 11 Vitis vinifera L. varieties (in blooming phenophase), were assayed for their chlorophyll (a+b) and carotenoid (x+c) concentration and their degradation after 30 days of cold (6°C) and dark storage. Peroxidase activity (EC 1.11.1.7) and total phenolic content (TP) of leaves were initially determined. We found an important linear correlation between peroxidase activity (PA) and the percentage of total chlorophyll (Chl) degraded (R^2=0.9243; p<0.05), and also a positive statistically significant relationship between PA and TP concentration (R^2=0.8389; p<0.05). Analyzing by fractions, the most important coefficient of determination was registered for the relationship PA – % of Chl a degraded (R^2=0.8389; p<0.05), with a poor correlation for PA – % of Chl b degraded and PA – % of carotenoid degraded relationships. Experimental data obtained indicates that peroxidase might be involved in chlorophyll bleaching in the presence of phenolic compounds, and might mediate in a lesser extent leaf carotenoids degradation.*
Key words: *peroxidase, chlorophyll, carotenoids, phenolic compounds, Vitis vinifera L. leaves*

Rezumat. *Cercetări anterioare au subliniat faptul că degradarea clorofilei şi a carotenoizilor este mediată de acțiunea unor enzime oxidative de tipul peroxidazei, în prezența compuşilor fenolici. În prezentul studiu, frunzele a 11 soiuri Vitis vinifera L. (în fenofaza de înflorire) au fost analizate în ceea ce priveşte concentrația de clorofilă (a+b) şi carotenoizi (x+c) şi degradarea acestor compuşi după 30 de zile de păstrare la rece (6°C) şi la întuneric. Activitatea peroxidazei (CE 1.11.1.7) şi conținutul de compuşi fenolici (CF) al frunzelor au fost de asemenea determinate. Astfel, a fost identificată o corelație liniară importantă între activitatea peroxidazei (AP) şi procentul de clorofilă (Cl.) degradat (R^2=0,9243; p<0,05) şi o relație pozitivă statistic semnificativă între AP şi concentrația de CF (R^2=0,8389; p<0,05). Analizând individual fracțiunile rezultate, cel mai important coeficient de determinare a fost înregistrat în cazul relației AP – % de Cl. a degradat (R^2=0,8389; p<0,05), cu o valoare mai redusă a acestuia în cazul relațiilor AP – % de Cl. b degradat şi AP – % de carotenoizi degradat. Datele experimentale obținute indică faptul că peroxidaza poate fi implicată în degradarea clorofilei în prezența*

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compuşilor fenolici şi poate media într-o măsură redusă degradarea carotenoizilor din frunzele soiurilor V. vinifera L.
Cuvinte cheie: *peroxidaza, clorofilă, carotenoizi, compuşi fenolici, frunze Vitis vinifera L.*

INTRODUCTION

Guaiacol (o-methoxyphenol) peroxidase (E.C. 1.11.1.7) is widely distributed in plants (chloroplasts, vacuoles, and cell walls) where they catalyze the reduction of hydrogen peroxide (H_2O_2) to water, rendering it harmless (Yamasaki et al., 1997; Bania and Mahanta, 2012), and that have been thought to be involved in the biodegradation pathway(s) of chlorophylls during leaf senescence (Matile and Hortensteiner, 1999; Yamauchi et al., 2004).

In the earlier literature was shown that chlorophylls and its derivatives are oxidized *in vitro* by the peroxidase-H_2O_2 system in the presence of a kind of phenolic compound (Kato and Shimizu, 1985), flavonols being able to act as electron donors for peroxidase (Miller and Schreier, 1985). Was also suggested that the phenolic compounds involved in chlorophyll degradation could be monophenols with a hydroxyl group at the *p*-position (Whitaker, 1994).

More recently, several studies have concluded that peroxidases can catalyze the type II degradation of chlorophylls („bleaching" or „photobleaching"), as an alternative route for chlorophyll biodegradation (Hynninen et al., 2010). According to Yamauchi et al. (2004), peroxidase oxidizes the phenolic compounds (which have the hydroxyl group at the *p*-position), to form the phenoxy radical and superoxide anion, that attacks chlorophyll *a*, which is degraded to colorless low molecular weight compounds. Data on the degradation of carotenoids by peroxidases are quite poor. Partial degradation of carotene by peroxidase was reported (Gelinas et al., 1998). Also, Matile and Martinoia (1982), highlighted that commercial peroxidase catalizes the oxidation of lutein to unknown colorless products.

However, the mechanism of chlorophyll and carotenoid degradation with the oxidation of the phenolic compounds is still unclear. Given the importance of peroxidases (in biological processes) and of chlorophyll and carotenoids, both as photosynthetic pigments and nutraceuticals, relationships that occur in their degradation must be known and understood.

MATERIAL AND METHOD

The research has been carried out on the leaves of 11 *Vitis vinifera* L. indigenous varieties (Gelu, Milcov, Cetățuia, Napoca, Someşan, Splendid, Transilvania, Coarnă neagră, Coarnă neagră selecționată, Purpuriu and Radames), growing in the Ampelographic Collection of the University of Agricultural Sciences and Veterinary Medicine Iasi, Romania. Leaves were harvested at vine flowering (on the 3th day after the beginning of flowering), on ice, between the nodes 1 and 5 of vine shoots (Zapatta et al., 1995), rapidly frozen (10 min) and analyzed in same day.

Peroxidase (guaiacol units) assay procedure was based on that of Bergmeyer (1974), in which the rate of decomposition of hydrogen peroxide by peroxidase, with guaiacol as hydrogen donor, is determined by measuring the rate of colour development spectrophotometrically at 436 nm and at 25°C (UV-vis Spectrostar Nano microplates spectrophotometer). A peroxidase unit (U) represents the amount of enzyme which catalyses the conversion of one micromole of H_2O_2 per minute at 25°C.

For assimilatory pigment extraction, frozen leaf samples (0.5 g) were grinded and washed with 10 mL of 99,98% v:v acetone in order to extract the compounds from the leaf tissue. The extract was placed in the refrigerator overnight to minimize phototransformation of chlorophyll and to complete extraction, and subsequently centrifuged (refrigerated laboratory centrifuge Nahita 2816) 15 min, 3000 rpm (10 °C). The analytical determination was conducted using a UV-vis Shimadzu 1700 Pharmaspec Spectrophotometer at the following wavelengths: 662 and 645 nm, for chlorophyll a and b and 470 nm for carotenoids (xanthophylls and carotenes). Photosynthetic pigment content was calculated in mg/g fresh weight (f.w.), using the equations proposed by Carnegie Institution for Science through Spectranomics Protocol in 2011. After 30 days of cold (6°C) and dark storage, extracts were reevaluated regarding the chlorophyll and carotenoid concentration.

Total phenolic content was determined by Folin-Ciocâlteu method, measuring the absorbance at 750 nm (Singleton and Rossi, 1965). A calibration curve using different concentrations of gallic acid solutions was used for expressing the results as gallic acid equivalent (GAE), with the equation y=0.8757x+0.0438 (R^2=0.991).

A one-way Analysis of variance (ANOVA) test was initiated to investigate significant differences between data. The method used to discriminate among the means was Fischer's least significant difference procedure at 95% confidence level. Simple regression analysis was performed to look for relationships between data.

RESULTS AND DISCUSSION

Moisture content and total dry matter of leaf samples (4 h at 105°C) at harvest was specific to the flowering phenophase, and varying from 71.99% to 77.48% (with a mean of 75.02%), and from 22.52% to 26.43% (with a mean of 24.98%), respectively, being in accordance to the data presented for *V. vinifera* L. varieties by Mustea (2004). Total mineral content of leaves, represented by ash (4 h at 510 °C), was within the range of 1.43% – 2.32% (with a mean of 1.97%), in accordance with data presented by Burzo et al. (2005).

Chlorophyll (*a* and *b*) and carotenoid content of Romanian grapevine leaves, initially and after 30 days of cold storage, are shown in table 1.

Table 1

Chlorophyll and carotenoid concentration (mg/g) in *V. vinifera* L. leaf extract, Initially (In.) and after the storage period (A.)

Variety	Carotenoids		Chlorophyll a		Chlorophyll b		Total Chl	
	In.	A.	In.	A.	In.	A.	In.	A.
Purpuriu	0.34^{000}	0.31^{000}	0.69^{000}	0.54^{000}	0.31^{000}	0.27^{000}	1.00^{000}	0.80^{000}
Splendid	0.40^{NS}	0.37^{NS}	0.75^{NS}	0.59^{NS}	0.35^{000}	0.29^{000}	1.10^{000}	0.88^{000}
Coarnă neagră	0.41^{NS}	0.37^{NS}	0.80^{NS}	0.62^{NS}	0.50^{NS}	0.41^{NS}	1.30^{NS}	1.03^{NS}
Coarnă neagră selecționată	0.42^{NS}	0.39^{NS}	0.80^{NS}	0.62^{NS}	0.46^{NS}	0.38^{NS}	1.26^{NS}	1.00^{NS}
Cetățuia	0.42^{NS}	0.38^{NS}	0.72^{000}	0.56^{NS}	0.53^{NS}	0.42^{NS}	1.25^{NS}	0.98^{NS}
Someşan	0.42^{NS}	0.39^{NS}	0.78^{NS}	0.62^{NS}	0.47^{NS}	0.40^{NS}	1.26^{NS}	1.00^{NS}
Gelu	0.43^{NS}	0.40^{NS}	0.86^{***}	0.67^{***}	0.50^{NS}	0.43^{NS}	1.36^{***}	1.09^{NS}
Milcov	0.43^{NS}	0.40^{NS}	0.79^{NS}	0.63^{NS}	0.73^{***}	0.64^{***}	1.52^{***}	1.24^{***}
Radames	0.43^{NS}	0.41^{NS}	0.78^{NS}	0.62^{NS}	0.45^{NS}	0.38^{NS}	1.22^{000}	0.99^{NS}
Transilvania	0.44^{NS}	0.41^{NS}	0.80^{NS}	0.63^{NS}	0.46^{NS}	0.38^{NS}	1.26^{NS}	1.01^{NS}
Napoca	0.45^{***}	0.41^{NS}	0.76^{NS}	0.60^{NS}	0.59^{***}	0.48^{***}	1.35^{***}	1.08^{NS}
Mean	0.42	0.39	0.78	0.61	0.49	0.41	1.26	1.01
St. error	0.03	0.03	0.04	0.04	0.11	0.10	0.14	0.11

Note: Data expressed as mean values (n = 3). $^{NS, *, **, ***}$ - indicate nonsignificant and positive significant at $p \leq 0.05, 0.01, 0.001$, respectively; $^{o, oo, ooo}$ - negative significant at $p \leq 0.05, 0.01, 0.001$, respectively.

Statistical analysis of the data revealed a high positive significance ($p<0.001$), compared to the mean, at the variety Gelu (chlorophyll *a*), Milcov and Napoca (chlorophyll *b*). Total chlorophyll (Chl) content ranged initially from 1.00 mg/g f.w. to 1.52 mg/g f.w. (with a mean of 1.26 mg/g f.w.), and decreasing to the range of 0.80 mg/g f.w. – 1.24 mg/g f.w. after 30 days of extract storage. Both in the case of chlorophylls and carotenoids a high initial content resulted in a lower degradation of these compounds, as was mentioned previously by Wilows (2004).

Percentage of carotenoids and chlorophylls in grapevine leaf extracts degraded after the storage period, varied in low limits between samples, from 5.86% to 8.52% in the case of carotenoids (mean 7.28±0.83%), and from 18.38% to 21.77% (mean 20.00±0.94%) for total chlorophylls (fig. 1).

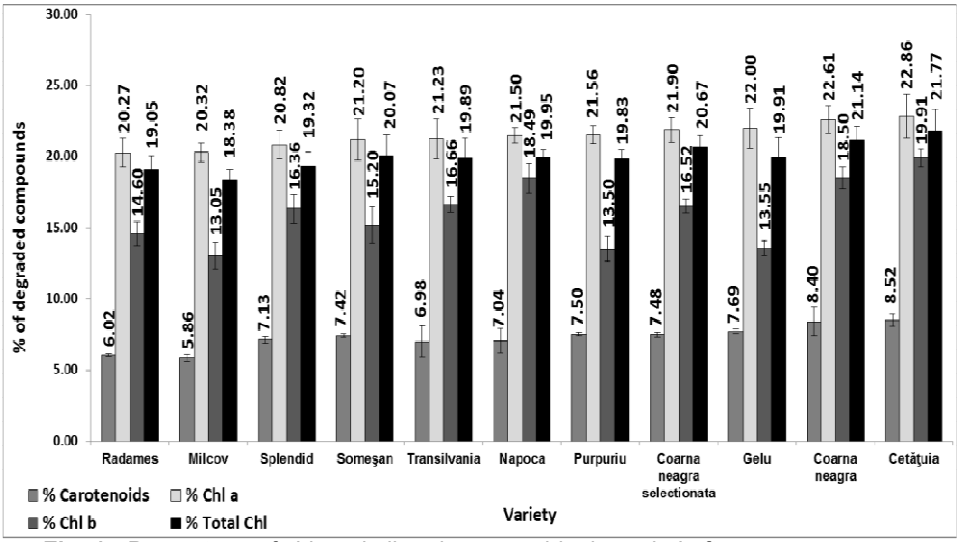

Fig. 1 - Percentage of chlorophyll and carotenoids degraded after extracts storage

Peroxidase activity (PA) in grapevine leaves ranging between 0.014 and 0.045 U/mg f.w., a higher phenolic concentration corresponding to a increased activity of the enzyme (fig. 2), as was earlier specified by Ghoul and Chebil (2012).

Involvement of peroxidase in assimilatory pigment degradation is demonstrated by the important coefficients of determination (R^2) registered for PA–TP relationship ($R^2=0.8389$; $p<0.05$), PA–% total Chl degraded ($R^2=0.9243$; $p<0.05$), and also for TP–% total Chl degraded ($R^2=0.8282$; $p<0.05$). Poor correlation of TP and % of Chl degraded after storage could be explain by the specificity of enzyme for certain phenolic compounds (with the hydroxyl group at the *p*-position) which might be not in adequate quantity in grapevine leaves. According to Katalinic et al. (2009), phenolic compounds in *V. vinifera* L. leaves were represented mainly by phenolic acids, flavonoids and stilbenes.

% of Chl *a* degraded was statistically significant correlated with the increase of PA ($R^2=0.8389$; $p<0.05$), while % of Chl *b* and carotenoids degraded after extract storage shown the same trend, but without a significant coefficient of determination ($R^2=0.7046$, and $R^2=0.7531$, respectively) (table 2).

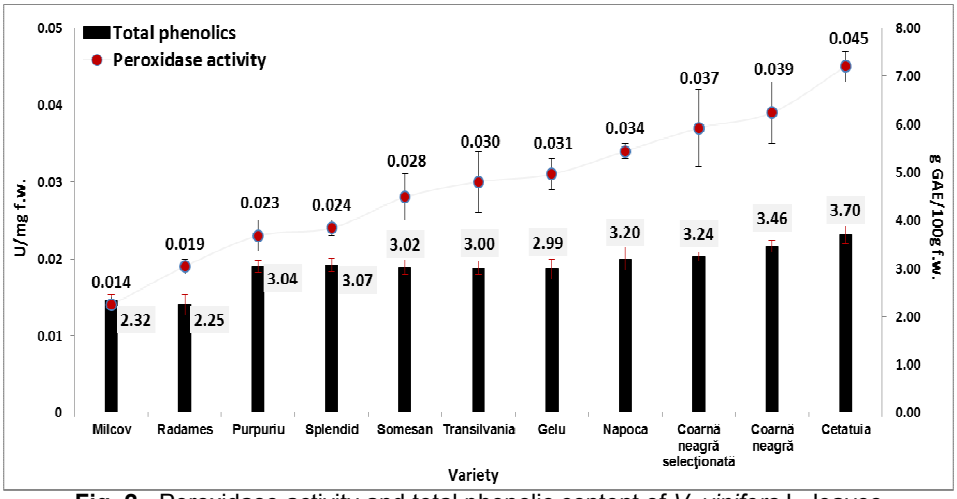

Fig. 2 - Peroxidase activity and total phenolic content of *V. vinifera* L. leaves

Table 2

Correlation (R^2) of phenolic content (TP) of leaves with peroxidase activity (PA) and the percentage of degraded pigments (%) after extract storage

V. vinifera L.	*TP*	*PA*	*% Carot.*	*% Chl a*	*% Chl b*	*% Total Chl*
TP	-					
PA	**0.8389**	-				
% Carot.	**0.8657**	0.7531	-			
% Chl a	**0.8162**	**0.8504**	**0.9058**	-		
% Chl b	0.6068	0.7046	0.3700	0.4128	-	
% Total Chl	**0.8282**	**0.9243**	**0.8453**	**0.8829**	0.5899	-

Note: Bolded values represent that correlation coefficients are statistically significant ($p<0.05$) in ANOVA test.

In the degradation process of assimilatory pigments, along with the activity of other specific enzyme (chlorophyllase, reductase) (Fang et al., 1998), Mg^{2+} degradation (Mg-dechelatase; phaeophytin formation) and the conversion of chlorophyll *b* to *a* (Matile and Hortensteiner, 1999), degradation of chlorophylls and carotenoids via peroxidase – phenolics– H_2O_2 system appears to be possible.

CONCLUSIONS

1. Percentage of carotenoids and chlorophylls in romanian *V. vinifera* L. varieties leaf extracts (at full flowering), degraded after 30 days of cold (6°C) and dark storage, varied in low limits, chlorophyll *a* being the most affected.

2. Experimental data obtained provide further evidence that peroxidase might be involved in chlorophyll bleaching in the presence of phenolic compounds, as an alternative route for the biodegradation of chlorophyll, and being able to mediate in a lesser extent leaf carotenoid degradation.

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