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MONITORING GRAPES' INFECTION WITH *Botrytis cinerea* BASED ON LACCASE ACTIVITY

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Abstract

Botrytis cinerea causes grey mould and is one of the most damaging disease occurring in vineyards worldwide, resulting in loss of grape production and wine quality. Laccase enzymes produced following fungal infection are involved in the oxidation of phenolic substances during the development of grey mould and has been used as an indicator of the degree of infection. Between the laccase activity of resulting juice of Botrytis cinerea infected bunches and the severity of infection observed in the vineyard a moderate to good correlation has been highlighted over the measurements carried out over veraison (BBCH81 – 88), and a strong correlation has been highlighted for the measurements carried out over the full ripening growth stage (BBCH 89). As a consequence of the degradation of polyphenolic compounds and the intensification of laccase activity in the infected musts, the change of chromatic characteristics, in the sense of increasing the values of the „Hue" parameter and the yellow color of musts was highlighted, in direct correlation with the oxidative processes.

Key words*: Botrytis cinerea, laccase, Vitis vinifera, polyphenolic compounds.*

INTRODUCTION

Gray mold caused by *Botrytis cinerea*, a necrotrophic fungus, is one of the most damaging grapevine diseases, along with downy mildew and powdery mildew. High values of relative humidity and a moderate temperature during the vegetative cycle of grapevine favour the development of the disease (Wan et al., 2015).

The infection with *Botrytis cinerea* on grapes determined the modification of their physico-chemical composition with important consequences on the quality of musts and wines (Bar Nun et al., 1988; Mayer, 2006).

Throughout the infection, numerous extracellular enzymes intervene, such as polygalacturonase (Johnston & Williamson, 1992; Have et al., 1998), xylanase (Brito et al., 2006), laccase (Grassin & Dubourdieu, 1984; 1989), as well as non-specific phytotoxic metabolites (microbial terpenes, botcinic acid) also known as virulence factors. As a results of their action, enzymatic degradation of cells and colonization of the host's substrate occurs (Breia et al., 2020). Among these enzymes, laccase (p-diphenol oxidoreductase) is considered to be the most important due to the negative effects on the quantity and quality of grape production.

This enzyme determines the protein oxidation of white wines and the color reduction of red wines as a result of the oxidation of phenolic compounds (Iland, 2000), the destruction of the aromatic and colouring substances in the grape berries (Kontek et al., 1997) affecting negatively the quality of wines (Grassin & Dubourdieu, 1989; Dewey et al., 2008).

Wines produced from grapes infected with *Botrytis* lack freshness and fruitiness and may present a predominant aromatic character of mold. Since correlations have been found between laccase activity and infection with *B. cinerea* (Roud et al., 1992; Dewey et al., 2008), laccase is considered an indicator of grape's infection with this fungus, activities higher than 3 U/mL is indicating the susceptibility for oxidative enzymatic

degradation of wine (Grassin & Dubourdieu, 1989).

The purpose of this study was to assess the risk of infection with *B. cinerea* in grapevine plantations based on the laccase activity and to monitor and evaluate from quantitative and qualitative point of view the grape harvest in order to apply an integrated management to combat gray mold.

MATERIALS AND METHODS

The biological material was represented by five wine grapevine cultivars: three varieties highly susceptible to gray mold - Chardonnay, Sauvignon blanc, Fetească albă, and two varieties with medium resistance - Fetească neagră and Cabernet Sauvignon.

Monitoring of the risk infection with *Botrytis cinerea* was performed during the growing season, special attention being paid to the stages of development represented by veraison, beginning of ripening (BBCH 81-88) and at harvest (BBCH 89) (Lorenz et al., 1995).

During beginning of ripening and full ripening phenophases grape bunches affected by *Botrytis* were hand harvested, the berries have been detached at the pedicel level and sorted according to the degree of infection into five categories of severity: 0 = no visible signs of infection/healthy grapes, 1 = up to 25% mycelium with visible spores, 2 = mycelium with spores affecting up to 50% of the berries, 3 = mycelium with spores affecting between 50% and 75% of the berries. Each sample unit was manually crushed into sterile plastic bag. The resulted must was analysed for soluble sugar content, total acidity, and laccase activity (Grassin & Dubourdieu, 1984; 1989).

The sugar content was measured by refractometry and expressed in g/L by conversion according to the OIV database (OIV-MA-AS313-01).

Total acidity was measured by titration using NaOH 0.1 N% phenolphthalein and the results were expressed in g/L^{-1} H_2SO_4 (OIV-MA-AS313-01 method).

Laccase activity was determined according to Grassin and Dubourdieu method (1984; 1989), slightly modified. The method is based on the use of syringaldazine as a substrate and the monitoring of the enzymatic reaction at 530 nm, where the extinction coefficient of oxidized syringaldazine is 65000 $nmol/ml^{-1}$.

In order to not inhibit the reaction, the phenolic compounds are fixed by using PVPP. A quantity of 1 mL of must containing laccase, as a result of the fungal attack with *Botrytis cinerea*, was filtered using a syringe containing 0.15 g of PVPP.

The results are expressed in units of laccase/mL, one unit of enzymatic activity being defined as the amount of enzyme that catalyzes the oxidation of a nanomole of syringaldazine per minute.

The production of laccase by *Botrytis cinerea* facilitates the infection process, the amount of laccase in the must and wine being considered an indicator of the degree of infection.

According to this method, musts with less than 0.2 UL/mL are considered to come from healthy grapes. The critical threshold is considered to have values higher than 3 UL/mL which will undergo to oxidative processes which lead to oxidative brownness of the wines.

In addition to the white varieties, the varieties for red wines were also analyzed in terms of chromatic parameters (color intensity, hue, % yellow, % red, % blue color), and polyphenolic potential, respectively, total polyphenol index, anthocyanin content and anthocyanin potential.

Chromatic parameters were determined according to the UV/Vis spectrophotometry method proposed by Glories (1984).

The phenolic potential was assessed by the standard ITV method (Cayla et al., 2002), based on the following analytical parameters: anthocyanins, total anthocyanins potential and total polyphenol index. The phenolic compounds were extracted from the grapes with an acidic aqueous solution. A quantity of 200 grape berries were randomly collected from each sample, weighed, then crushed and macerated for one hour at room temperature.

Fifty grams of the resulting must were treated with a solution composed of 85 mL of 0.1% HCl (37%) and 15 mL of ethanol (95%) and shacked for one minute every fifteen minutes. For obtaining clear solutions the coloured extracts were filtered through glass wool.

The sample were diluted in distilled water to 1/100, then the absorbance was measured at an optical density of 280 nm against a blank of

distilled water, and total polyphenolic index (TPI) was determined as follow:
Total polyphenol index (TPI) = OD 280 x 100 x [(weight of grape juice + 100)/ weight of grape juice].
Other samples were diluted 1/20 in 1% hydrochloric acid solution and absorbance was measured at OD 520nm against a blank of distilled water. The concentration of anthocyanin and the total anthocyanin potential were determined by using the following formulas:
Anthocyanins (mg/L) = OD 520 x 22.75 x 20
Total anthocyanins potential (mg/kg) = anthocyanins (mg/L) x [(weight of grape juice + 100)/ weight of grape juice].
Data was statistically analysed by applying the Dunnett test, in order to compare multiple groups with a single control group (Dunnett, 1964).

RESULTS AND DISCUSSIONS

Climatic conditions favorable for the *Botrytis cinerea* attack

Taking into account the average temperature during the period of leaf wetness and duration of leaf wetness, the quantity of precipitation and the relative humidity value, favorable conditions for the gray mold development were in June (127.7 hours), July (166.1 hours), and August (127.5 hours) of 2020, on the optimal temperature background of 22.7°C, 24.2°C, respectively 23.8°C, and in the months of June (95.4 hours) and August (68.8 hours) of 2021, based on the optimal temperature background of 19.4°C, respectively 21.4°C (Table 1).

Table 1. Climatic parameters favorable for the *Botrytis cinerea* development

Month	Medium temperature during the leaf wetness (°C)		Duration of leaf wetness (hours)		Conditions for *Botrytis cinerea* development (hours)	
	2020	2021	2020	2021	2020	2021
April	17.9	9.5	2.2	276.5	2.2	276.5
May	19.6	18.1	1.3	43.8	1.3	43.8
June	22.7	19.4	127.7	95.4	127.7	95.4
July	24.2	24.2	97.9	41.0	166.1	41.0
August	23.8	21.4	85.5	68.8	127.5	68.8
September	20.8	16.9	155.9	11.7	203.4	11.7

Monitoring of *Botrytis cinerea* infection risk through the attack degree evaluation

In accordance with the OIV methodology (2009) regarding the determination of grape resistance degree against the *Botrytis cinerea* fungus attack, in the climatic conditions of 2020 and 2021 and with the phytosanitary treatments applied, the grapes varieties studied presented a good and very good resistance, the attack degree on the grape production being very low. Thus, only a few isolated attacks of low intensity were reported, both on leaves and on grapes, these being mainly influenced by the sensitivity of the variety and the microclimate of the plantations (Table 2).

Table 2. Analysis of the resistance degree of *Vinifera* cultivars against the *Botrytis cinerea* attack

Variety	Veraison (BBCH 81-88)			Ripening, harvest (BBCH 89)		
	Frequency of attack (%)	Intensity of attack (%)	Degree of attack (%)	Frequency of attack (%)	Intensity of attack (%)	Degree of attack (%)
2020						
Chardonnay	3	0.16	0.01	19	6.3	1.2
Sauvignon	6	0.32	0.02	15	7.3	1.1
Fetească albă	15	5.7	0.86	16	5.9	0.95
Fetească neagră	8	5.63	0.45	20	2.8	0.56
Cabernet Sauvignon	10	0.86	0.09	15	4.7	0.71
2021						
Chardonnay	21	9.52	2	15	6.3	0.95
Sauvignon blanc	18	4.6	0.83	17	7.3	1.24
Fetească albă	12	5.45	0.65	18	5.9	1.06
Fetească neagră	8	0.45	0.04	10	2.55	0.25
Cabernet Sauvignon	10	0.86	0.09	11	4.7	0.52

Monitoring of *Botrytis cinerea* infection risk through the laccase analysis

The determinations made during the veraison phenophase hightlighed a moderate to good correlation (R ranged between 0.64 and 0.70) between the laccase activity of resulting juice from *Botrytis cinerea* infected bunches and the severity of infection observed in the vineyard. The laccase activity registered values ranging between 1.02 U/mL in case of 10% degree of the attack and 5.42 U/mL for 25% degree of attack (Figure 1a).
A very good correlation (R ranged between 0.6485 and 0.91) has been highlighted for the measurements carried out over the full ripening growth stage, at harvest. The laccase activity registered values ranging between 6.20 U/mL in case of 10% degree of attack and 16.24 U/mL for 75% degree of attack (Figure 1b).

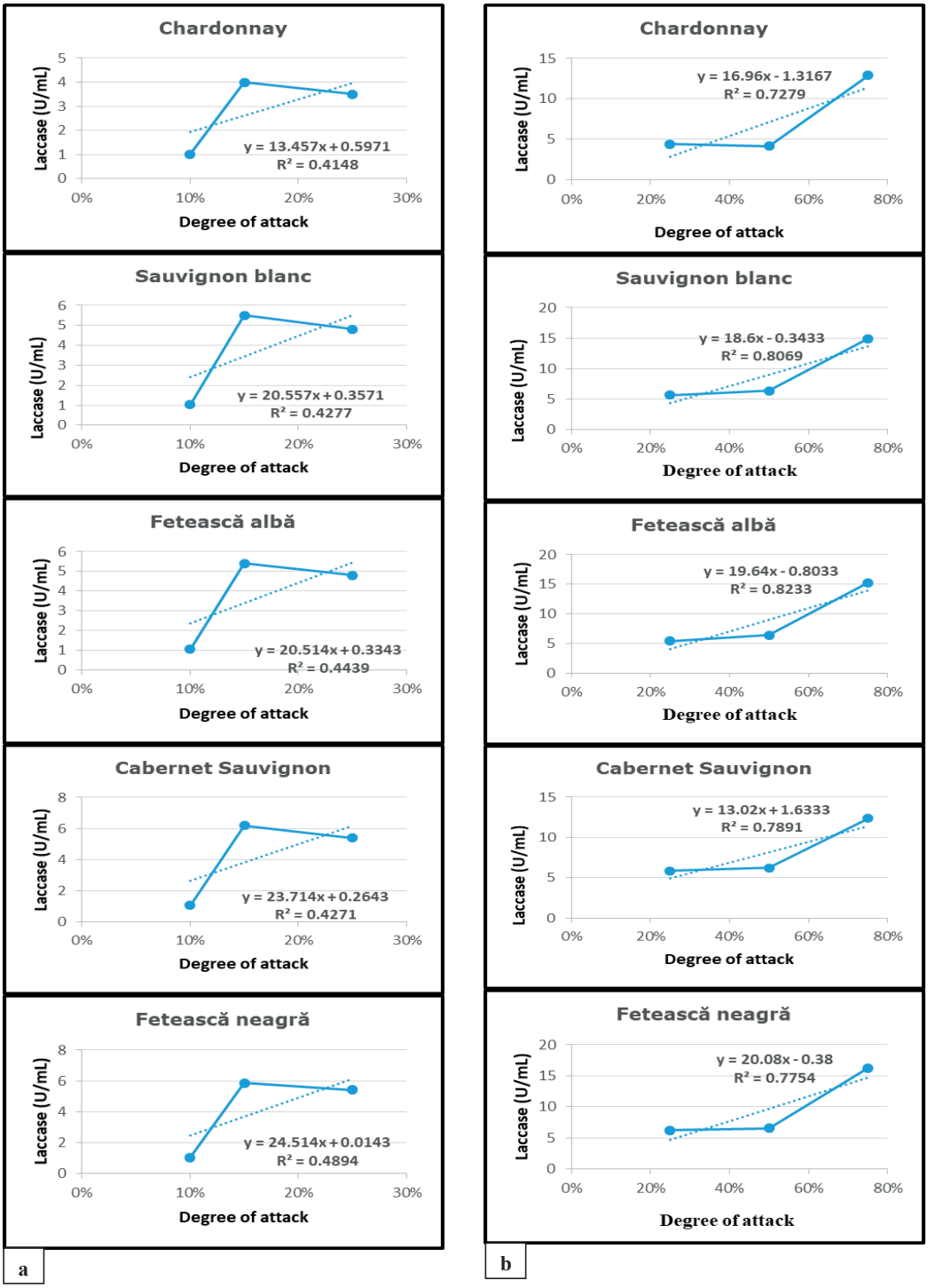

Figure 1. Level of laccase activity function of the attack degree with *Botrytis* at veraison (a) and at harvest (b)

Characterization of grapes and musts affected by the gray mold

In the veraison phenophase, in isolate cases, grapes affected by the *Botrytis cinerea* attack were identified, but with a degree of infection rate less than 25%. At harvesting, the attack frequency had values close to those recorded in the veraison phenophase but, the infection level increased up to 75%, as an effect of the change in the intensity of the attack. The soluble sugar content and total acidity of the must in the veraison phenophase registered values with max differences of +/- 5% compared with the control but even these small differences were statisticaly significant in terms of total acididy. At harvesting the sugar content from the must increase significative depending on the infection level of the grapes (Table 3).

Table 3. General physicochemical composition of grapes

Variety-Variant	Veraison				Harvesting			
	Sugar (g/L)	% +/-	Total acidity (g/L acid tartaric)	% +/-	Sugar (g/L)	% +/-	Total acidity (g/L acid tartaric)	% +/-
Chardonnay								
Control IA* 0%	114		14.32		209		7.79	
IA < 25%	114	0	14.35**	0.21	243**	16.27	7.95**	2.05
IA 25 – 50%	116	1.75	14.72**	2.79	247**	18.18	7.98**	2.44
IA 51 – 75%					252**	20.57	8.05**	-0.9
Sauvignon blanc								
Control IA* 0%	90		17.42		219		7.42	
IA < 25%	89	-1.11	18.2**	4.48	241**	10.05	7.95	7.14
IA 25 – 50%	90	0	18.53**	4.59	247**	12.79	7.08	-4.58
IA 51 – 75%					249**	13.7	6.98	-5.93
Fetească albă								
Control IA* 0%	115		14.24		200		6.44	
IA < 25%	116	0.87	14.56	2.23	237**	18.5	6.75**	4.81
IA 25 – 50%					241**	20.5	7.13**	10.71
IA 51 – 75%					249**	24.5	7.29**	13.2
Fetească neagră								
Control IA* 0%	162		8.22		219		7.16	
IA < 25%	164	1.23	8.12	-1.22	232**	5.94	7.2	0.56
IA 25 – 50%					268**	22.37	7.53**	5.17
IA 51 – 75%					272**	24.2	7.68**	7.26
Cabernet Sauvignon								
Control IA* 0%	154		9.18		213		7.2	
IA < 25%	153	-0.65	8.73	-4.9	232**	8.92	7.48**	3.89
IA 25 – 50%					238**	11.74	8.23**	14.31
IA 51 – 75%					242**	13.62	8.25**	14.58

**The mean difference is significant at the level P< 0.001 according to Dunnett t-tests.

Diffrences between -0.90% and 14.58% were observed in the total acidity values registred in the healthy must compared with the grapes affected by *Botrytis* attack.

The phenolic potential of grapes at harvesting

The phenolic potential of grapes changes significantly depending on the degree of harvest damage. The total polyphenol index (IPT) of the must resulted from the infected grapes is almost twice lower compared with the one registered in the healthy must. It was observed that amount of anthocyanins and the anthocyanin potential are reducing significatively depending on the attack degree with approximately 32-39%, at an attack intensity of 25%, reaching a reduction of 47-50% at an attack intensity of 51-75% (Table 4).

Table 4. Phenolic potential of grapes at harvest

Variety/ Attack intensity (AI)	Polyphenolic index		Anthocyanins (mg/L)		Anthocyanin potential (mg/kg)	
	Value	% reduction	Value	% reduction	Value	% reduction
Fetească neagră						
Control – AI 0%	55		258		774	
AI < 25%	38**	30,91	175**	32.17	525**	32.82
AI 25 – 50%	29**	47,27	131**	49.22	394**	49.10
AI 51 – 75%	26**	52,73	127**	50.78	382**	50.65
Cabernet Sauvignon						
Control – AI 0%	66		317		952	
AI < 25%	41**	37,88	193**	39.12	580**	39.08
AI 25 – 50%	39**	40,91	166**	47.63	498**	47.69
AI 51 – 75%	33**	50,0	166**	47.63	497**	47.79

**The mean difference is significant at the level P< 0.001 according to Dunnett t-tests.

The degradation of poliphenolic compunds in infected must can be explained by the increasing of laccase activity in the infected must with Botrytis from 5.86 U/mL 6.2 U/mL (Cabernet Sauvignon) and 6.2 U/mL (Fetească neagră), at an attack intensity of 25%, at values between 12.37 U/mL (Cabernet Sauvignon) and 16.24 U/mL (Fetească neagră) at an attack intensity of 51-75%. As a cosequence of the degradation of poliphenolic compunds and the intensification of laccase activity in the infected musts, the change of chromatic characteristics, in the sense of increasing the values of the „Hue" parameter and the yellow color of musts was highlighted, in direct correlation with the oxidative processes. The color intensity was also significatively affected by the *Botrytis cinerea* attack. The percentage of the yellow pigment in the must increases with the degree of fungal attack while the percentage share of the red pigment decreased (Table 5).

Table 5. Chromatic properties of grape juice

Variety/ Attack intensity (AI)	Color intensity	Color hue	% yellow pigments	% red pigments	% blue pigments
Fetească neagră					
Control - AI 0%	7.33	7.227	36.9	51.16	11.87
AI < 25%	9.99**	14.841**	51.25	34.53	14.21
AI 25 - 50%	9.20**	15.686**	53.17	33.90	12.93
AI 51 - 75%	8.83**	14.219**	51.53	36.24	12.33
Cabernet Sauvignon					
Control - IA 0%	8.27	6.082	33.98	55.86	10.16
AI < 25%	7.41**	12.345**	48.31	39.14	12.55
AI 25 - 50%	8.45**	14.487**	53.49	36.92	9.59
AI 51 - 75%	8.85**	14.308**	52.54	36.72	10.73

**The mean difference is significant at the level P< 0.001 according to Dunnett t-tests.

CONCLUSIONS

Botrytis cinerea causes gray mold disease on grapevine, determining the reduction of grape production and modification of wine quality. The infection with *Botrytis cinerea* on grapes determined the modification of their physico-chemical composition with important consequences on the quality of musts and wines As a consequence of degradation of the polyphenolic compounds and the intensification of laccase activity in the infected musts, the change of chromatic characteristics, in the sense of increasing the values of the „Hue" parameter and the yellow color of musts was highlighted, in direct correlation with the oxidative processes.

Laccase activity may be used as an indicator of grape's fungal infection with *Botrytis cinerea,* strong correlations being found between laccase activity and the infection produced by *B. cinerea*. Evidence of the presence of laccase in grapes and musts can be used to detect *Botrytis* infection and to monitor its evolution, as well as for the timely application of corrective measures.

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