

## Phytoplasma solani', Bois Noir 'Candidatus

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### Evaluation of the influence of Bois Noir 'Candidatus Phytoplasma solani', on the quality parameters of grape must and wine

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#### SUMMARY

2014-2016 . - In the period 2014-2016, a  
Bois noir (BN) - comparative study was conducted in IVE -  
Pleven to determine the effect of  
phytoplasma disease Bois noir (BN) on  
some indicators characterizing grape must  
and Chardonnay wine. Diseased and  
healthy vines are diagnosed by visual  
examination of symptoms, and the presence  
of phytoplasma has been proved by DNA  
identification with direct and nested PCR.  
A chemical analysis of the grape must  
was carried out and the main  
technological parameters were  
determined: sugars, glucose, fructose,  
titratable acids, tartaric and malic acids,  
pH. The chemical composition and  
organoleptic characteristics of the wines  
obtained have been analyzed. The  
comparative analysis of the experimental  
data for grape must and wines, vintage  
2014 and 2015, shows that there are no  
significant differences in the mean values  
of the main characteristic indicators, with  
the exception of tartaric acid, which has a  
significantly lower average value of the  
diseased vines variant compared to

BN  
,  
,  
:  
, Bois noir, 'Candidatus Phytoplasma solani'

*Tenericutes*;  
*Mollicutes*; *Acholeplasmatales*,  
*Acholeplasmataceae*; :  
'Candidatus Phytoplasma' (IRPCM, 2004).  
"Flavescence dorée" (FD)  
(BN).  
BN (EPPO, 2006;  
Sakaliev et al., 2007; Avramov et al.,  
2008). BN, 'Candidatus  
Phytoplasma solani,'  
(16SrXII-A)

(*Convolvulus arvensis*)  
(*Urtica dioica*) (Maixner, 2011).  
*Hyalesthes obsoletus* (Hemiptera,  
Cixiidae),  
2012 ..  
BN  
'Candidatus Phytoplasma solani', (Genov,  
2012).

healthy control. This study shows the lack of significant differences in the main indicators of grape must and wines obtained from diseased (BN) and healthy vines. The probable cause is that the disease affects individual shoots on which the grapes dries immediately after flowering or later before reaching of technological maturity.

**Key words:** grapevine, Chardonnay, wine, Bois noir, 'Candidatus Phytoplasma solani'

## INTRODUCTION

Grapevine yellows (GY) is a complex disease that causes changes in the leaves color and rolling downward of their periphery; drying the flowers or clusters; uneven ripening of the shoots and lack of lignification, giving them the appearance of rubber. Causing agent of GY are phytoplasmas – prokaryotic bacteria related to the Phylum: *Tenericutes*; Class *Mollicutes*; Order *Acholeplasmatales*, family *Acholeplasmataceae*; Genus: 'Candidatus Phytoplasma' (IRPCM, 2004). "Flavescence dorée" (FD) and "Bois noir" (BN) are of the greatest importance for the grapevine. Of these, only BN has been detected in Bulgaria up to now (EPPO, 2006; Sakaliev et al., 2007; Avramov et al., 2008). The cause of BN, 'Candidatus Phytoplasma solani', belongs to the Stolbur group (16SrXII-A) and besides the vine attacks a wide range of hosts such as vegetables – tomatoes, potatoes, carrots, celery; field crops and some weeds. The sources of infection in vineyards are considered to be the bindweed (*Convolvulus arvensis*) and the nettle (*Urtica dioica*) (Maixner, 2011). The main vector of stolbur in Europe is the leafhopper *Hyalesthes obsoletus* (Hemiptera, Cixiidae), which is a polyphage and prefers grass weeds. In 2012, a vineyard with Chardonnay variety from the Pleven region was found to be contaminated and identified the causative agent of BN 'Candidatus Phytoplasma solani,' (Genov, 2012).

The quality of wine is one of the most important criteria determining its price and profitability. Achieving high results in this direction requires above all a high quality raw material. Many are the abiotic and biotic factors that influence the quality characteristics of grape must and the resulting wines. The impact of most of them: soil, climate, exposure, load, formations, etc., has been the subject of a study by a number of researchers over the years. Also, the influence of some economically important diseases on the vine on the quantity and quality of grape harvest has also been studied. Relatively fewer studies have been conducted on the effects of systemic diseases, including viral and bacterial, which, in addition to yield, have an impact on the longevity of vineyards.

This study aimed to assess the impact of Bois noir disease on some quality characteristics of Chardonnay grape must and wine.

## MATERIAL AND METHODS

In 2014, a survey and marking of vines showing symptoms of grapevine yellows was carried out in a Chardonnay vineyard. Diseased and healthy vines were diagnosed by visual examination of symptoms, and the presence of phytoplasma agent was approved by DNA identification in direct and nested PCR, as described in the relevant protocols (Angelini et al., 2001).

In 2014 and 2015, when the grape reached technological maturity, samples were picked from experimental vines by variants (diseased and healthy vines) in three replications. The grape was processed according to the classic scheme for the production of white dry wines under the conditions of microvinification (Yankov, 1992). A chemical analysis of the grape must has been carried out and the main technological

Bois noir

2014

PCR,

(Angelini et al., 2001).

2014 2015

( )

(Yankov, 1992).

parameters are defined: sugars, glucose, fructose, titratable acids, tartaric and malic acids, pH. After clarification, the must was sulphited with 50 mg/dm<sup>3</sup> SO<sub>2</sub> and seeded with pure culture of dry yeast *Saccharomyces cerevisiae* strain Vitilevure B+C, 20 g/hl. Alcoholic fermentation was carried out at a temperature of 20° C and daily control. At the end of the process the wines were decanted and sulfated. The chemical composition of grape must and the resulting wines were analyzed according to the methods commonly used in wine-making practice (Ivanov et al., 1979). The organoleptic characteristics of the test samples are determined by a 100-point scale, according to the indicators: color, aroma, taste and general impressions (Tsvetanov, 2001; Prodanova, 2008). The comparative analysis of the results for the parameters characterizing the grape must and the obtained wines was made using the methods of the descriptive statistics, and the differences in the average values of the individual indicators were assessed by *Student test* (*t* - test).

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## RESULTS AND DISCUSSION

The quality of the raw material in 2014 reflects the unfavorable climatic conditions during the vegetation and the early autumn. The grapes are harvested by technological maturity. The content of sugars in the variant repetitions ranges from 208.0 g/L to 219.0 g/L. The titratable acids (7.73 - 8.33 g/L), the tartaric acid and the malic acid are slightly elevated and the pH values are normal for the white varieties (3.14 - 3.20). The analysis of the survey results in 2014 shows that there are no proven differences in the mean values of the main technological indexes of musts between variants (Table 1).

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1. , 2014 . 2015 .  
**Table 1. Chemical content of grape must, vintage 2014 and 2015**

Indexes (g/L)	/ Variants			
	2014		2015	
	BN Diseased BN	Healthy	BN Diseased BN	Healthy
Sugars	213.0 ± 0.00	214.0 ± 0.31	235.67 ± 3.28	229.67 ± 5.49
Glucose	89.27 ± 3.26	88.80 ± 0.79	95.58 ± 0.30	91.74 ± 1.33
Fructose	123.73 ± 3.26	125.53 ± 3.78	140.09 ± 3.50	137.93 ± 4.30
Titratable acids	8.03 ± 0.04	7.95 ± 0.19	6.40 ± 0.15	6.65 ± 0.13
Tartaric acid	4.11 ± 0.52	4.35 ± 0.02	2.78 ± 0.33	2.34 ± 0.37
Malic acid	6.39 ± 0.21	6.10 ± 0.15	4.19 ± 0.28	4.64 ± 0.40
	3.19 ± 0.01	3.16 ± 0.01	3.13 ± 0.01	3.12 ± 0.01

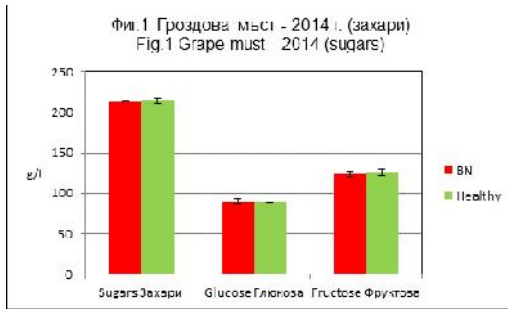
• [ ± SE] Mean ± Standard Error of mean

2015 . ( 1),  
 220.00 g/L  
 242.0 g/L BN.  
 6.15 g/L  
 ( . BN, . 2) 6.90 g/L ( .  
 , . 2).  
 2015 .  
 2014 .  
 ..

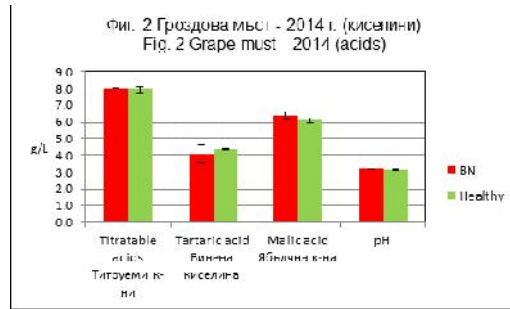
The chemical composition of grape must harvested in 2015 (Table 1) shows that the sugar content of the grapes in the test variants varies from 220.0 g/L in the case of healthy vines to 242.0 g/L in the BN variant. The grape harvest was performed at a technological maturity with a quantitative predominance of fructose.

The titratable acid values range from 6.15 g/L (var. BN, rep. 2) to 6.90 g/L (var. healthy, rep. 2). Of the analyzed organic acids in the must, the malic acid predominate over the tartaric acid in all experimental variants.

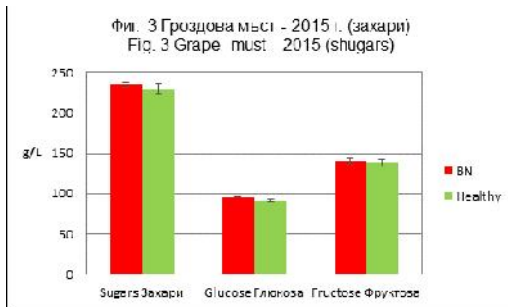
The analysis showed that the 2015 results are one-way with those from 2014 and there are no proven differences in the average values of the key technological indicators between the variants.



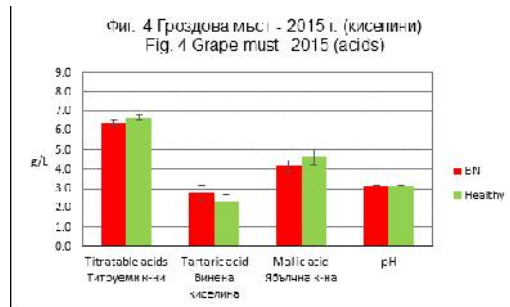
1. 2014



2. 2014 pH



3. 2015



4. 2015 pH

Year	Variant	Alcohol Content (%)	SFE (g/L)
2014	BN	12.88	18.26
	Healthy	12.74	18.96
2015	BN	12.51	19.80
	Healthy	12.66	18.96

The chemical composition of Chardonnay wines from the experimental variants, 2014 and 2015 vintages is presented in Table 2. The 2014 wines have a similar alcohol content ranging from 12.51% vol. to 12.88% vol. for the healthy variants and within a narrower range of 12.66% vol. % to 12.74% vol. with the BN variant. This is due to the small differences between the variants with respect to sugar in the grapes. For the complete course of alcoholic fermentation, with no deviations from its normal course are judged by the amount of residual sugars: from 1.54 g/L to 2.80 g/L in the BN variant and 1.68 g/L to 1.94 g/L in healthy. An important indicator of the composition of the wine is the content of the Sugar-free extract (SFE), which determines the density in their taste. For samples from the vintage 2014, its value is within the range typical for white wines of this variety - from 18.26 g/L to 18.90 g/L for BN and from 18.96 to 19.16 g/L in healthy ones. In the both variants, the

19.16 g/L

7.00 g/L

( )

(0.70 g/L),

(0.88 g/L).

(77.71 )

titratable acidity is above 7.00 g/L and determines the freshness in their taste. There are no significant differences between the acids of the test samples. The data show that, from the analyzed organic acids, the malic acid predominates several times over the tartaric acid.

The content of common phenolic compounds (CPC) of the samples varies within the narrow range typical of the variety. The lowest value is one of the wines of the healthy vines variant (0.70 g/L), and with the highest - wine of the diseased vine variant (0.88 g/L). The same wine has the highest NSE, titratable acidity and CPC, and is rated with the highest score in organoleptic analysis (77.71 points). Due to its aromatic and taste characteristics, the wine is defined as being typical of the variety, harmonious and balanced. In the other replications of the variants there is no relation between the studied chemical composition and the tasting evaluation.

## 2.

2014 . 2015 .

**Table 2. Chemical content of trial wines vintage 2014 and 2015**

(g/L)	/ Indexes	/ Variants			
		2014		2015	
		Diseased BN	Healthy	Diseased BN	Healthy
Alcohol vol. %		12.68 ± 0.03	12.73 ± 0.11	12.92 ± 0.14	12.74 ± 0.08
Sugars		21.07 ± 0.82	20.87 ± 0.12	2.73 ± 0.24	2.47 ± 0.18
Total extract		18.95 ± 0.45	19.08 ± 0.06	20.77 ± 0.27	20.57 ± 0.34
Sugar-free extr.		2.11 ± 0.37	1.79 ± 0.08	18.04 ± 0.47	18.10 ± 0.26
Titrateable acids		7.45 ± 0.19	7.38 ± 0.11	6.18 ± 0.09	6.15 ± 0.04
Volatile acids		0.44 ± 0.02	0.40 ± 0.02	0.48 ± 0.06	0.54 ± 0.03
Tartaric acid		1.78 ± 0.11	2.05 ± 0.21	1.32 ± 0.09	1.74 ± 0.09
Malic acid		5.48 ± 0.21	5.25 ± 0.32	3.61 ± 0.31	4.15 ± 0.23
Total phenol comp.		0.84 ± 0.02	0.80 ± 0.05	0.46 ± 0.02	0.39 ± 0.05
		3.03 ± 0.01	3.03 ± 0.01	3.09 ± 0.03	3.04 ± 0.01
Tasting evaluation		77.00 ± 0.36	76.85 ± 0.14	76.33 ± 1.24	75.57 ± 0.62

• [ ± SE] mean ± Standard Error of mean







(2007).

Garau et al.

regarding the lack of influence of the disease on the chemical composition of the grape must are in accordance with those of Garau et al. (2007). One of the main reasons for the decline in yield in diseased vines is the drying of the grapes, some of which are still inflorescence phase. Our goal was to find out if the other bunches formed a different chemical composition and quality under the influence of the disease, and whether this impact affects the chemical composition and organoleptic characteristics of the wines obtained.

## CONCLUSIONS

1.

2014 2015

1. The comparative analysis of the chemical data for grape must from the harvest 2014 and 2015 shows that there are no significant differences in the mean values of the main indicators between the variants of diseased and healthy vines;

2.

2014 2015

2. The wines, vintage 2014 and 2015, also have no significant differences in the average values of the indicators except for the tartaric acid, which has a significantly lower average value in the wine from diseased vines as compared to the healthy control.

3.

BN

3. The probable reason of the absence of significant differences in the main indicators of grape must and wine obtained from BN and healthy vines is that the disease affects individual shoots to which the grapes dries immediately after flowering or at a later stage before reaching a technological maturity.

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## Occurrence and diagnostic of grapevine yellows on Chardonnay variety in the region of Pleven, Bulgaria

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### SUMMARY

2012  
"Bois noir (BN) "  
nested PCR,  
(RFLP)  
BN  
'*Candidatus Phytoplasma solani*' (16S rXII-A stolbur subgroup)  
RFLP  
tuf -  
PCR/RFLP  
*TaqMan* real-time PCR  
BN

In 2012, six years after the first report of the Stolbur phytoplasma, as a cause of Bois noir (BN) on grapevine in Bulgaria, vines with symptoms of grapevine yellows were found in a vineyard with Chardonnay variety. Phytoplasma detection and characterization were carried out by means of DNA amplification with nested PCR, followed by restriction fragments length polymorphism (RFLP) analysis of amplicons. Results showed that three of the four samples were collected from BN diseased grapevines. This was the first detection of '*Candidatus Phytoplasma solani*' (16S rXII-A stolbur subgroup) in grapevine tissue in the region of Pleven.

The conducted RFLP analysis showed that the isolates belong to tuf-B type, that is common for isolates from Eastern Europe. The results from detection with conventional PCR/RLFP assays were confirmed by *TaqMan* real-time PCR. Over the next years the spatial spread of the disease in the vineyard has been traced and new samples were tested and confirmed the diagnosis of Bois noir and

'*Candidatus* Phytoplasma solani'..  
:  
, Bois noir,  
'*Candidatus* Phytoplasma solani',  
PCR/RLFP , *TaqMan* PCR

*Candidatus* Phytoplasma solani.  
**Key words:** grapevine yellows,  
Bois noir, '*Candidatus* Phytoplasma  
solani', PCR/RLFP assays, *TaqMan* real-  
time PCR

(GY) -  
,  
-  
,  
*Mollicutes*, *Acholeplasmataceae*,  
: '*Candidatus* Phytoplasma' (IRPCM.  
2004).  
(Doi et al., 1967)  
( mycoplasma-  
like organisms – MLOs),  
(Kovachevsky et al., 1995).

## INTRODUCTION

Grapevine yellows (GY) are serious diseases, spread worldwide in vine growing countries. They are caused by phytoplasmas – small bacteria that refer to the Class Mollicutes, family Acholeplasmataceae, Genus: '*Candidatus* Phytoplasma' (IRPCM, 2004). After their discovering in the 1960s (Doi et al., 1967), they were originally called mycoplasma-like organisms (MLOs) because of their similarity to mycoplasma-causing diseases in animals (Kovachevsky et al., 1995). Phytoplasmas lives in the phloem tissues of plants and is transferred by insects suckling plant sap.

: *Flavescence dorée* (FD),  
FD  
'*Candidatus* Phytoplasma vitis' ( -  
16Sr V -  
)  
*Scaphoideus titanus* Ball.  
*Bois noir* (BN)  
e  
(STOL) ( -  
XII 16Sr )  
*Hyalesthes*  
*obsoletus* Signoret.

GY present in Europe are essentially two: *Flavescence dorée* (FD), a quarantine disease in the European Community, associated to FD phytoplasma - '*Candidatus* Phytoplasma vitis' (phylogenetically belonging to 16SrV ribosomal group). It is specifically transmitted by *Scaphoideus titanus* Ball. The disease shows an epidemical behaviour.

*Bois noir* (BN), associated to Stolbur (STOL) phytoplasma (phylogenetically belonging to 16SrXII ribosomal group). It is specifically transmitted by *Hyalesthes obsoletus* Signoret. Usually the disease shows an endemic behaviour.

. FD -  
20  
, BN  
(Borgo et al., 2005;  
Borgo and Angelini, 2007; Bertaccini et al.,  
2008; Belli et al., 2010). FD

In Italy both diseases occur. FD and its vector have been spread to North and Central Italy in the last 20 years, while BN is common in all Italian regions (Borgo et al., 2005; Borgo and Angelini, 2007; Bertaccini et al., 2008; Belli et al., 2010). Arrival of FD in Italy caused huge damages to Italian viticulture, especially

BN  
*H. obsoletus* (EPPO, 2006; Sakalieva et al., 2007; Avramov et al., 2008).  
 FD  
*S. titanus* (Avramov et al., 2011).  
 FD  
*S. titanus* 2003  
 (Duduk et al., 2003; Magud and Toševski, 2003; Duduk et al., 2004),

FD

GY.

GY

FD.

GY

nested real-time PCR.

2012

BN

due to the lack of knowledge on the correct disease control strategies. Preventive control strategies include early diagnosis of the disease, carried out by field observation and molecular diagnosis.

In Bulgaria the presence of BN and its vector *H. obsoletus* has been reported (EPPO, 2006; Sakalieva et al., 2007; Avramov et al., 2008). FD have not been yet detected, but its vector *S. titanus* was recently found in the country (Avramov et al., 2011). Moreover, FD and its insect vector have been present at least since 2003 in the nearby Serbia (Duduk et al., 2003; Magud and Toševski, 2003; Duduk et al., 2004), where they caused very serious economical losses to vine growers. Therefore, there is the serious and real risk that FD spreads out in Bulgaria soon, causing epidemics in vineyards. Thus, a strict surveillance and a joint effort by all Bulgarian teams working on grapevine are necessary.

The main purpose of this work was molecular detection and identification of phytoplasmas in samples from grapevines expressing symptoms of GY. It is very precious in order to face the problem of GY in the grapevine germplasm collection at IVE and, more generally, in Bulgaria, and to allow the survey of the country for the possible entry of FD.

To be achieved the purpose, activities including field observation of GY symptoms and molecular diagnosis of phytoplasmas were implemented. This comprised recognizing symptoms of grapevine phytoplasma diseases; collecting and maintenance of samples; isolation of DNA from samples; detection of grapevine phytoplasmas with nested and real-time PCR.

## MATERIAL AND METHODS

A routine survey for identification of GY symptoms on different varieties in vineyards near Pleven were performed in 2012, six years after the first report of the Stulbur phytoplasma, as a cause of BN on

GY

grapevine in Bulgaria.

Vines with symptoms of grapevine yellows (changes the color of leaves, downward rolling of leaf edges to form a characteristic triangular shape (Figure 1), lack of lignification – “rubber shoots” (Figure 2), and flower or cluster shriveling) were found in a vineyard with Chardonnay variety. Distinction of grapevine yellows symptoms from different symptoms associated to other biotic (such as viruses and leafhopper damages) or abiotic pathologies were also implemented. The samples were maintained in fridge until they were processed



**Fig. 1. Symptoms of Grapevine yellows on Chardonnay variety – a characteristic triangular shape of the leaves.**



**Fig. 2. Symptoms of Grapevine yellows on Chardonnay variety – lack of lignification “rubber shoots”.**

CTAB  
Angelini et al. (2001)

Molecular analyses were carried out for detecting presence of phytoplasmas in four Bulgarian grapevine samples. DNA extractions from plant tissues and insects were performed by using the CTAB procedures as described

PCR RFLP  
 PCR  
 PCR P1/P7 (Deng and Hiruki, 1991; Smart et al., 1996).  
 1:50,  
 -PCR :  
 16r758f/M23Sr (Gibb et al., 1995; Padovan et al., 1995); R16(V) F1/R1 R16 (I)F1/R1 (Lee et al., 1994),  
 16SrV and 16SrI/16SrXII  
*tuf*  
 16SrI/16SrXII  
 PCR,  
 Tuf1/rTuf1 fTufAy/rTufAy (Schneider et al., 1997; Langer and Maixner, 2004).  
 1% -  
 GelRed™ (Biotium)  
 UV  
 (RFLP)  
*Taq* I  
 16r758f/M23Sr *Hpa* II  
 fTufAy/rTufAy  
 (Angelini et al., 2001; Langer and Maixner, 2004; Botti and Bertaccini, 2007). RFLP  
 13%  
 (PAGE),  
 GelRed™ (Biotium)  
 UV

in Angelini et al. (2001). Phytoplasma detection and characterization were carried out by means of DNA amplification with nested PCR, followed by restriction fragments length polymorphism (RFLP) analysis of amplicons. Ribosomal DNA was amplified in nested-PCR procedure with universal and specific primer pairs for phytoplasmas. The first direct PCR was performed with universal primer pair P1/P7 (Deng and Hiruki, 1991; Smart et al., 1996). The obtained amplicons, after dilution 1:50 in water, were used as target DNA in three different nested-PCR amplification: with 16r758f/M23Sr primers (Gibb et al., 1995; Padovan et al., 1995) for universal detection of phytoplasmas; with R16(V)F1/R1 and R16(I)F1/R1 primer pairs (Lee et al., 1994), which are specific for phytoplasmas belonging to 16SrV and 16SrI/16SrXII groups, respectively.

Primers targeting the nonribosomal *tuf* gene of phytoplasmas belonging to 16SrI/16SrXII groups were also used in direct and nested PCR using primer pairs fTuf1/rTuf1 and fTufAy/rTufAy respectively (Schneider et al., 1997; Langer and Maixner, 2004). The PCR products were analyzed by electrophoresis in 1% agarose gel, stained with GelRed™ Nucleic Acid Gel Stain (Biotium) and visualized in UV transilluminator.

The phytoplasma group in the positive DNA samples was determined by means of RFLP analyses after enzymatic digestions with the restriction endonucleases *Taq* I for 16r758f/M23Sr amplicons and *Hpa* II for fTufAy/rTufAy amplicons (Angelini et al., 2001; Langer and Maixner, 2004; Botti and Bertaccini, 2007). The products of digestions were processed on 13% polyacrylamide gel electrophoresis (PAGE), stained with Gel Red™ Stain and visualized in UV transilluminator. (

All DNA samples were diluted 1:50



1:50 1:500  
*TaqMan* real-time PCR (Angelini et al., 2007).  
 96-  
 Bio-Rad 25µl  
 5µl 2X Platinum qPCR Supermix-UDG (Invitrogen).  
 0.15µM,  
 0.2µM.  
 3 min 50°C  
 UDG 3 min 95°C 50  
 15 s 95°C  
 1 min / 60°C  
 (Angelini et al., 2007).

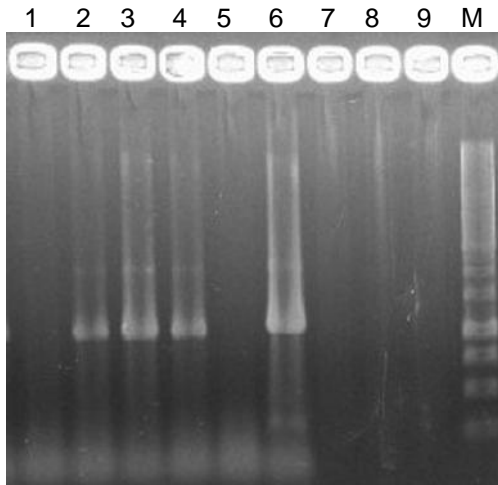
and 1:500 prior to amplification and were put to test by *TaqMan* real-time PCR (Angelini et al., 2007). Reactions were performed in 96-well plates using Bio-Rad thermal cycler in 25µl total volume, including 5µl of DNA and 2X Platinum qPCR Supermix-UDG (Invitrogen). The concentration of primers was 0.15µM and of the probe 0.2µM. The program of thermal cycler included a decontamination step of 3 min at 50°C for optimal UDG enzymatic activity, followed by 3 min at 95°C and 50 cycles of two-step protocol including 15 s of denaturation at 95°C and 1 min of annealing/extension at 60°C (Angelini et al., 2007).

## RESULTS AND DISCUSSION

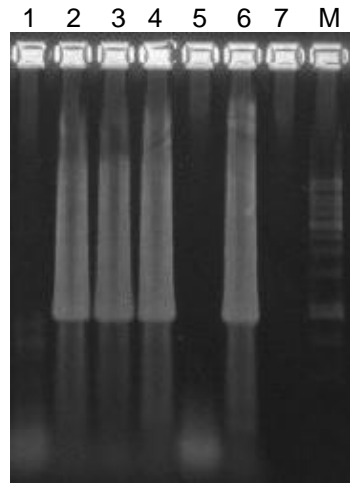
PCR  
 16r758f/M23Sr  
 1050 ( )  
 ( 3). PCR  
 R16(V)F1/R1 R16(I)F1/R1  
 R16(I)F1/R,  
 16SrI/16SrXII  
 BN  
*tuf* (Tuf1/rTuf1  
 16Sr  
 I XII  
 ( 4).

The performed nested PCR with universal for phytoplasma primers 16r758f/M23Sr yielded an amplification product of approximately 1050 base pair (bp) in three of the four DNA samples isolated from vines with symptoms of grapevine yellows (Figure 3). PCR reactions with specific primer pairs R16 (V) F1/R1 and R16 (I) F1/R1 gave positive result only at R16 (I) F1/R, which determines isolates as belonging to 16SrI/16Sr XII groups. Results showed that three of the four Bulgarian samples were collected from BN diseased grapevines.

The polymerase reactions with the primers targeting the amplification of the nonribosomal *tuf* gene (Tuf1/rTuf1 and fTufAy/rTufAy), of the phytoplasmas belonging to 16Sr I and XII groups showed positive results in the same three samples (Figure 4).



**Fig. 3. Agarose gel showing the fragments from nested PCR with primers 16r758f/M23Sr.** Lines - 1-4 - samples 59, 60, 61, 62; 5 - B (negative control); 6 - positive control; 7-9 - empty; empty wells; M - 1 Kb ladder.



**Fig. 4. Agarose gel showing the fragments from nested PCR with primers fTufAy/rTufAy.** Lines - 1 - 4 - samples 59, 60, 61, 62;; 5 - B (negative control); 6 - positive control; M - 1 Kb ladder.

. 3.  
**16r758f/M23Sr.** - 1-4 - samples 59, 60, 61, 62; 5 - B (negative control); 6 - positive control; 7-9 - empty; empty wells; M - 1 Kb ladder.

. 4.  
**fTufAy/rTufAy.** - 1 - 4 - samples 59, 60, 61, 62;; 5 - B (negative control); 6 - positive control; M - 1 Kb ladder.

RFLP  
 PCR  
 16r758f/M23Sr *Taq* I  
 16SrXII-A  
 (5).  
 Langer *Hpa*II  
 Mainxner (2004),  
 stolbur  
 aster yellows  
 stolbur,  
 (a, b, c)  
 RFLP  
 b (6).  
 Schneider et al. (1997), *Hpa* II - RFLP

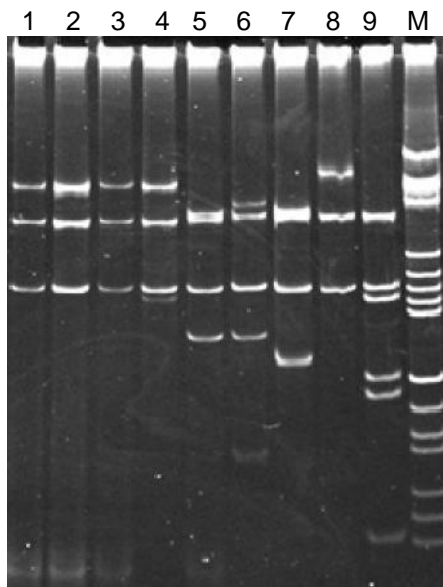
The results of RFLP analysis of amplicons, obtained from PCR with the universal primer pair 16r758f/M23Sr and *Taq* I (Figure 5), showed that the DNA profiles of the three positive samples were phytoplasmas, belonging to the 16SrXII-A group of "stolbur".

According to Langer and Mainxner (2004), digestion with *Hpa*II allows the differentiation of stolbur and aster yellows isolates and leads to three different restriction profiles (Type a, b, c) of stolbur that can be detected in grapevine, herbaceous plants and the vectors. In our case the profiles obtained from the enzymatic digestion of *tuf* amplicons by *Hpa* II in the RFLP assay were the same of the three samples and corresponded to the *tuf*-type b profile (Figure 6). These results agree also with those obtained from Schneider et al. (1997) of *Hpa* II - RFLP profiles of a fragment, amplified

fTufAy/rTufAy.  
 PCR/RFLP  
*TaqMan* real-time  
 PCR,

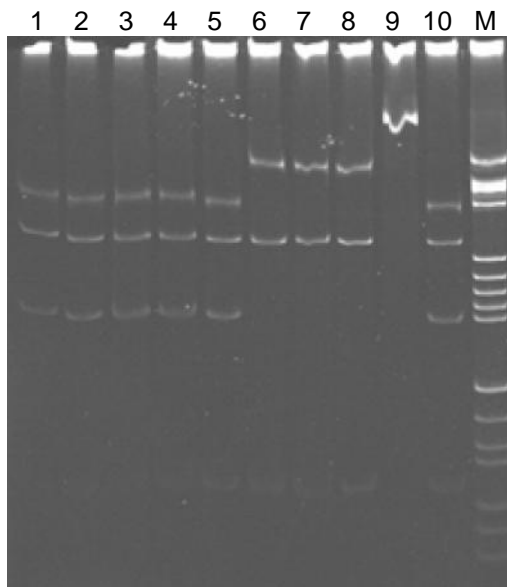
from "stolbur" phytoplasmic DNA with primer fTufAy/rTufAy.

The results from detection with conventional PCR/RFLP assays were confirmed by *TaqMan* real-time PCR (Angelini *et al.*, 2007), which also showed the presence of phytoplasmic DNA in the samples tested.



**Fig. 5. RFLP profiles of nested PCR amplicons (16r758f/M23Sr), digested with *TaqI* and divided in 13% PAGE. Lines - 1 - 3 - 60, 61, 62; 4 - 9 - phytoplasma reference isolates, subgroups 16SrXII-A; I-B; I-C; II-C; V-A; X; M - DNA ladder (pBR322 *Hae* III digest).**

**5. RFLP PCR (16r758f/M23Sr), *TaqI* 13% PAGE.**  
 - 1 - 3 60, 61, 62;  
 4 - 9 - 16SrXII-A; I-B; I-C; II-C; V-A; X; 10 - 34; 11 - (pBR322 *Hae* III).



**Fig. 6. RFLP profiles of nested PCR amplicons (fTufAy/rTufAy), digested with *HpaII* and divided in 13% PAGE. Lines - 1 - 5 samples 29, 30, 31, 32, 33; 6 - 8 - 60, 61, 62; 9 - phytoplasma reference isolate subgroup 16SrI-C; 10 - 34; M - DNA ladder (pBR322 *Hae* III digest).**

**6. RFLP PCR (fTufAy/rTufAy), *HpaII* 13% PAGE.**  
 - 1 - 5 29, 30, 31, 32, 33; 6 - 8 - 60, 61, 62; 9 - 16SrI-C; 10 - 34; 11 - (pBR322 *Hae* III).

BN  
 'Candidatus Phytoplasma solani'.

Over the next years the spatial spread of the disease in the vineyard has been traced and new samples were tested and confirmed the diagnosis of Bois noir and *Candidatus Phytoplasma solani*.

" " (16SrXII-A),  
 " " (BN)  
 2006 . (EPPO,  
 2006; Sakalieva et al., 2007; Avramov et al.,  
 2008).

- *Hyalesthes obsoletus* Signoret  
*Reptalus panzeri*

'*Candidatus* Phytoplasma solani' (16S rXII-A  
 stolbur subgroup)

RFLP

*tuf-b*

(Maixner, 2011).

## CONCLUSIONS

As a result of the molecular analysis carried out in three of the Chardonnay variety samples from the Pleven region, a phytoplasma of the "stolbur" group (16SrXII-A), a causative agent of "bois noir" on the grapevine, was found. The disease was first reported in Bulgaria in 2006 (EPPO, 2006, Sakalieva et al., 2007, Avramov et al., 2008). So far, only insect vectors - *Hyalesthes obsoletus* Signoret and *Reptalus panzeri* have been reported in the Pleven region and as far as we know that was the first detection of '*Candidatus* Phytoplasma solani' (16S rXII-A stolbur subgroup) in grapevine tissue in the region of Pleven. The Bulgarian isolates were determined to belong to *tuf-b* type, which is common in Eastern Europe countries (Maixner, 2011).

As a further result, it was complemented the possibilities of the IVE plant protection group to study and protect sanitary state of the grapevine germplasm in Bulgaria. This collaboration will lead to a better control and protection against GY epidemics in Bulgaria and in particular in the IVE grapevine germplasm collection.

## ACKNOWLEDGEMENTS

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(CREA-VIT),

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COST-STSM-ECOST-STSM-FA0807-240912-018471 COST Action FA0807 "

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## Study of resveratrol content in grapes and wine of the varieties Storgozia, Kaylashki Rubin, Trapezitsa, Rubin, Bouquet and Pinot Noir

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### SUMMARY

50mg/dm<sup>3</sup>  
SIHA RUBINO CRU.  
25-28  
(1,14-2,41 mg/dm<sup>3</sup>  
3,53mg/dm<sup>3</sup>  
1,87-3,27 mg/dm<sup>3</sup>,  
3,22mg/dm<sup>3</sup>)  
Vitis vinifera

The chemical composition of grapes and wine was analyzed. The obtained grapes indicators were a precondition for making quality red regional wine. The grapes from each variety were mashed and vinified. The resulting grape pulp was sulphitated with 50 mg/dm<sup>3</sup> of sulfur dioxide. After 2 hours the medium was inactivated with selected, dry yeast strain SIHA RUBINO CRU. The alcoholic fermentation was carried out at 25-28 °C. The values of the chemical indicators and resveratrol (for grapes 1,14-2,41 mg/dm<sup>3</sup> at 3,53 mg/dm<sup>3</sup> for the Pino noir control and for wine respectively 1,87-3,27 mg/dm<sup>3</sup>, at the control of 3,22 mg/dm<sup>3</sup>) of the studied varieties in the grapes and wine were comparable to those of *Vitis vinifera* grown in the region of Pleven.

**Key words:** grapes, wine, chemical composition, resveratrol, intraspecific and interspecific hybridization

1990

## INTRODUCTION

Resveratrol as a chemical compound in grapes and wine has been paid attention by the scientific community since 1990 when the compound has been shown to have cardioprotective properties. Resveratrol as a gift of nature has a wide range of health-promoting effects, including antibacterial, antifungal, antioxidant, anti-inflammatory, cardioprotective and anti-tumor functions. It protects vine from UV rays, viruses, bacteria, fungi and acts as a natural antibiotic (Fartsov et al. 2013).

(Fartsov et al. 2013 .).

Resveratrol is an extremely rare substance. In addition to grapes and wine, certain amounts of it are contained only peanuts and some rare species of forest fruits such as blueberries, blackberries, strawberries, mulberries and cherries.

Chemically, it is a type of natural polyphenol produced by some plants (as in the skin of red grapes) when they are attacked by pathogens such as bacteria or fungi. Therefore, that substance is the plant response to overcoming the unfavorable external conditions and possesses strong antibiotic and fungicidal properties, respectively.

Resveratrol is a powerful antioxidant. It has a high ability to capture and block the free oxidative radicals and prevents cells from malignant alterations.

The substance has a number of benefits: anticancer and antiviral effect, it increases the energy and improves heart function, it normalizes blood glucose levels and stimulates the nervous system and significantly improves the sport achievements. Another very interesting feature of resveratrol is that it stimulates the producing of a specific gene in the cells, the so called "anti-age" gene by the scientist that participates in the mechanisms for repairing damaged DNA



(Fartsov et al., 2013).

( ) - 2016 .  
(Ivanov, 2016).  
( , , , ) .  
0,2ha  
3,00m/1,20m,  
SO4.  
3535-4500<sup>0</sup> .  
20<sup>0</sup> . 20<sup>0</sup>  
30<sup>0</sup> ,  
(Haygarov, 2012).

and in fat metabolism and thus improves the health of the whole organism. It creates prerequisites for extending life. Resveratrol is an antidepressant containing vitamin E and reduces bad cholesterol. It is also thought to block the formation of COX-2 enzyme that promotes the development of colon cancer (Fartsov et al., 2013).

The objective of this study was to determine resveratrol content in grapes and wine from varieties selected at the Institute of Viticulture and Enology - Pleven.

## MATERIAL AND METHODS

The study was carried out at the Institute of Viticulture and Enology (IVE) - Pleven, vintage 2016. The object of the study were the red wine grapevine varieties Storgozia, Kaylashki Rubin, Trapezitsa, Rubin and Bouquet, selected at IVE-Pleven by way of intraspecies and interspecies hybridization (Ivanov, 2011; 2016). They are distributed in separate micro-regions all over the country (Pleven, Sadovets, Barkach, Suhindol, Pavlikeni, Brestovitsa, Karnobat, Burgas, Blagoevgrad and Sandanski).

The vineyard was located at the experimental base of IVE, on an area of 0.2 ha of each studied variety. The vineyards were fruit-bearing, grown on medium stem training system, planting distance 3.00m/1.20m, Berlandieri x Riparia SO4 rootstock. During the vegetation period, standard agrotechnical and plant protection operations were performed.

The climate is continental, characterized by cold winters and hot summers. The sum of the average daily temperatures during the vegetation period varied from 3535-4500<sup>0</sup> . The average temperature of the warmest month was always above 20°C. There were no temperatures below 20°C and over 30°C to make the physiological processes in the grapevine difficult (Hyagarov, 2012).

(  
 ) (Ivanov, 2016).

12375;  
 V -2-15  
 Vitis amurensis;

12375;  
 Vitis vinifera,  
 (Videnova,  
 2017).

(Ivanov, 1981; Yankov, 1992;  
 Mandjukov, 2010):  
 (50 mg/kg SO<sub>2</sub>),  
*Saccharomyces cerevisiae* (20 g/hl),  
 (25-28 ),  
 30 kg.

- These data showed that Pleven region has suitable conditions for obtaining quality red grapes as a raw material for the production of red regional table wines.

- The soil is leached chernozem on a loess basis, suitable for grapevine growth.

- The interspecific varieties Storgozia, Kaylashki Rubin and Trapezitsa and the intraspecific variety Bouquet have increased resistance to stress factors (low winter temperatures and downy mildew (Ivanov M., 2016).

- The parental forms of the studied varieties are as follows:

Storgozia – Bouquet x Villard Blanc 12375;  
 Kaylashki Rubin – Pamid x Hybrid V -2-15  
 Gamay Noir Vitis amurensis;  
 Trapezitsa – Dunavska Gamza Marseilles early;  
 Dunavska Gamza – Bouquet Villard Blanc 12375;  
 Rubin – Nebbiolo x Syrah;  
 Bouquet – Mavrud x Pinot Noir.

- Pinot Noir of *Vitis vinifera* was used for control as according to the literature data it had the highest resveratrol content (Videnova, 2017).

- The dynamics of grapes ripening was followed. The grapes were picked upon reaching technological maturity and vinified at the Experimental wine cellar of IVE-Pleven.

- Vinification was carried out in accordance with the classical technology for dry red winemaking at the Experimental wine cellar of IVE=Pleven (Ivanov, 1081, Yankov et al., 1992): removing the berries, crushing, sulfating (50 mg/kg SO<sub>2</sub>), adding pure culture dry wine yeast *Saccharomyces cerevisiae* (20 g/hl), fermentation temperature (25-28 ), separation of solid particles, malic-lactic acid fermentation, further sulfating, storage. The grapes from each variety were 30 kg.

- The main chemical indicators of the grape pulp composition and the obtained experimental wines from the studied varieties were determined by the general methods used in winemaking (Ivanov et

(Ivanov et al., 1979; Chobanova, 2007; Pandeliev et al., 2010).

HPLC  
2006)

(Anli et al.,

100-

(Prodanova, 2008;

Marni Old, 2016)

Microsoft office Excel

al., 1979; Chobanova, 2007; Pandeliev et al., 2010).

For the determination of the resveratrol in the grapes, two-fold extraction of the grape pulp with methanol was performed for each sample. High-pressure liquid chromatography (HPLC) according to the modified method was used (Anli et al., 2006) for the determination of resveratrol in the grape and wine samples.

A 100-score scale was used for evaluating the organoleptic properties of the wines (Prodanova, 2008; Marni Olt, 2016) as data were processed with Microsoft Office Excel and the standard deviations were specified.

## RESULTS AND DISCUSSION

The dynamics of grapes ripening and the vintage date for each of the studied varieties since the beginning of August were followed. Data on the chemical composition and vintage date are resented in Table 1.

1. **2016**  
**Table 1. Chemical composition of grapes, vintage 2016**

Grape variety	Storgozia	Kailashky rubin	Trapezitsa	Rubin	Bouquet	Pino noar
Date of harvest	10.09.	11.09.	30.08.	31.08.	12.09.	30.08.
Reducing sugars, g/dm <sup>3</sup>	229	223	200	233	210	235
Titratable acids, g/dm <sup>3</sup>	7,55	6,68	5,55	7,00	6,00	6,53
Tartaric acid, g/dm <sup>3</sup>	5,70	5,78	3,56	5,44	6,11	1,39
Malic acid, g/dm <sup>3</sup>	4,05	5,26	5,19	4,92	3,62	3,26
	3,56	3,29	3,21	3,46	3,36	3,26
Resveratrol, mg/dm <sup>3</sup>	1,21	1,29	1,58	2,41	1,14	3,53

(21-23%)

Upon reaching technological maturity (21-23%) the grapes were

(Marinov, 1990; Yankov, 1992).

50 mg/dm<sup>3</sup>

2

SIHA RUBINO CRU.

25-28

1000

30mg/dm<sup>3</sup>

200g/dm<sup>3</sup>

235g/dm<sup>3</sup>

5,55g/dm<sup>3</sup>

6,68g/dm<sup>3</sup>

1

30.08.2016 .

30.08.2016 .

31.08.2016 .

10.09.2016 .

11.09.2016 .

12.09.2016 .

1

2,41 mg/dm<sup>3</sup>;

2. 1,58 mg/dm<sup>3</sup>;

3. 1,29 mg/dm<sup>3</sup>;

harvested and vinification was carried out by the classic red wine technology (Marinov, 1990, Yankov, 1992). A grinder was used for crushing the grapes and separating the rachis. The resulting grape pulp was sulphitated with 50 mg/dm<sup>3</sup> of sulfur dioxide. After 2 hours the medium was inactivated with selected, dry yeast strain SIHA RUBINO CRU. The alcoholic fermentation was carried out at 25 - 28 . When the relative density of must had reached 1000, the solids were removed from the ready wine. Then the wines were poured, decanted and filled up. They were further sulphitated with 30 mg/dm<sup>3</sup> of free sulfur dioxide. After the wines had been clarified, a chemical and organoleptic analysis was carried out.

From the indicators presented in Table 1, it was evident that grapes had accumulated sugars from 200 g/dm<sup>3</sup> for Trapezitsa variety to 235 g/dm<sup>3</sup> for Pinot Noir. The fructose predominated in all varieties indicating good ripening of the grapes. The titratable acids were within the range from 5.55 g/dm<sup>3</sup> for Storgozia and Trapezitsa to 6.68 g/dm<sup>3</sup> for Kaylashki Rubin, characteristic for that variety. The tartaric and malic acids and pH were typical for a well-ripened grape in the region of Pleven. These indicators of grapes were a prerequisite for obtaining quality red regional wines.

The grapes were harvested as follows for:

Pinot Noir on August 30, 2016  
 Trapezitsa on August 30, 2016  
 Rubin on August 31, 2016  
 Storgozia on September 10, 2016  
 Kaylashki Rubin on September 11, 2016  
 Bouquet on September 12, 2016

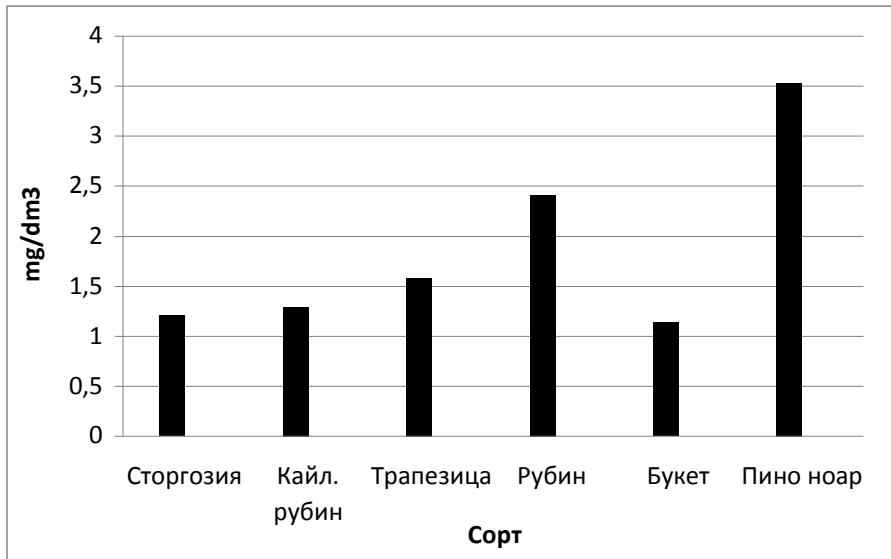
Figure 1 shows the resveratrol rates in the grapes of the studied varieties. The highest resveratrol content was found in Rubin – 2.41 mg/dm<sup>3</sup>;

followed by:

2. Trapezitsa – 1.58 mg/dm<sup>3</sup>;  
 3. Kaylashki Rubin – 1.29mg/dm<sup>3</sup>;

4. 1,21 mg/dm<sup>3</sup>;  
 5. 1,14 mg/dm<sup>3</sup>;  
 mg/dm<sup>3</sup>.

4. Storgozia – 1.21 mg/dm<sup>3</sup>;  
 5. Bouquet – 1.14 mg/dm<sup>3</sup>;  
 The rate of the control Pinot Noir was 3.53 mg/dm<sup>3</sup>.



1.  
**Fig. 1. Resveratrol content in grapes**

Vitis vinifera.  
 2016 .  
 13,19  
 % 13,52 %  
 1,44  
 g/dm<sup>3</sup> 2,52 g/dm<sup>3</sup>  
 23,10 g/dm<sup>3</sup>  
 g/dm<sup>3</sup> 28,40  
 p  
 2-3 (457,18 mg/dm<sup>3</sup>  
 114,34mg/dm<sup>3</sup> ).

The data revealed that the amount of resveratrol in the grapes from the studied varieties in the region of Pleven was almost similar and comparable to those of the control Pinot Noir from Vitis vinifera.

Table 2 present the chemical indicators of the wines from the studied varieties, vintage 2016.

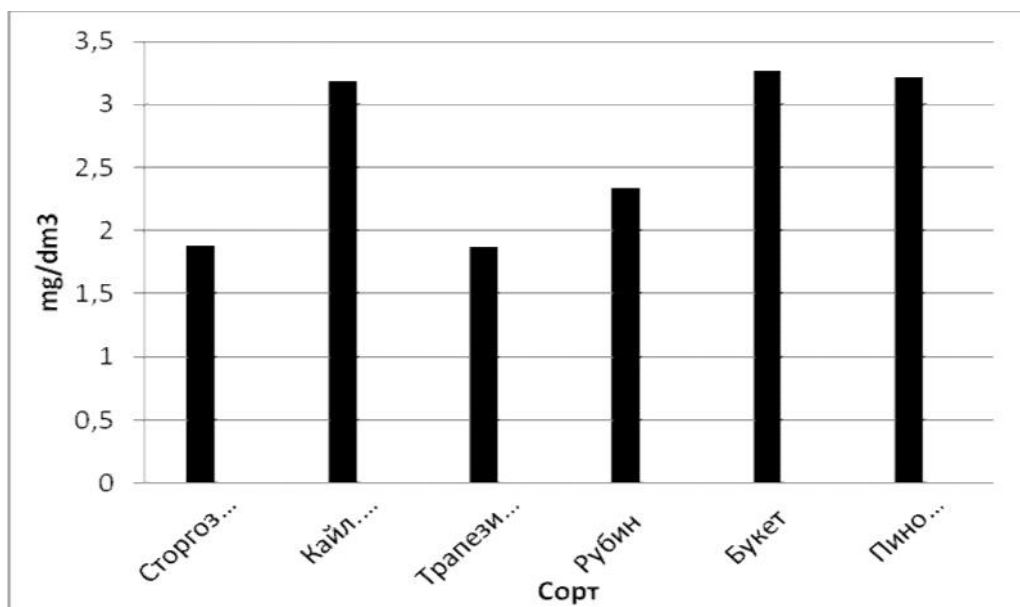
Alcohol ranged from 13.19 vol.% in Rubin to 13.52 vol.% in Pinot Noir. Wines were well fermented and as being dry, the sugars varied from 1.44 g/dm<sup>3</sup> in Storgozia to 2.52 g/dm<sup>3</sup> in Kaylashki Rubin. The total extract was 23.10 g/dm<sup>3</sup> for Trapezitsa to 28.40 g/dm<sup>3</sup> for Rubin.

The titratable and organic acids and pH were within the standard rates for dry wines produced in the region of Pleven. Referring the anthocyanins, the studied varieties exceeded the control 2-3 times (457.18 mg/dm<sup>3</sup> for Bouquet and 114.34 mg/dm<sup>3</sup> for Pinot Noir). The color

- characteristics corresponded to the more dyed wines of the studied wines. The rates of phenolic substances in the control and the studied varieties were within the same range with few exceptions. For these varieties (Rubin, Kaylashki Rubin, Trapezitsa and Bouquet) this was a varietal feature.

**2. 2016**  
**Table 2. Chemical composition of wine, vintage 2016**

Indicators	Storgozia	Kailashki rubin	Trapezitsa	Rubin	Bouquet	Pino noar
1. Alcohol, vol. %	12,89	12,65	11,46	13,19	11,72	13,52
2. Reducing sugars, g/dm <sup>3</sup>	1,44	2,52	1,61	1,98	1,78	2,32
3. Relative density	0,9920	0,9949	0,9936	0,9937	0,9942	0,9934
4. Total extract, g/dm <sup>3</sup>	23,30	26,90	23,10	28,40	25,40	26,70
5. Titratable acids, g/dm <sup>3</sup>	5,30	6,23	5,65	7,05	6,65	6,35
6. Tartaric acid, g/dm <sup>3</sup>	1,12	1,19	1,37	1,26	1,50	1,41
7. Malic acid, g/dm <sup>3</sup>	3,68	4,25	3,75	3,36	3,06	3,60
8. Free SO <sub>2</sub> , mg/dm <sup>3</sup>	18,00	22,00	19,00	24,00	21,00	28,00
9. Total SO <sub>2</sub> , mg/dm <sup>3</sup>	57,00	60,00	42,00	72,00	59,00	50,00
10. Anthocyanins, mg/dm <sup>3</sup>	345,44	434,47	216,66	444,37	457,18	114,34
11. Intensity	9,94	9,21	3,13	13,17	11,88	5,84
12. Nuance	0,52	0,64	0,87	0,60	0,47	0,68
13.	3,56	3,22	3,06	3,19	3,42	3,50
14. TPS, g/dm <sup>3</sup>	1,91	2,02	1,54	3,44	1,94	3,54
15. FPS, mg/dm <sup>3</sup>	3055	3757	1390	3292	2329	3425
16. NPS, mg/dm <sup>3</sup>	233	508	188	222	243	182
17. Resveratrol, mg/dm <sup>3</sup>	1,88	3,18	1,8	2,34	3,27	3,22



. 2.

**Fig. 2. Resveratrol content in wine**

2  
-  
( 3  
(3,18mg/dm<sup>3</sup>)  
-  
1,88mg/dm<sup>3</sup>.

2)  
(3,27mg/dm<sup>3</sup>,  
(2,34mg/dm<sup>3</sup>).  
1,87mg/dm<sup>3</sup>

Figure 2 presents the wine resveratrol content. The highest resveratrol content (Table 3 and Figure 2) had wine from Bouquet variety (3.27mg/dm<sup>3</sup> with parental forms Mavrud x Pinot Noir) followed by Kaylashki Rubin (3.18mg/dm<sup>3</sup>) and Rubin (2.34mg/dm<sup>3</sup>). The least resveratrol content from the studied varieties had Trapezitsa and Storgozia, 1.87mg/dm<sup>3</sup> and 1.88mg/dm<sup>3</sup>, respectively.

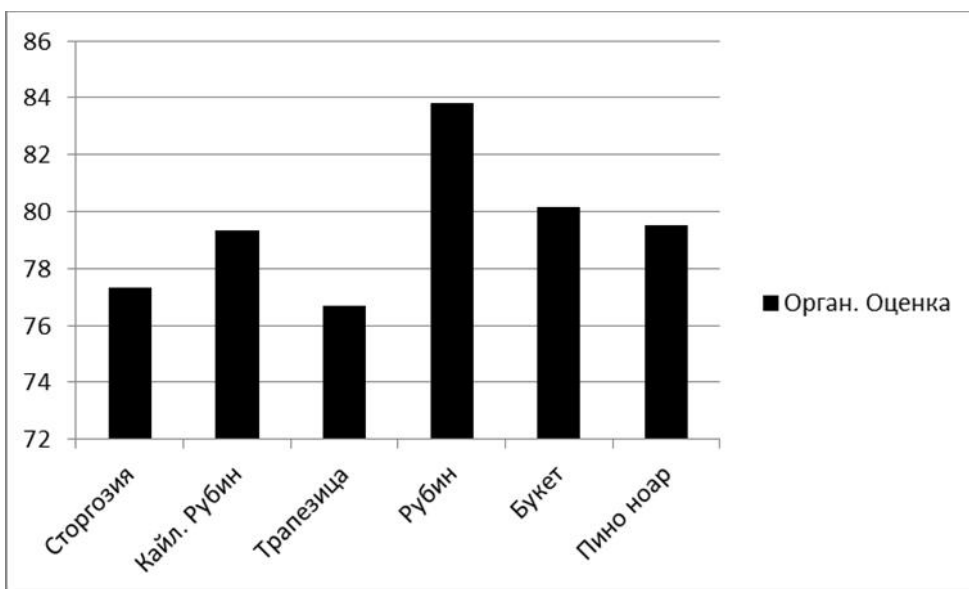


Fig. 3. Organoleptic evaluation of wine

2016 .  
 83,83  
 80,17,  
 79,33  
 76,67.  
 77,33  
 79,50.

Figure 3 shows the organoleptic evaluation of a wine win s, vintage 2016. With the greatest organoleptic rating, Rubin wine was distinguishes with 83.83 p., characterized by good clarity, ruby-red color, characteristic fruity aroma, with pleasant harmony, fullness and a long final. Followed by Bouquet - one excellent wine with a score of 80,17 p., Kailashky rubin characterized by a pleasant aroma with a score of 79,33 p., and finally Storgozia and Trapezitsa with 77,33 p. and 76,67 p. respectively. The Pino noar control has an average tasting score of 79,50 p.

### CONCLUSIONS

Vitis vinifera.

(3,27mg/dm<sup>3</sup>)

(3,18mg/dm<sup>3</sup>),

Referring the grapes and wine chemical indicators the studied varieties selected through intraspecific and interspecific hybridization did not differ from those of the Vitis vinifera control.

The highest resveratrol content had Bouquet wine (3.27mg/dm<sup>3</sup>) with parental forms Mavrud x Pinot Noir, followed by Kaylashki Rubin (3.18mg/dm<sup>3</sup>) with parental forms Pamid x Hybrid VI-2-15 x



2-15	Vitis amurensis (2,34mg/dm <sup>3</sup> )	V -	Gamay Noir x Vitis amurensis and Rubin (2.34mg/dm <sup>3</sup> ) with parental forms Nebbiolo x Syrah.
	-		
	(1,88mg/dm <sup>3</sup> )		The least resveratrol content of the studied varieties had Storgozia (1.88mg/dm <sup>3</sup> ) with parental forms Bouquet x Villard Blanc 12375 and Trapezitsa
	(1,87mg/dm <sup>3</sup> )	12375	(1.87mg/dm <sup>3</sup> ) with parental forms Dunavska Gamza Marseilles early.
			The amount of resveratrol in grapes and wine from the studied varieties grown in the region of Pleven was almost similar and comparable to those of Pinot noir control from Vitis vinifera.
vinifera.		Vitis	

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## Labels and brands in Bulgarian wine tourism: opportunities and future

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### SUMMARY

Over the last two decades global wine tourism has grown rapidly and is important not only for the wineries that offer it, but also for the regions where they are located for various reasons: increasing income, creating jobs, preserving heritage, development of local culture and many others. They create synergies attracting more and more tourists and investment, and this is particularly relevant for those regions where other types of tourism are missing. In Bulgaria the officially registered wineries are over 270, half of them have opened doors for visitors. A small number of them are visited all year round. Each winery offers different wines, made from different varieties, with little known brands for consumers, and very few of them look forward to developing their own wine tourism label and brand.

The paper discusses some opportunities for the development of labels and brands related to wine tourism, through specialization and diversification. The specialization is most often based on variety, terroir and production of quality

270,

20–25  
10

wines, while the diversification of the tourist product can be connected with nature, local food, wine and tourism education, organizing cultural events, links with other types of tourism. Once developed individual labels of wine tourism have to be united in larger brands – regional wine routes/ tours. In the near future, to attract more and more visitors, Bulgarian wine tourism should create and develop about 20-25 regional brands, united in 7 to 10 Wine Roads.

**Key words:** wine tourism, label, brand, specialization, diversification, wine roads

## INTRODUCTION

Wine tourism in many wine-growing regions around the world has developed rather quickly over the past two decades. Initially some wineries opened doors for visitors, but gradually that relatively new type of tourism gained important economic and social significance not only for them, but also for the communities where they were located, and especially where there were no other types of tourism. The positive impacts of the enotourism development, as it is also known, are indisputable. As a result of the visit of tourists and extending the length of their stay, income increases, new jobs are created, tangible and intangible cultural heritage is preserved, local culture is developing, etc. That in turn creates synergies, with opportunities for more investment and attracting more tourists.

The viticulture and winemaking sector is characterized by a combination of complex and interrelated activities, ranging from cultivation of the grape varieties to wine processing, storage, bottling, export and marketing. In the era of globalization and the rapid development of communications, transport, technology, electronics, the wine producers both from the Old and the

New World, use all possible and available competitive advantages without any problems. Their purpose is to make their own labels easily recognizable and marketable. The economic logic points that as in the case of many other goods and services, nowadays it is easier to produce wine than to sell it, which in turn developed labels into brands, using all available marketing tools in the location where it is produced.

On the other hand, the modern logic of tourism development has shown that it is certainly not enough to visit a given location if there is only a museum or a hotel. Therefore, it should be paid attention to all possible available resources and factors that could be utilized and combined so as to make the travel agencies' products attractive and profitable, and the location where they were situated to be established as a preferred destination. This is the current problem to be solved by the Bulgarian wine producers who offer wine tourism.

Apart from them, wine tourism is proposed by some hotels, guest houses, wine restaurants and bars, indicating on the one hand, a greater variety and specialization of tourist services, and on the other hand, a growing interest of visitors to such type of services. It provokes the search for greater opportunities in the wine-growing regions, deriving all tourist assets, services and leisure activities, combining history, traditions, winemaking, culture, culinary, customs, arts and cultural programmes. During the last 10 years, in Bulgaria, as in most wine-producing regions in the world, annual wine festivals and the combination of wine, arts, crafts and food have been organized.

All this shows that wine tourism is still not well developed market in Bulgaria, and attention should be paid to the opportunities and prospects for its growth in the next 5 to 10 years.

The objective of this paper was to

present the opportunities for short and medium term development of wine tourist labels and brands that would make certain wine-growing regions of Bulgaria into sustainable and attractive destination for wine tourism. The main objects and events of wine tourism, their specialization and diversification have been discussed for the achievement of this objective.

## MATERIAL AND METHODS

An inductive and deductive method, conceptual modeling; theoretical, marketing and comparative analysis; official data, reports and statistics of Bulgarian, European and world government and international organizations: Ministry of Agriculture and Food (MAF) Agricultural Statistics Department, Executive Agency on Vine and Wine (EAVW), International Organization of Vine and Wine (O.I.V.) have been used. Some examples of Bulgarian wineries based on their official websites have been shown.

## RESULTS AND DISCUSSION

Viticulture in Bulgaria has been developing in a negative direction. There was a decrease in the total area of vineyards (in holdings and not included in them), as in 2006 it was 128,857 ha while in 2015 it was reduced twice – 62,791 ha in 2015 (MAF, Agricultural statistics, 2016). The largest share of the grapes-growing holdings – about 70% are located in the Southeast and the South Central Region (Report on Agriculture, 2016, MAF). That has determined the concentration of a large part of the Bulgarian wineries in these two regions.

Bulgaria has registered at the International Organization of Vine and Wine (O.I.V.) a total of 52 appellations for the production of quality wines (white wine; red wine; rose wine, dry wine; semi-dry wine; semi-sweet wine; sweet wine) with a sign of Protected Designation of

(O.I.V.).

70%

'2016, MAF).

(O.I.V.) 52

(white wine; red wine; rose wine, dry wine; semi-dry wine; semi-sweet wine; sweet wine)



2016 ., 270

– 20 50 000

(Concours Mondial de Bruxelles, 2016).

70-

(Tsakov, 2011).

80-

90-

20

(2008-2013 .) 50

(Dimitrov and Dimitrova, 2016).

2009) value. Just because of the specific way of winemaking, its usefulness for human health and the cultural experiences it could provide, it was paid special attention to the benefits of offering wine tourism in some wine-growing regions of the country.

In Bulgaria, according to EAVW data for 2016, over 270 wine producers were registered. Most of them have a small and medium output capacity between 20 and 50,000 bottles of wine. Not a small part of their wines are quality, boutique, in limited series and enjoy ever growing attention. That is evidenced by the awards from specialized competitions and world fairs (Concours Mondial de Bruxelles, 2016).

Wine tourism has been offered since the 1970-ies. Then through the state tour operator *Balkantourist* groups have visited some wineries (called at the time “vinprom”), especially those near the big resorts (Tsakov, 2011). Following the economic changes in the late 1980-ies and the privatization of almost all state-owned wineries, some owners looked at the opportunities offered by the wine tourism market. In the 1990-ies, almost 20 wineries opened doors for visits. Until the economic crisis (2008-2013), about 50 enterprises have provided that type of service, and afterwards approximately half of the registered wineries in the country. In some municipalities of Bulgaria there have been initiatives in support of wine tourism, most often related to wine festivals and celebrations. That was a proof of the growing interest in this type of cultural events as well as the development of wine and gourmet culture. However, there have not been joint tourist products of wine tourism known throughout the world, as *Wine Route* (Dimitrov and Dimitrova, 2016). These positive trends have suggested that for the future success of individual wine tourism products and entire regions it should be paid attention to these factors and resources that might improve the



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2017).

– *winescape* (Peters, 1997).

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supply and make Bulgaria into an international destination for wine tourism. Above all, it should be directed to the formation, production and supply of diverse tourism products, based on the reputation of the wine and the wine-producing region where they were made. The individual brands of wine in combination with the traditions, history and culture, might become an appropriate marketing tool for attracting tourists. Actually, it is necessary all available local assets to be used (connected simultaneously with winemaking and tourism), through which a positive and memorable image can be imposed in order the wine tourism product to be made marketable and recognizable.

#### Labels and brands of wine tourism

The basis of quality wines production is the cultivation of grapes on terrains, where due to the specific natural and climatic conditions, wines with unique organoleptic flavors and tastes are obtained. Such wine-growing micro-regions are known as *terroir*, combining the terrain, soils, climate and the production technologies (Levine, 2017). The concept of the *terroir* is discussed from the point of view of tourism, and in particular in terms of the recreational opportunities it can offer like rural and ecological tourism. Such opportunities include, for example, strolls among well-kept vineyards, visiting wineries and tasting, attractive settlements, and near them landscapes overlooking mountains, forests, rivers, lakes and other landmarks. For this reason, the boom of wine tourism imposed the term – *winescape* (Peters, 1997).

The classification of the quality wines, their production technology and grape variety represent the so-called label (brand). For the first time in France, in the middle of the 19th century, an official name was given of the wines, the wine-growing regions from which they

al., 2000).

(Hall et

originated and their identity. This system has been still used nowadays for the production of quality wines, determining their appellation (location) and protection against unfair competition. It turned out that was a good marketing tool applied by most wineries to attract tourists (Hall et al., 2000).

Labels in wine tourism are associated with the reputation of a particular brand of wine and the winery where it is produced. Namely, a certain brand of wine, well-known among the consumers is the main reason for them to undertake a trip to visit the places where it is produced and where they can try it just "*from the spring*". The two main factors – the quality wine and the concept of terroir represent the core of the wine tourism label. However, it could not be relied only on these two factors to be offered quality wine tourism. Quality tourism products of wine tourism should be combined with the typical tourist services (e.g. accommodation, meals, transportation, animation). They provide added value, synergies are created and sustainability of the destination(s) is ensured. The more varied in quantity and quality of the tourist services, the more preferred they would be.

Vlachvei et al. (2012)

Vlachvei et al. (2012) had emphasized that the *wine brand* was much more than the label in the buyer's mind. The wine brand helps the buyer to distinguish individual brands of wine and facilitates its choice. The wine brand is a synthesis of marketing and advertising messages that help in their uniformity to be distinguished their own wine from the other products supplied on the extremely difficult wine market. Therefore, and with a view of the opportunities provided by tourism, a great number of the wine producers and the wine-growing regions have turned to wine tourism.

In the current situation of the tourism market, the establishment, imposing and positioning of a brand

(Ilieva, 2014).  
Rafailova and Dzhabarova  
(2014) „

(Ilieva, 2014) is essential for the competitiveness of the individual companies and destinations. According to Rafailova and Dzhabarova (2014) “branding is a process of creating and using the common brand of the region or promoting the region as a destination for unique and very specific wines and food, boutique wineries, historical architecture and a crossroad of cultures shaping the local tradition”. While wine tourism labels could be linked to company tourism products, the concept of which is based on the synthesis of “quality wines – terroir” and all typical tourism activities and services that could be offered along this line. Therefore, the branding of the wine tourism product uses all possible available resources and factors of the whole wine-growing region, which create a positive and attractive image of a wine tourism destination.

Wine tourism labels and brands could be developed simultaneously in two directions – through specialization and diversification.

*The specialization* in the tourist business related to wine tourism (not only in Bulgaria, but also globally) is getting in and imposing more and more. It is dictated by the increasing demand for tourism products related to ecology, healthy foods, peacefulness, unpolluted environment, fashion, visiting not so well known regions. On the other hand, tourist products simultaneously providing recreation and cultural-informative opportunities are sought. Wine tourism in Bulgaria undoubtedly offers such rich prospects: a large number of wineries have opened doors for visits; most of them are located in picturesque areas with a lot of places for recreation and other types of tourism nearby; wine festivals and feasts are organized; wine and its cultural transformations attract an increasing number of visitors. However, although it is quite well developed by some wineries, there are still not enough

utilized resources and unimplemented policies that have the potential to turn some regions into sustainable destinations for wine tourism.

From the Bulgarian wine-making practice, the following types of sites offering wine tourism could be distinguished on the basis of their production, material base and specialization:

*Wineries (enterprises)* – the tour is organized within the winery and its vicinity. Wine tourism is related to the presentation of all technological processes – from grapes processing to bottling as it ends with tasting. Typically, these are large-capacity wineries (e.g. “Asenovgrad”, “Black Sea Gold”, Pomorie) and are well established on the domestic and international markets. In addition to a wide range of wines, the connoisseurs are also offered other derivatives (brandy, cognac, sparkling wine, liqueurs) produced by these wineries.

*“Chateau” type wineries* – these are boutique wineries with their own vineyards from several tens to several hundred hectares. They offer the visitors various activities – ranging from a tour of the vineyards, presentation of the wines production and storage and tasting, to accommodation in own hotels and restaurants. As this type of sites are located on *winescape* they have the greatest charm and preference, especially among tourists, admirers of nature.

More famous ones that have established their names not only with their wines, but also with wine tourism are “Chateau Burgozene”, Oryahovo; “Chateau Des Bergers”, Targovishte; “Ruse” Wine House with a boutique restaurant located on a former military fortress “Levent Tabia”; Chateau “Polichoronoff”, Varna; “Zlaten Rojen” Winery; Chateau “Medovo”; “Bessa Valley”, Pazardzhik; “Vila Justina” and much more. “Vila Justina” organizes in its annual calendar a variety of gourmet experiences,

*winescape*

(Wine & veal meat; Wine & boer goad meat; Wine & pasta (guest chief); Folklore BBQ party in the Park-vineyard),

„Todoroff Wine & Spa”,

combining traditional Bulgarian and international cuisine (Wine & veal meat; Wine & boer goat meat; Wine & pasta (guest chief); Folklore BBQ party in the Park-vineyard), folklore events, picnics and celebrations of various holidays.

In some of the wineries, such as “Todoroff Wine & Spa”, Plovdiv and “Starosel” Winery, guests can enjoy wine-spa programmes in addition to tasting and accommodation.

*Family wineries* – they continue the winemaking of several generations. Some of them have arranged a museum exhibition room, displaying old objects and tools used by the past generations. They usually produce small quantities of boutique wines but are renowned in the area where they are located. Such wineries are suitable for visitors looking for home-like coziness and rural idyll. Such family wineries are Winery in the village of Nisovo, Ruse; “Maryan”, Elena; “Salla Estate”, Provadia; Villa “Melnik”, town of Melnik; “Kyosev”.

*Wine museums* – exhibiting artifacts and objects, customs and traditions related to wine-growing at a regional level. Most of them also have tasting halls where typical regional wines can be tasted. Some of them are located in natural caves, such as “Magura” and Chateau “Kailaka”, while others are located in antique houses, set in special thematic exhibition halls. These are the wine museums in the towns of Lyaskovets, Suhindol and Melnik.

Hotels and guest houses – it becomes increasingly more common in the Bulgarian tourist practice to invest in special rooms – tasting and enotheques, where guests are offered wine tasting and local food. They are situated in places – centers of cultural, historical, spa or other types of tourism. Such are, for example, “Sopot” Winery with a guest house and a restaurant.;



(14.02.),  
 (Rose Wine Expo)  
 " (Dimitrov and  
 Dimitrova, 2015).  
 (Dimitrov, 2014).

various thematic concepts based on local food and beverages. That has led to the organization of wine and culinary festivals and feasts. The calendar of events related to viticulture and winemaking is becoming more and more intense. The most famous feast is the *Day of the Vine-grower and Wine-maker* in honour of St. Trifon-Zarezan (February, 14), symbolizing the awakening of nature, giving new life and strength. The better known wine festivals are the *Defile of Young Wine*, Plovdiv; *Avgustiada* in Stara Zagora, *Rose Wine Expo* in Kazanlak and "*Perperikon*" Festival (Dimitrov and Dimitrova, 2015).

*Diversification* as a strategic marketing approach is very appropriate in offering wine tourism in Bulgaria (Dimitrov, 2014). Through the wine product diversification, the range of specific products and services can be improved and expanded to promote the competitiveness and generate a synergy effect. The sites of wine tourism above mentioned could develop products through diversification to expand, diversify, enrich and complement the existing ones with other products and services of specialized tourist products. For the company products, diversification is in three main directions:

- *at the entrance and inside the enterprise*: These are the physical means and resources – the vineyards, the infrastructure, the buildings and the facilities for quality wines producing. They are the "face" of the wine tourism product. The quality wines making and everything attractively related to them are the leading motivation for the visit. That's the reason for the wineries to adapt their base for the tourists and the place for the presentation – the tasting halls. But to attract more tourists, they should offer more services and facilities typical of tourism – such as a restaurant or a bar; a hotel or a guest





On the basis of the advantages of the wine-growing region and tourism, brands of tourist products can be established using the following interrelation: "variety-terroir-quality wines (with their production technology) – local traditions-history-culture-tourism".

It should be looked for opportunities to reveal the potential of the local varieties and Bulgarian selection varieties in the medium and long term aspect. It is these varieties that could contribute to the strengthening of the market positions of the small and medium-sized wineries according to their output capacity. Interesting and demanded by wine lovers and wine tourists are just such wines that are different in taste and uniqueness from, for example, some famous French (Chardonnay, Sauvignon Blanc, Merlot, Cabernet), German (Riesling) and Italian (San Jovese) varieties. That opinion was expressed by the managers of some Bulgarian wineries. In our survey (Dimitrov, 2014, p.130), they stated that wines produced from Bulgarian varieties (45% of the respondents) were almost equally demanded on the domestic and international market as the introduced varieties (55% of the respondents). Namely, local varieties and varieties of Bulgarian selection should become a regional brand in the production of quality wines with PDO and PGI. They might be imposed more efficiently and lasting by organizing joint business initiatives and advertising, on the one hand and on the other they would contribute to the establishing the unique image of a given region and attract more tourists. Such well-known regions are Vidin and Suhindol with wines from the village of Gamza; Melnik with Keratsuda and Shiroka Melnishka Loza, Asenovgrad with v. Mavrud, where the famous "Stanimashka Malaga" wine was produced in the past; Varna and Bourgas with white wines from the v. Dimiat. The varieties of Vrachanski Muscat, Varnenski Muscat, Tamianka, Rubin, Pamid could be added to them that can enrich the image of the Bulgarian wines.

Another opportunity for wine tourism development is presented by the production of a certain type of wine. Such a strategic marketing approach is implemented by "Orbelus" Winery, focusing solely on the production of bio-wines, being sold in the European Union, North America and the Asia-Pacific markets.

The wines of the "Minkov Brothers" Winery are also an increasingly recognizable brand. The winery has focused on the middle and high price classes, producing several varietal wines and their coupages - white, rosé and red wines in the "Cycle" series. The success of the winery was largely due to maintaining the quality of the wine during the last 5-6 vintages.

At present the Bulgarian wine producers rely mainly on products based on the coupage between Bulgarian and foreign varieties. The production of wines from introduced varieties takes the leading position. The pathway of the Bulgarian winemaking and, respectively, of Bulgarian wine tourism in the coming years should be under the motto "*back to the roots*". The wineries investing in the Bulgarian varieties would undertake a higher risk however that is a market niche with a great potential, especially from the point of view of the tourist industry – hotels and restaurants. The tourists visiting our resorts demand to taste typical Bulgarian products and Bulgarian wine is one of these convertible products.

In the short and medium term prognosis, all business entities interested in offering wine tourism (wineries, communities, tourist companies, museums, crafts, transport, etc.) should look for integration opportunities and jointly create and offer larger regional tourism products, turning the wine-growing region into a preferred destination. Such associations are *Wine Roads*. There are no such networks yet in the country, but considering the good European and global practices, there is

- potential for creating such regional products over the next few years. The core of this product is the region with its natural and anthropogenic advantages. The associations of all involved in the wine tourism in a regional destination could more easily advertise and impose their own brands, tourist services, trade, etc. under one common brand. The quality wine tourism sites are the ones that offer the most rest options and interesting experiences for the tourists. They are also the most visited and are the advertising media for the region. Therefore, the individual business labels should be the leading and the most recognizable among the visitors. The image, of the company and the region, is the leading one in recognizing and remembering the destination's branding. Branding, in turn, is of paramount significance in the imposing and implementation of the tourist product.

( ) - According to the Secretary General of the World Tourism Organization (WTO), Mr. Taleb Rifai, *Bulgarian tourism should be advertised with an emphasis on the Thracians and the wine*. Cultural tourism could increase the tourists flow and be offered all year round, overcoming seasonality. The numerous fortresses, architecture, cultural monuments and artifacts might be combined with wine tours, balneo and SPA, rural, eco and other types of tourism. Thus added value and sustainability would be achieved, including in the wine-growing regions.

- Wine tourism in Bulgaria has been currently developed mainly in two directions – at company level; and organization of wine related festivals and feasts. The companies, based on specialization related to local varieties and quality wines production and their combination with various tourist services and products are among the most visited and preferred. In the future, the leading factors in the choice of wine tourism destination would be the quality wines production and the branding, respectively

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the marketing of the individual regions.

Therefore, it is necessary tourism products to be developed based on the existing appellations for quality and regional wines that are maintained. These 52 appellations and the characteristic grape varieties as well as the wines made from them could be distributed into 20-25 regional brands, covering 2-3 neighboring municipalities where routes/tours to be developed. They can in turn merge into larger networks – for example from 7 to 10 Wine Roads.

The wine routes actually would comprise everything possible – from variety, terroir and quality wines production, to the diversification of the tourist product related to nature, history, local food, events organization, joint advertising, links with other types of tourism.

Tourists could be attracted by organizing wine festivals and feasts and the regions to be advertised as destinations. This is particularly important for the wine-growing regions of Northern Bulgaria, where there are almost no such events in comparison with Southern Bulgaria. The attraction of tourists for wine tourism would greatly depend on the calendar of cultural events in a given region. The more cultural events are included in the calendar, the greater is the chance to be visited. More attractive cultural events will be those related to history, viticulture and wine, culinary, folklore, musical days, etc. The opportunities offered by the operational programs of the European Union could be used to finance these events.

## CONCLUSIONS

The number of wineries offering wine tourism in Bulgaria has been constantly increasing while the implementation of such a product is

carried out also by other entities – wine-museums, restaurants and hotels. Local festivals are also organized. Actually, within the territory of the whole country, and especially in some of the wine-growing regions well-known in the past (Melnik, Plovdiv, Haskovo, Ruse, Pleven, etc.), there is a potential for their recognition as destinations for wine tourism. In search of synergies for sustainable rural development, conditions might be created where the existing family hotels and guest houses could diversify their activities through the production of limited wine quantities to be offered to the tourists.

Thus, more wineries and especially family vineyard farms that have difficulty in the sale of their produce in particular years could be included in the supply of wine tourism. This is related to certain changes in the existing normative regulation of wine-making in the country.

An important condition for the success and establishment of the separate wine-growing destinations in the short-term prospect is the local support by the municipalities. That support could generally be expressed in the construction of the appropriate infrastructure; organization of holidays and events related to wine and tourism; advertising and promotion of the communities as destinations. A key role in the implementation of this policy is the involvement of professionals and specialists in the field of tourism, winemaking, history, culture, marketing and advertising. Hence the need of vocational training and the training of staff with secondary and higher education, as well as support for career development in this branch.

The government should redirect and reconsider its policy towards the branding and advertising of tourism in

Bulgaria. The opportunities for development of many types of tourism have not been implemented yet due to their insufficient advertising on the world tourist fairs. Wine tourism is one of those opportunities that still lacks the necessary popularity and development support.

Through the organization of *Wine Roads* in the wine-producing regions based on quality wines and all available resources to attract tourists, much more conditions will be created to support the implementation of the wine tourism product as well as the recognizability of our country on the domestic and international tourist market would be promoted.

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