GOCE DELCEV UNIVERSITY - STIP
FACULTY OF AGRICULTURE

JOURNAL OF AGRICULTURE AND PLANT SCIENCES

YEAR 2021 VOLUME 19, Number 2

Journal of Agriculture and Plant Sciences, JAPS Vol. 19, No. 2

ISSN 2545-4447 print
ISSN 2545-4455 on line
Vol. 19, No. 2, Year 2021

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doi.org/10.46763/JAPS201

ISSN 2545-4447 print
ISSN 2545-4455 on line
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EDITORIAL BOARD

CONTENT

INTORDUCTION

The end of 2021 will be remembered by the world for the new variant of the SARAS-CoV-2 virus called omicron, which gave the covid-19 pandemic a new chapter. The human population, since the beginning of the pandemic which occurred at the end of 2019 and continues for two years, adapts all mechanisms of operation in conditions of pandemic, mainly with online communication and systems of enhanced internet communication and minimization of personal contacts.

Agricultural production seems to have been hit hardest. Except in conditions of pandemic and economic crisis, the production of food of plant origin is also facing serious problem of global warming. A rise in temperature of 1.5^{0}C is a threat to all ecosystems with long-term consequences of declining and even extinction of some endangered species. The ago-ecosystem is not spared from this effect either. In food production in the last decade the challenges of the scientific and professional community were focused on systems of soilless production and the use of contemporary biotechnological methods. Even if the covid-19 pandemic is brought under control, the world will not be able to keep global warming at 1.5^{0}C. Despite the promises of all countries that they will reduce pollution, rising of temperatures will continue with forecast that the end of the century will end with a rise in global temperatures of up to 2.4^{0}C. This is an additional threat, but at the same time a challenge, for the scientific community to deal with food production in conditions of pandemic, global warming and rapid growth of human population.

Only science, with scientifically proven methods, is able to find appropriate solutions to deal with the global problems that plague the world since the beginning of the XXI century. New trends in science are not unknown for local research centers either. As a small but well-established research center, the Faculty of Agriculture at the Goce Delcev University - Stip strives to be in trend with the novel trends, to implement all data and available methods in agricultural production and to offer the economy scientifically and professionally proven methods in the processes of agricultural production.

In the conditions of a covid-19 pandemic, the Editorial Board of JAPS continuously had published all journal issues in order to share with the scientific and professional community the new research results in the field of agricultural production and plant sciences. We are honored and pleased to share with you five peer-reviewed scientific papers in JAPS issue No. 19., Vol.2 and also to encourage our colleagues from the Republic of Northern Macedonia, the region and wider to publish the results from their research in JAPS.

Editorial Board,
December, 2021

Editor in chief,
Prof. Liljana Koleva Gudeva, PhD

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 19, No. 2, 2021

Manuscript received: 22.11.2021
Accepted: 10.12.2021

In print: ISSN 2545-4447
On line: ISSN 2545-4455
doi: https://doi.org/10.46763/JAPS2119209a
Original scientific paper

OBSERVATION OF THE CHEMICAL PARAMETERS ON IMPORTED AND DOMESTIC WINES FOUND ON THE MARKET IN THE REPUBLIC OF NORTH MACEDONIA

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Abstract

In the Republic of North Macedonia, the production of wine is very well known, but in the markets, there is also a variety of imported wines. Therefore, the aim of our research was to examine the basic parameters which determine the quality of the wine. A total of 106 domestic and imported wines were included in this research. The basic chemical parameters for each of the samples were examined by using standard OIV methods. Verification of the methods was done by determining its accuracy, precision, repeatability and reproducibility using standard reference material and proficiency testing. Depending on the sugar content, wines were divided into 4 groups: dry, semi dry, semi-sweet and sweet wines. The highest alcohol content was observed in dry wines originating from all countries that were subject of this research (up to 13.54 vol %) and the lowest was observed in wines originating from Italy which are mostly used as dessert wines (5.07 vol %). The semi-dry wines originating from France showed the slightest value (min.12.18 mg/L free SO_2 and min.60.20 mg/L total SO_2), which corresponds to their high quality and price on the market. This research is of great interest for the needs of the market and the price of the wine, due to the wine quality standards under the law of Republic of North Macedonia.

Keywords: *wine quality, OIV methods, descriptive analysis, method verification*

INTRODUCTION

As there are several varieties of apple, tomato, etc., there are also several varieties of grapes. But over the years, it was determined which varieties of grapes are most suitable and possess all the necessary characteristics for producing quality wine (pleasant taste, resistance to various diseases and pests, yielding high yields, etc.). The type of grapes used for production largely determines both the quality and the specific characteristics of the wine, such as the taste and colour of the wine, the presence of residual sugar, the content of alcohol, acidity and the presence of tannins. Recently, mostly used grape varieties for production of red wines are Shiraz, Pinot Noir, Cabernet Sauvignon and Merlo and for white wines, those are Sauvignon Blanc and Chardonnay. But there are other important factors that determine the quality and style of the wine. In order to obtain a healthy harvest, grapes need factors that influence and improve the quality, such as favourable climate, enough sunny days, moderate amount of water, heat and proper soil with balanced content of all nutrients. Vineyards are very tolerant and grow on all types of soils, but without proper nutrient content in the soil itself, the product obtained will be of lower quality. Also, in the process of winemaking the most important part is the fermentation, where the grape juice changes the flavours into those of wine and knowing when to end the fermentation process determines the type and the quality of the final product. In the Republic of North Macedonia, the production of wine is very well known and it exists more than 4000 years in this area, but in the markets, there is also a variety of imported wines that can be found. Therefore, the aim of our research was to examine the basic parameters which determine the quality of wine, such as total alcohol content, total and free SO_2, total and volatile acids, reduced sugars, specific gravity and total dry extract, by using standard accredited methods.

MATERIAL AND METHODS

Wine is an alcoholic drink made during the fermentation process from grape juice. The quality of the wine is directly related to the quality of the grape variety and is represented as complex set of interactions, so its quality is easier to detect than define.

A total of 106 samples of red, rose and white imported and domestic wines from different manufacturers were included in this research, originating from Italy (60 samples – 34 white wines, 2 rose wine, 24 red wines), France (20 samples – 8 white wines, 2 rose wines, 10 red wines), Spain (5 white wines), Serbia (16 samples – 7 red wines, 2 rose wines, 7 white wines) and North Macedonia (5 samples – 3 red wines, 2 white wines).

During the research, standard accredited methods were used according to the Law on Wine and Wine Products of the Republic of North Macedonia, as follows: for determining the alcoholic strength in volume percentages OIV-A2 (MA-EAS312-01-TALVOL), (IOVW) method was used by using a pycnometer to measure the distillate density obtained after the distillation of the wine at 20° C. The presence of acids in wine is very important in the process of winemaking and the finished product of wine. They have direct influences on the colour, the balance of the wine and gives fresh and sour taste of the final product. The measurement of the acidity (g/L) in wine is usually known as "total acidity" or "titratable acidity" which originates mainly from the presence of citric, tartaric and malic acid. The method used for determining the content of total acids (such as tartaric acid) was OIV-A10 (MA-EAS313-01-ACITOT), (IOVW). This method includes potentiometric titration with 0.1M NaOH by using standard Titrino Plus titrators. To determine the content of volatile acids (such as acetic acid) the OIV- A11 (MA-EAS313-02-ACITVOL), (IOVW) method was used. This method includes primary distillation of the sample and double titration by using NaOH and Iodine standard solutions. The sweetness is a main indicator of how much sugar wine contains (primarily glucose) and depending on that the wines are classified as dry, semi dry, semi-sweet and sweet wines. The residual sugar is the one that remains after the fermentation stops and usually is measured in g/L. So, for determining the content of reducing sugars the OIV-A4 (MA-EAS311- 01-SUCRED), (IOVW) method was used. This method is based on the reducing characteristics of the sugars present in the grapes by using Fehling solution and then titration with Iodine standard solution. The usage of sulphur dioxide is very critical in the process of winemaking. The presence of free sulphur dioxide keeps the wine from spoilage and oxidation, but too much SO_2 can mask the fruity aromas of the wine and gives metallic, sharp and bitter flavour to the wine which has negative effect on the quality. The presence of total sulphur dioxide is the total amount of free sulphur dioxide plus the one that is bound to sugars, pigment, aldehydes. It is very important the concentration (mg/L) of total and free sulphur dioxide to be in balance due to the quality characteristics of the wine. The OIV-A17 (MA-EAS323-04-DIOSOU), (IOVW) official method was used to determine the content of total and free sulphur dioxide. For determination of free SO_2, standard H_2SO_4 solution is used and for determining the total SO_2 content, standard solutions of NaOH and H_2SO_4 are used and then the samples are titrated by using standard Iodine solution on Titrino plus titrators. The density and specific gravity analysis is used for determining the total alcohol content in g/L and vol. %. For this reason, OIV-A1 (MA-EAS2-01- MASVOL), (IOVW) standard method was used. OIV-A3 (MA-EAS2-03-EXTSEC), (IOVW) method was used to determine the total dry extract content (g/L), by direct evaporation of the volume of the sample.

Before the analysis, verification on each method was performed by determining accuracy, precision (standard deviation and relative standard deviation), repeatability and reproducibility by using standard reference material and proficiency testing.

The measurement of the control reference material (PT FAPAS 1389 - set 1 and 2, Quality indicators in wine) was performed in 10 repetitions for each method separately and for the calculation of the extended measurement uncertainty as a source of uncertainty were taken into account the repetition, bias, as well as errors arising from the equipment used.

The results for the extended measurement

uncertainty for each method are as follows: volatile acidity ± 6.27 %, total dry extract ± 4.92 %, total SO_2 ± 1.07 %, total acidity ± 1.87 %, sugar content ± 6.06 %, free SO_2 ± 5.33 %, total alcohol content ± 6.19 % and specific gravity ± 0.20 %. (Extended measurement uncertainty for k = 2, 95 % probability level).

RESULTS AND DISCUSSION

Depending on the content of sugars in the wine, they were first divided into four basic groups as dry (up to 4 g/L), semi dry (up to 12 g/L), semi-sweet (up to 45 g/L) and sweet wines (more than 45 g/L) and also, depending of the country of origin. The results shown that most of the wines included in this research belong to the group of semi dry wines (n=62 or 65.72 %) and least in the group of sweet wines (n=6 or 6.36 %). Residual sugar is one that remains in the wine after alcoholic fermentation. Then, each wine group was examined on the basic chemical parameters that were subject of this research. The results showed differences in almost all examined parameters.

Table 1. Reducing sugar content (g/L)

	Type of wine			
Country	Dry	Semi dry	Semi Sweet	Sweet
Italy	10	32	13	5
France	2	17	1	/
Spain	/	/	5	/
Serbia	2	11	2	1
North Macedonia	3	2	/	/
Total number of samples	17= 18.02 %	62 = 65.72 %	21 = 22.26 %	6 = 6.36 %

The sugar content of the grapes is closely related to the alcohol content of the wine. Fermentation is a process where under the action of the yeast (mostly *Saccharomyces cerevisiae*) comes to the utilization of the sugar present in the grape juice, which produces alcohol and carbon dioxide, with at least 12 enzymes included in the process. The longer the fermentation, the higher the alcohol and lower the sugar level. So, this is very important step in the process of winemaking because of the different flavours produced which directly affect the taste of the wine. Therefore, from the results shown we can notice that dry wines originating from all countries included in the research, contain the highest alcohol content (up to 13.54 ± 0.83 vol %), with some minor exceptions, such as two samples of semi dry wine originating from North Macedonia, one sample of semi-sweet wine originating from France and one sample of sweet wine originating from Serbia – vermouth wine. The lowest alcohol content was observed in wines originating from Italy and they are mostly used as dessert wines (5.07 ± 0.31 vol %).

The total acidity in wine usually depends on the presence of non-volatile acids, such as mallic, tartaric or citric acid plus the volatile acids such as acetic acid. These components directly affect the smell and the taste of the wine. Determination of volatile acidity is used routinely as an indicator of wine spoilage. The results shown no significant difference between all groups of wine and the countries of origin such as, for total acidity between 4.81 ± 0.09 g/L - 6.70 ± 0.12 g/L and for volatile acidity between 0.26 ± 0.02 - 0.39 ± 0.02 g/L.

Table 1.1. Mean values of physical-chemical parameters in dry wines from different countries

	Dry wines						
Country	Total alcohol content (vol %)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total dry extract (g/L)	Specific gravity (MU = ± 0.0019 for all wines)
Italy	14.55 ± 0.90	4.98 ± 0.09	0.44 ± 0.03	66.75 ± 3.55	150.80 ± 1.61	32.25 ± 1.58	0.9796
	14.00 ± 0.86	5.92 ± 0.11	0.37 ± 0.02	38.16 ± 2.03	90.66 ± 0.97	25.85 ± 1.27	0.9801
	12.50 ± 0.77	4.85 ± 0.09	0.22 ± 0.02	18.85 ± 1.00	88.21 ± 0.94	20.40 ± 1.00	0.9810
	12.78 ± 0.79	4.46 ± 0.08	0.22 ± 0.02	30.41 ± 1.62	98.82 ± 1.05	18.39 ± 0.90	0.9816
	14.12 ± 0.87	4.15 ± 0.08	0.20 ± 0.02	24.68 ± 1.31	68.17 ± 0.72	26.02 ± 1.28	0.9801
	14.55 ± 0.90	4.25 ± 0.08	0.26 ± 0.02	25.70 ± 1.36	75.50 ± 0.80	25.50 ± 1.25	0.9796
	13.55 ± 0.83	5.30 ± 0.10	0.26 ± 0.02	28.15 ± 1.50	69.25 ± 0.74	24.12 ± 1.18	0.9807
	13.30 ± 0.82	5.17 ± 0.10	0.25 ± 0.02	27.03 ± 1.44	68.35 ± 0.73	23.52 ± 1.15	0.9810
	13.50 ± 0.83	5.10 ± 0.10	0.36 ± 0.02	27.20 ± 1.45	60.20 ± 0.64	20.22 ± 0.99	0.9807
	12.55 ± 0.77	6.10 ± 0.11	0.33 ± 0.02	30.20 ± 1.61	105.19 ± 1.12	20.25 ± 0.99	0.9818
	MV=	**MV =**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	13.54 ± 0.83	**5.03 ± 0.09**	**0.29 ± 0.02**	**31.71 ± 1.69**	**87.51 ± 0.93**	**23.65 ± 1.16**	**0.9806**
France	12.55 ± 0.77	6.01 ± 0.11	0.38 ± 0.02	44.80 ± 2.38	97.56 ± 1.04	15.70 ± 0.77	0.9818
	13.00 ± 0.80	5.43 ± 0.10	0.40 ± 0.03	38.71 ± 2.06	132.94 ± 1.42	16.70 ± 0.82	0.9813
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	12.77 ± 0.79	**5.72 ± 0.11**	**0.39 ± 0.02**	**41.75 ± 2.22**	**115.25 ± 1.23**	**16.20 ± 0.79**	**0.9815**
Spain	/						
Serbia	13.50 ± 0.83	4.78 ± 0.09	0.25 ± 0.02	20.80 ± 1.10	139.52 ± 1.49	17.25 ± 0.84	0.9807
	13.05 ± 0.80	4.85 ± 0.09	0.35 ± 0.02	30.52 ± 1.62	75.90 ± 0.81	19.55 ± 0.96	0.9813
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	13.27 ± 0.82	**4.81 ± 0.09**	**0.30 ± 0.02**	**25.66 ± 1.36**	**107.71 ± 1.15**	**18.40 ± 0.90**	**0.9810**
North Macedonia	11.83 ± 0.73	5.62 ± 0.10	0.25 ± 0.02	28.98 ± 1.54	85.20 ± 0.91	21.20 ± 1.04	0.9827
	13.77 ± 0.85	5.25 ± 0.10	0.27 ± 0.02	36.66 ± 1.95	101.17 ± 1.08	17.56 ± 0.86	0.9805
	13.86 ± 0.85	5.08 ± 0.09	0.25 ± 0.02	29.57 ± 1.57	84.76 ± 0.90	16.87 ± 0.83	0.9804
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	13.15 ± 0.81	**5.31 ± 0.10**	**0.26 ± 0.02**	**31.73 ± 1.69**	**90.37 ± 0.96**	**18.54 ± 0.91**	**0.9812**

Table 1.2. Mean values of physical-chemical parameters in semi dry wines from different countries

	Semi dry wines						
Country	Total alcohol content (vol %)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total dry extract (g/L)	Specific gravity (MU = ± 0.0019 for all wines)
Italy	11.00 ± 0.68	5.46 ± 0.10	0.28 ± 0.02	30.14 ± 1.60	90.20 ± 0.96	26.55 ± 1.30	0.9836
	11.10 ± 0.68	5.95 ± 0.11	0.31 ± 0.02	26.50 ± 1.41	86.65 ± 0.92	27.66 ± 1.36	0.9836
	11.00 ± 0.68	5.87 ± 0.11	0.30 ± 0.02	27.18 ± 1.44	89.76 ± 0.96	28.20 ± 1.38	0.9836
	11.50 ± 0.71	5.82 ± 0.11	0.25 ± 0.02	42.15 ± 2.24	140.55 ± 1.50	25.15 ± 1.23	0.9830
	12.00 ± 0.74	5.82 ± 0.11	0.41 ± 0.03	30.25 ± 1.61	150.94 ± 1.61	33.75 ± 1.66	0.9824
	12.50 ± 0.77	5.12 ± 0.10	0.25 ± 0.02	22.84 ± 1.21	98.50 ± 1.05	21.71 ± 1.06	0.9810
	15.95 ± 0.98	5.49 ± 0.10	0.31 ± 0.02	30.11 ± 1.60	151.01 ± 1.61	38.86 ± 1.91	0.9781
	12.00 ± 0.74	5.18 ± 0.10	0.29 ± 0.02	28.20 ± 1.50	120.82 ± 1.29	23.65 ± 1.16	0.9824
	12.17 ± 0.75	5.28 ± 0.10	0.30 ± 0.02	21.40 ± 1.14	100.53 ± 1.07	21.12 ± 1.03	0.9823
	11.74 ± 0.72	5.57 ± 0.10	0.32 ± 0.02	19.84 ± 1.05	73.58 ± 0.78	24.62 ± 1.21	0.9828
	13.05 ± 0.80	4.90 ± 0.09	0.34 ± 0.02	26.76 ± 1.42	78.10 ± 0.83	27.18 ± 1.33	0.9813
	11.95 ± 0.73	4.95 ± 0.09	0.30 ± 0.02	28.16 ± 1.50	119.40 ± 1.27	23.76 ± 1.16	0.9824
	12.45 ± 0.77	5.10 ± 0.10	0.25 ± 0.02	22.85 ± 1.21	98.42 ± 1.05	21.70 ± 1.06	0.9810
	12.50 ± 0.77	7.77 ± 0.14	0.40 ± 0.03	13.11 ± 0.69	74.75 ± 0.79	27.21 ± 1.33	0.9819
	13.00 ± 0.80	4.80 ± 0.09	0.28 ± 0.02	25.60 ± 1.36	60.75 ± 0.65	24.95 ± 1.22	0.9813
	12.00 ± 0.74	5.15 ± 0.10	0.30 ± 0.02	28.85 ± 1.52	120.80 ± 1.29	24.50 ± 1.20	0.9824
	15.23 ± 0.94	6.18 ± 0.11	0.32 ± 0.02	25.55 ± 1.36	98.00 ± 1.04	33.37 ± 1.20	0.9789
	14.06 ± 0.87	4.90 ± 0.09	0.30 ± 0.02	27.18 ± 1.44	60.75 ± 0.65	26.60 ± 1.30	0.9801
	12.50 ± 0.77	4.90 ± 0.09	0.22 ± 0.02	20.22 ± 1.07	85.16 ± 0.91	20.16 ± 0.99	0.9818
	13.05 ± 0.80	4.85 ± 0.09	0.28 ± 0.02	25.55 ± 1.36	59.62 ± 0.63	25.18 ± 1.23	0.9813
	12.00 ± 0.74	5.12 ± 0.10	0.30 ± 0.02	29.00 ± 1.54	123.98 ± 1.32	24.78 ± 1.21	0.9824
	12.54 ± 0.77	4.87 ± 0.09	0.20 ± 0.02	19.87 ± 1.05	90.52 ± 0.96	19.21 ± 0.94	0.9818
	14.00 ± 0.86	4.78 ± 0.09	0.29 ± 0.02	24.66 ± 1.31	58.80 ± 0.62	25.88 ± 1.27	0.9801
	13.50 ± 0.83	5.10 ± 0.10	0.33 ± 0.02	30.16 ± 1.60	80.02 ± 0.85	26.68 ± 1.31	0.9807
	12.00 ± 0.74	5.90 ± 0.11	0.42 ± 0.02	31.25 ± 1.66	152.80 ± 1.63	33.80 ± 1.66	0.9824
	14.56 ± 0.90	4.63 ± 0.09	0.26 ± 0.02	30.69 ± 1.63	81.10 ± 0.86	27.20 ± 1.33	0.9796
	14.00 ± 0.86	4.95 ± 0.09	0.22 ± 0.02	25.80 ± 1.37	80.10 ± 0.85	25.38 ± 1.24	0.9801
	13.56 ± 0.83	5.07 ± 0.10	0.20 ± 0.02	27.60 ± 1.47	65.59 ± 0.70	26.35 ± 1.29	0.9807
	11.00 ± 0.68	5.13 ± 0.10	0.23 ± 0.02	23.32 ± 1.24	142.08 ± 1.52	25.12 ± 1.23	0.9836
	11.05 ± 0.68	6.00 ± 0.11	0.34 ± 0.02	32.87 ± 1.75	108.50 ± 1.16	26.30 ± 1.29	0.9836
	9.50 ± 0.58	4.87 ± 0.09	0.30 ± 0.02	27.58 ± 1.47	112.75 ± 1.20	28.10 ± 1.38	0.9854
	11.50 ± 0.71	5.55 ± 0.10	0.42 ± 0.03	40.16 ± 2.14	133.00 ± 1.42	35.18 ± 1.73	0.9830
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	12.50 ± 0.77	**5.34 ± 0.10**	**0.30 ± 0.02**	**27.04 ± 1.44**	**99.61 ± 1.06**	**26.55 ± 1.30**	**0.9818**

France	12.00 ± 0.74	7.33 ± 0.14	0.32 ± 0.02	13.20 ± 0.70	66.65 ± 0.71	21.90 ± 1.07	0.9824
	12.00 ± 0.74	7.59 ± 0.14	0.30 ± 0.02	12.18 ± 0.64	60.20 ± 0.64	22.20 ± 1.09	0.9824
	12.00 ± 0.74	7.60 ± 0.14	0.33 ± 0.02	15.16 ± 0.80	62.18 ± 0.66	23.10 ± 1.13	0.9824
	12.54 ± 0.77	6.75 ± 0.12	0.24 ± 0.02	15.20 ± 0.81	78.80 ± 0.84	24.18 ± 1.18	0.9818
	12.50 ± 0.77	6.90 ± 0.13	0.28 ± 0.02	16.18 ± 0.86	79.92 ± 0.85	23.75 ± 1.16	0.9818
	14.77 ± 0.91	4.50 ± 0.09	0.28 ± 0.02	26.27 ± 1.40	83.48 ± 0.89	27.28 ± 1.34	0.9794
	15.05 ± 0.93	4.33 ± 0.09	0.26 ± 0.02	27.21 ± 1.45	96.69 ± 1.03	27.96 ± 1.37	0.9791
	13.55 ± 0.83	5.06 ± 0.10	0.40 ± 0.03	37.48 ± 1.99	118.66 ± 1.26	26.53 ± 1.30	0.9807
	13.50 ± 0.83	4.27 ± 0.08	0.38 ± 0.02	45.52 ± 2.42	90.78 ± 0.97	29.82 ± 1.46	0.9807
	13.00 ± 0.80	4.70 ± 0.09	0.41 ± 0.03	38.15 ± 2.03	107.20 ± 1.14	25.20 ± 1.23	0.9813
	15.05 ± 0.93	4.68 ± 0.09	0.35 ± 0.02	38.78 ± 2.06	132.94 ± 1.42	29.71 ± 1.46	0.9790
	12.50 ± 0.77	6.86 ± 0.13	0.37 ± 0.02	19.76 ± 1.05	105.65 ± 1.13	23.09 ± 1.13	0.9818
	12.50 ± 0.77	4.19 ± 0.08	0.44 ± 0.03	31.95 ± 1.70	79.87 ± 0.85	23.05 ± 1.13	0.9818
	12.52 ± 0.77	4.33 ± 0.09	0.42 ± 0.03	32.20 ± 1.71	81.15 ± 0.86	24.80 ± 1.22	0.9818
	12.55 ± 0.77	4.58 ± 0.09	0.30 ± 0.02	50.18 ± 2.67	105.16 ± 1.12	30.88 ± 1.51	0.9818
	11.00 ± 0.68	6.60 ± 0.12	0.40 ± 0.03	44.59 ± 2.37	125.77 ± 1.34	25.65 ± 1.26	0.9836
	11.00 ± 0.68	5.90 ± 0.11	0.39 ± 0.02	40.90 ± 2.17	120.15 ± 1.28	32.82 ± 1.61	0.9836
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	12.82 ± 0.79	**5.65 ± 0.11**	**0.33 ± 0.02**	**29.70 ± 1.58**	**93.83 ± 1.00**	**25.99 ± 1.27**	**0.9777**
Spain	**/**						
Serbia	12.50 ± 0.77	4.48 ± 0.09	0.28 ± 0.02	28.31 ± 1.50	53.79 ± 0.57	24.42 ± 1.20	0.9818
	12.05 ± 0.74	5.41 ± 0.10	0.32 ± 0.02	18.48 ± 0.98	127.82 ± 1.36	22.12 ± 1.08	0.9824
	11.53 ± 0.71	4.90 ± 0.09	0.29 ± 0.02	20.80 ± 1.10	110.50 ± 1.03	21.60 ± 1.06	0.9830
	13.43 ± 0.83	5.89 ± 0.11	0.35 ± 0.02	44.24 ± 2.35	139.11 ± 1.48	21.32 ± 1.04	0.9808
	12.00 ± 0.74	7.03 ± 0.13	0.40 ± 0.03	61.49 ± 3.27	136.52 ± 1.46	22.56 ± 1.10	0.9824
	14.05 ± 0.86	6.76 ± 0.12	0.39 ± 0.02	48.97 ± 2.61	115.02 ± 1.60	27.49 ± 1.35	0.9801
	13.30 ± 0.82	5.62 ± 0.10	0.33 ± 0.02	43.65 ± 2.32	130.79 ± 1.39	32.41 ± 1.59	0.9810
	14.00 ± 0.86	6.23 ± 0.12	0.37 ± 0.02	51.61 ± 2.75	136.78 ± 1.46	33.04 ± 1.62	0.9801
	11.40 ± 0.70	5.02 ± 0.09	0.31 ± 0.02	28.22 ± 1.50	73.16 ± 0.78	30.78 ± 1.51	0.9831
	12.00 ± 0.74	4.80 ± 0.09	0.28 ± 0.02	30.13 ± 1.60	70.88 ± 0.75	31.85 ± 1.56	0.9824
	12.45 ± 0.77	5.20 ± 0.10	0.30 ± 0.02	23.20 ± 1.23	69.20 ± 1.55	32.80 ± 1.61	0.9819
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	12.61 ± 0.78	**5.57 ± 0.10**	**0.33 ± 0.02**	**36.28 ± 1.93**	**105.77 ± 1.13**	**27.30 ± 1.34**	**0.9817**
North Macedonia	14.57 ± 0.90	5.71 ± 0.11	0.28 ± 0.02	40.24 ± 2.14	90.29 ± 0.96	33.99 ± 1.67	0.9796
	15.61 ± 0.96	5.99 ± 0.11	0.38 ± 0.02	42.55 ± 2.26	92.24 ± 0.98	38.67 ± 1.90	0.9785
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	15.09 ± 0.93	**5.85 ± 0.11**	**0.33 ± 0.02**	**41.39 ± 2.20**	**91.26 ± 0.97**	**36.33 ± 1.78**	**0.9790**

Table 1.3. Mean values of physical-chemical parameters in semi-sweet wines from different countries

	Semi sweet wines						
Country	Total alcohol content (vol %)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total dry extract (g/L)	Specific gravity (MU = ± 0.0019 for all wines)
Italy	11.50 ± 0.71	5.55 ± 0.10	0.41 ± 0.03	40.22 ± 2.14	120.55 ± 1.28	34.88 ± 1.71	0.9830
	11.60 ± 0.71	6.19 ± 0.12	0.40 ± 0.03	55.20 ± 2.94	140.10 ± 1.49	31.20 ± 1.53	0.9904
	11.50 ± 0.71	6.12 ± 0.12	0.35 ± 0.02	38.15 ± 2.03	140.16 ± 1.49	38.20 ± 1.87	0.9831
	9.65 ± 0.59	6.80 ± 0.13	0.36 ± 0.02	12.50 ± 0.66	128.66 ± 1.37	55.60 ± 2.73	0.9850
	11.00 ± 0.68	5.80 ± 0.11	0.40 ± 0.03	30.10 ± 1.60	150.75 ± 3.08	35.15 ± 1.72	0.9836
	10.05 ± 0.62	6.50 ± 0.12	0.38 ± 0.02	29.50 ± 1.57	130.88 ± 1.40	76.15 ± 3.74	0.9848
	10.02 ± 0.62	5.94 ± 0.11	0.40 ± 0.03	29.20 ± 1.55	180.14 ± 1.92	66.80 ± 3.28	0.9848
	12.03 ± 0.74	5.07 ± 0.10	0.30 ± 0.02	28.67 ± 1.52	100.53 ± 1.07	29.33 ± 1.44	0.9813
	11.00 ± 0.68	5.66 ± 0.11	0.40 ± 0.03	30.15 ± 1.60	154.18 ± 1.64	35.20 ± 1.73	0.9836
	9.55 ± 0.59	5.07 ± 0.09	0.35 ± 0.02	26.88 ± 1.43	130.20 ± 1.39	60.80 ± 2.99	0.9804
	10.85 ± 0.67	5.37 ± 0.10	0.22 ± 0.02	16.36 ± 0.87	109.29 ± 1.16	28.01 ± 1.37	0.9845
	18.10 ± 1.12	4.10 ± 0.08	0.15 ± 0.02	15.22 ± 0.81	58.18 ± 0.62	55.18 ± 2.71	0.9850
	7.55 ± 0.46	5.38 ± 0.10	0.40 ± 0.03	40.60 ± 2.16	128.16 ± 1.37	34.65 ± 1.70	0.9878
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	11.10 ± 0.68	**5.65 ± 0.10**	**0.34 ± 0.02**	**30.21 ± 1.61**	**128.59 ± 1.37**	**44.70 ± 2.19**	**0.9844**
France	13.40 ± 0.82	6.70 ± 0.12	0.36 ± 0.02	32.10 ± 1.71	110.28 ± 1.17	41.25 ± 2.02	0.9808
Spain	11.53 ± 0.71	5.24 ± 0.10	0.22 ± 0.02	18.12 ± 0.96	115.69 ± 1.23	36.55 ± 1.79	0.9830
	11.50 ± 0.71	5.11 ± 0.10	0.32 ± 0.02	14.49 ± 0.77	103.53 ± 1.10	52.51 ± 2.58	0.9830
	11.50 ± 0.71	5.24 ± 0.10	0.32 ± 0.02	16.30 ± 0.86	103.53 ± 1.10	52.50 ± 2.58	0.9830
	11.50 ± 0.71	5.11 ± 0.10	0.22 ± 0.02	14.49 ± 0.77	115.68 ± 1.23	36.54 ± 1.78	0.9830
	11.50 ± 0.71	5.17 ± 0.10	0.27 ± 0.02	18.12 ± 0.96	109.60 ± 1.17	44.52 ± 2.19	0.9830
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	11.50 ± 0.70	**5.17 ± 0.10**	**0.27 ± 0.02**	**16.30 ± 0.86**	**109.60 ± 1.17**	**44.52 ± 2.19**	**0.9830**
Serbia	12.16 ± 0.75	6.65 ± 0.12	0.38 ± 0.02	72.83 ± 3.88	145.92 ± 1.56	27.53 ± 1.35	0.9823
	12.03 ± 0.74	6.38 ± 0.12	0.36 ± 0.02	63.67 ± 3.39	123.34 ± 1.31	24.16 ± 1.18	0.9824
	MV=	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**	**MV=**
	12.10 ± 0.75	**6.51 ± 0.12**	**0.37 ± 0.02**	**68.25 ± 3.63**	**134.63 ± 1.44**	**25.84 ± 1.27**	**0.9824**
North Macedonia	/						

Table 1.4. Mean values of physical-chemical parameters in sweet wines from different countries

	Sweet wines						
Country	Total alcohol content (vol %)	Total acidity (g/L)	Volatile acidity (g/L)	Free SO_2 (mg/L)	Total SO_2 (mg/L)	Total dry extract (g/L)	Specific gravity (MU = ± 0.0019 for all wines)
Italy	15.00 ± 0.92 12.34 ± 0.76 7.38 ± 0.45 12.03 ± 0.74 5.07 ± 0.31 **MV=** **10.36 ± 0.64**	5.60 ± 0.10 4.93 ± 0.09 6.43 ± 0.12 5.10 ± 0.10 5.83 ± 0.11 **MV=** **5.57 ± 0.10**	0.27 ± 0.02 0.21 ± 0.02 0.38± 0.02 0.31 ± 0.02 0.22 ± 0.02 **MV=** **0.27 ± 0.02**	20.20 ± 1.07 12.62 ± 0.67 33.74 ± 1.79 28.66 ± 1.52 38.78 ± 2.06 **MV=** **26.80 ± 1.42**	98.18 ± 1.05 70.94 ± 0.75 146.56 ± 1.56 100.51 ± 1.07 181.09 ± 1.93 **MV=** **119.45 ± 1.27**	61.10 ± 3.00 185.76 ±9.13 78.03 ± 3.83 79.35 ± 3.90 177.26 ±8.72 **MV=** **116.30 ±5.72**	0.9790 0.9821 0.9881 0.9813 0.9911 **MV=** **0.9843**
France	/						
Spain	/						
Serbia	16.09 ± 0.99	4.88 ± 0.09	0.30 ± 0.02	9.11 ± 0.48	91.19 ± 0.97	170.55 ± 8.39	0.9779
North Macedonia	/						

Table 2. The mean values, standard deviation and relative standard deviation were calculated for each physical-chemical parameter depending on the type of wine

Parameters	Dry wine	Semi dry wine	Semi sweet wine	Sweet wine	Standard deviation (SD)	Relative standard deviation (RSD)
Total alcohol content (vol %)	12.77-13.54	12.50-12.82 15.09*	11.10-12.10 13.40*	10.36 – 16.09	0.38 %	3.09 %
Total acidity (g/L)	4.81-5.72	5.34-5.85	5.17-6.70	4.88-5.57	0.03 g/L	0.68 %
Volatile acidity (g/L)	0.26-0.39	0.30-0.33	0.27-0.37	0.27-0.30	0.01 g/L	3.12 %
Free SO_2 (mg/L)	25.66-41.75	27.04-41.39	16.30-68.25	9.11-26.80	0.19 mg/L	0.56 %
Total SO_2 (mg/L)	87.51-115.25	91.26-105.77	109.60-134.63	91.19-119.45	0.22 mg/L	0.38 %
Total dry extract (g/L)	16.20-23.65	25.99-36.33	25.84-44.70	116.30-170.55	0.58 g/L	2.42 %
Reduced sugar content (g/L)	up to 4 g/L	up to 12 g/L	up to 45 g/L	more than 45 g/L	0.04 g/L	1.74 %

* exceptions from the mean values

The usage of sulphur dioxide (SO_2) as preservative in the winemaking industry is known for a long time. It has a direct impact on the wine quality and is used to ensure microbial, oxidative and antiseptic stability. The presence of total SO_2 in wine is usually the total amount of free and bound SO_2 and also there is a molecular form of SO_2. Molecular SO_2 has broad-spectrum of antimicrobial properties (Divol du Toit et al., 2012), so it can kill or inhibit most of the spoilage yeast and bacteria that could affect wine. The free SO_2 concentration is defined as molecular SO_2 plus bisulphites and gives oxidative stability in concentrations between 20-40 mg/L. But its use is of crucial importance and must be regulated because too much SO_2 can mask the fruity aromas and gives metallic, sharp and bitter flavour to the wine which has negative effect on the quality. In our research, the concentrations of free and total sulphur dioxide in all samples are in balance, but there were semi-dry wines originating from France which showed the slightest value (min.12.18 ± 0.64 mg/L free SO_2 and min.60.20 ± 0.64 mg/L total SO_2), which corresponds to their high quality and price on the market.

In the past, the content of the total dry extract was considered as a basic parameter for determining the possible falsification of the wine, or it's dilution with water. But nowadays it is generally accepted that the content of the total dry extract depends mostly on the variety of grapes, seasonal variations as well as the method of wine production. The composition of the total dry extract represents all non-volatile matter which in specific conditions do not volatilize (Florin Dumitru BORA et al., 2015). From the chemical aspect, the total dry extract consists of: sugars, tannins and dyes, organic acids such as (tartaric, malic, succinic acid, lactic acid), glycerol, 2,3 butylene glycol, nitrogen, pectin, gums, etc. The higher the extract, the fuller the body and greater aroma and flavour of the wine. In ideal conditions, the dry extract should be in balance with the sugar, acidity and alcohol levels in wine. In our research, we can notice that the content of the total extract is continuously growing as the content of sugars in the samples increases. So, the lowest content is observed in the dry wines originating from all countries (16.20 ± 0.79 g/L) and the highest in the sweet wines (up to 170.55 ± 8.39 g/L).

CONCLUDING REMARKS

Based on the results from the research, we came to the conclusion that all types of white, rose and red imported and domestic dry, semi dry, semi-sweet and sweet wine, satisfy the quality standards prescribed in Law of wine and wine products of the Republic of North Macedonia and the Regulations of wine of the European Commission.

In the markets across the country, many types of wine of different quality, price, and different countries of origin, can be found. Our research included wines commonly found on the market and by examining their basic parameters that determine the quality, we managed to establish that most of the imported and domestic wines satisfy the quality standards prescribed in the Law of wine and wine products of the Republic of North Macedonia, although some have a lower, and others have a higher market price.

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ОПРЕДЕЛУВАЊЕ НА ХЕМИСКИТЕ ПАРАМЕТРИ НА УВЕЗЕНИ И ДОМАШНИ ВИНА НА ПАЗАРОТ ВО РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

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Резиме

Во Република Северна Македонија производството на вино е многу добро познато, но на пазарите има и разновидни увозни вина. Значи, целта на нашето истражување беше да ги испитаме основните параметри кои го одредуваат квалитетот на виното. Во ова истражување беа опфатени вкупно 106 домашни и увозни вина. Основните хемиски параметри за секој од примероците беа испитани со користење на стандардни OIV методи. Верификацијата на методите беше направена со одредување на точноста, прецизноста, повторливоста и репродуктивноста со користење на стандарден референтен материјал и тестови на оспособеност. Во зависност од содржината на шеќер, вината беа поделени во 4 групи: суви, полусуви, полуслатки и слатки вина. Највисока содржина на алкохол е забележана кај сувите вина со потекло од сите земји кои беа предмет на ова истражување (до 13,54 вол.%), а најниска е забележана кај вината со потекло од Италија кои најчесто се користат како десертни вина (5,07 вол.%). Полусувите вина со потекло од Франција покажаа најмала вредност (min.12,18 mg/L слободен SO_2 и мин.60,20 mg/L вкупен SO_2), што одговара на нивниот висок квалитет и цена на пазарот. Ова истражување е од голем интерес за потребите на пазарот и цената на виното, поради стандардите за квалитет на виното според законот на Република Северна Македонија.

Клучни зборови: *квалитет на вино, OIV методи, описна анализа, верификација на метод.*

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 19, No. 2, 2021

Manuscript received: 24.11.2021
Accepted: 02.12.2021

In print: ISSN 2545-4447
On line: ISSN 2545-4455
doi: https://doi.org/10.46763/JAPS21192019kv
Original scientific paper

TOTAL BACTERIAL COUNT, SOMATIC CELL COUNT AND PRESENCE OF AFLATOXIN M1 IN RAW MILK FROM THE "OVČE POLE" REGION, REPUBLIC OF NORTH MACEDONIA

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Abstract

Dairy product quality monitoring begins at the farm and ends in the hands of the consumer. Raw milk must also meet other quality standards; it should be free of drug residues, free of added water and free of sediment, contaminants and other abnormalities. In our researches, is taken somatic cells count, the number of bacteria and Aflatoxins as indicators of the quality of raw milk from the Ovče Pole region in the period January-June, 2018. For the needs of this research, an analysis was made of 1320 samples for the presence of bacteria in raw milk, determination of somatic cell count in 478 samples as well as identification and quantification of aflatoxin M1 in 60 samples.

The results from this study indicated determination acceptable count of somatic cells in 95.5 % of the samples from raw milk while in 2 samples of raw milk, the amount of aflatoxin M1 was above limits with highest amount of 0.58 mg/kg raw milk. According to European milk quality standards, in the biggest part of the samples (89.55 %), presence of bacteria does not meet the standard. From the analyses made by the milk producers that were the subject of analysis in this research, it can be concluded that they do not adhere to good agricultural practice, the level of milk contamination is high due to poor hygiene, improper handling of milk after milking and insufficient education of farmers for hygiene in primary production.

Keywords: *raw milk, total bacteria count, somatic cells, aflatoxin M1*

INTRODUCTION

Somatic cell count is the common method for determination of raw milk quality (Bansal et al., 2005). An increased amount of somatic cells results either from an inflammatory process due to the presence of an intramammary infection or, under non-pathological conditions, from physiological processes such as estrus or advanced stage of lactation (Batavani et al., 2007). Monitoring of somatic cell numbers has been simplified by automated cell counters that allow large numbers of milk samples to be evaluated quickly. The number of somatic cells, usually called somatic cell count, in milk is used as an important indicator of udder health since somatic cells are involved in protecting the mammary gland from infection as part of the innate immune system. SCC in milk is influenced by many factors, such as animal species, milk production level, lactation stage, and also the individual and environmental factors as well as management practices. The selection of dairy animals for greater milk production and the removal of milk by machine milking impose unnatural stress on the bovine udder. This has increased the chances of mammary infections in these animals. To defend against the mammary infections, somatic cells (SCs) are released into the milk. These cells not only fight infection but also repair tissue damage. All the developed countries are using milk somatic cell counts (SCCs) as a marker to monitor the prevalence of mastitis in dairy herds, as an indicator of raw milk quality to processors, and also as a more general indicator of the hygienic conditions of milk production on farms. Though somatic cell count is subjected to variation, it is still used as

an indicator of milk quality in several species, especially in ruminant and human. Generally, SCs until now have been considered as negative. High SCC is associated with udder inflammation, which leads to bacteriological problems in milk, an alteration of milk composition, and finally, the major modifications of dairy product characteristics compared to the normal values. The role of SCs is generally assessed as a global effect, although the influence of the other factors has not been considered separately, and then, includes intrinsic characteristics of milk modified by the inflammation of the mammary gland, consequences on milk biosynthesis and secretion, and bacterial count. Aflatoxins (AFs) are secondary metabolites produced by *Aspergillus flavus*, *Aspergillus parasiticus*, and *Aspergillus nominus* fungi under impropriate growing and storage conditions (Applebaum et al., 1982). There are 18 different known AFs, and AFB1 is the most toxic and can contaminate various foods (Aycicek et al., 2005). After ingestion, its high fat solubility favours gastrointestinal absorption and can reach the liver (Battacone et al., 2003), where it is metabolized by the cytochrome P450 enzyme family and is hydrolysed into Aflatoxin M1 (AFM1) or milk Aflatoxin (Decastelli et al., 2007). It is then transferred to milk (Diener et al., 2001) and, thus, to milk derivatives and products (Dragacci et al., 2001). The most common aflatoxin with proven cancerogenic effect in raw milk is aflatoxin M1. European Community (EC) and Codex Alimentarius prescribe a limit of 50 ng/kg AFM1 in milk and 25 ng/kg for infant milk products. However, US regulation fixed the limit to a maximum of 500 ng/kg for milk and 25 ng/kg for infant milk products. The microbial milk contamination source comes from herd hygiene and health status, mastitis prevalence, production environment, and milking parlour and milk conserving practices in dairy farm. Other microbial contamination of milk possibility may occur during the long milk storage, under low insufficient temperature (Lin H et al., 2016). Usually, contaminated environments are a potential source of food-borne pathogens and spoilage bacteria present in raw milk bulk tank in the dairy farm, which are affecting the milk quality and emerging public health risk (Van Kessel JS et al., 2008, Viljoen BC,2001, Kagkli DM et al., 2007. External contamination of the udder can have a huge impact on the total number of bacteria. (Bramley and McKinnon. 1990). The study of risk factors associated with contamination of raw milk from Listeria monocytopenia's milk showed that insufficient cleanliness of cows, inadequate lighting of milking parlours and barns may be an indication of neglect of milking hygiene. Inadequate disinfection of towels used to dry the udder can significantly increase the likelihood of contamination (Sana et al., 2003).

Silage is also an important source of contamination with Listeria spp., including *L. monocytogenes* and other potential human pathogens such as *Yersinia enterocolitica* and *Aeromonas hydrophila* (Sana et al., 2003).

The aim of this work was screening of the quality of the raw milk for the period of January-June, 2018 by determination of total bacteria and somatic cell counts as well as quantification of aflatoxine M1. For this purpose, 1316 samples of raw milk were selected for investigation of total bacterial count, 478 samples were selected for determination of somatic cell count and 60 samples were collected for identification and quantification of aflatoxine M1.

MATERIAL AND METHODS

Determination of somatic cell count and aflatoxin M1 in raw milk

60 samples of raw milk from the farm in the region of "Ovče Pole" were the subject of the presence of aflatoxin M1 and somatic cell counts. All samples were stored at 2-8^{0}C and tested for 24 hours. Some samples that we were not able to analyse within 24 hours were stored at -20^{0}C. To determine the total number of bacteria in the period from January to June 2018, 1316 samples were taken, while to determine the number of somatic cells, 478 samples of raw milk from producers in the Ovce Pole region. Samples are taken and delivered in sterile plastic cups with a volume of 50 ml canned by Adizol (Sigma-Aldrich vol. 25 ml). After taking, they were transported at a temperature of 4^{o}C in the laboratory for testing the quality of raw milk at the Faculty of Veterinary Medicine Skopje. All samples are

analysed by accredited method in accordance with ISO 21187: 2004. The instrument used for the tests was the Bactoscan 8000S (Foss Electric Denmark). The BMO procedure was performed according to the Milk-Quantitative determination of bacteriological quality, IDF Standard 161A: 1995. This device works on the principle of staining bacteria with fluorescent dye. In the procedure after staining the bacteria, a thin film of the milk sample is placed on a rotating disk under the lens of a fluorescent microscope. This microscope counts coloured bacteria as light pulses that are electronically converted and displayed as numerical data.

Before somatic cell counting, the samples are heated to 40^{0}C and analysed twice on a Fossomatic 5000 (FossElectric, Denmark). The somatic cell counting procedure was performed in accordance with the accredited method ISO 17025-FVM-SOP-398 according to references from ISO 13366-2: 2006. ELISA equipment Immunoscreen AFM1 (Tecna, s.r.l, Trieste, Italy) and HPLC equipped by fluorescence detector (Waters Alliance 2695) were applied for determination of Aflatoxin M1 in 60 samples of raw milk. All standard controls were duplicated on a 96-well plate coated with anti-AFM1 antibodies. After colorization, using the appropriate chromogen, the samples were weighed using a microplate Bio-Rad Model 680 (Philadelphia, USA) photometer set at 450 nm. The measured absorption was inversely proportional to the AFM1 concentration in the sample and the measured apparatus ranging from 5 to 250 ng/kg.

Statistical analyses

The experimental results of the quality of raw milk samples were subject to independent one-way analysis of variance (ANOVA) to examine the impact of each fixed factor (i.e., raw milk samples), on the dependent variables (i.e., the somatic cell count and the amount of adlatoxin M1). The level of significant differences of the mean values (p-value) used was 5% for all the performed one-way ANOVA and Tukey's tests. When the F-tests resulted in significant differences, the mean values were further subjected to Tukey's-HSD post-doc tests for a comparison of the mean differences between groups of the independent variables (i.e., the total somatic cells and the amount of aflatoxin M1) could be undertaken. The IBM SPSS Statistics v.16.0 software (IBM Corporation, Chicago, IL, USA) was used for the statistical analyses.

RESULTS AND DISCUSSION

Determination of somatic cell count

Besides the immune defence role in the udder, SCs can continue their protective function in milk (Gera & Guha, 2011). Additionally, some components identified as being from SCs are present in milk and also help to enhance the host defence. For example, PMNs have bactericidal and respiratory burst activities and they can eliminate the invading bacteria by releasing reactive oxygen species (ROS) and granular enzymes. According to the results obtained from 482 samples taken once per month, 462 samples meet the National and EU standards for the total number of somatic cells as a parameter for milk quality. The measured average values showed that the highest value of somatic cells count was 277743.90 scc/mL in June 2018 and the lowest measured average value was detected for March 2018 (233701.3 scc/mL). Furthermore, from 482 samples collected in the "Ovče Pole" region, 95.8% met the criteria prescribed in the milk and dairy products regulative of 2016 where the maximum number of somatic cells can be 400,000 cfu/ml in raw cow's milk and are also satisfied and EU milk quality standards. Identification of area-specific and farm-specific risk factors was crucial in cow mastitis control programmes. As we can see from the figure 2, only 6 samples from 72 samples collected in January was above limit of 400,000 cfu/ml. Furthermore, 6 from 76 samples in total collected in February had higher number of somatic cells in raw milk samples. Only one sample from 78 samples of raw milk collected in March and one sample from 84 samples of April did not meet regulative for somatic cells count. The results from examined samples in May indicated 6 samples with higher number of somatic cells and the number of somatic cells in all 83 samples from raw milk collected in June was below 400,000 cfu/ml. From the results above, it can be concluded that the raw milk had good quality (in relation to somatic cell count) and selected exclusively healthy head

of cattle had a low percentage of mastitis and good control of the mammary gland.

Determination of the amount of Aflatoxin M1

Determination of the amount of aflatoxin M1 was determined in 60 samples of raw milk. In two samples a concentration higher than 0.05 ng/kg was detected by ELISA method. The amount of aflatoxin A1M1 in those two samples were additionally analysed by HPLC with fluorescent detector, as a confirmation method (Galvano et al., 1996). AFM1 concentrations in both samples (3.3%) exceeded the maximum permissible levels, and the highest detected concentration was 0.58 ng/ kg, which is 0.08 ng/ kg above the permissible limit (Ghorbanian et al., 2008).

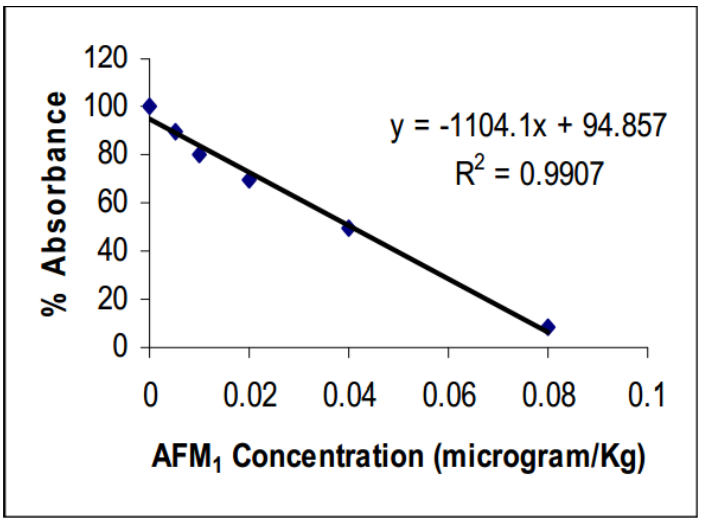

Figure 1. A sample enzyme-linked immunosorbent assay calibration curve.

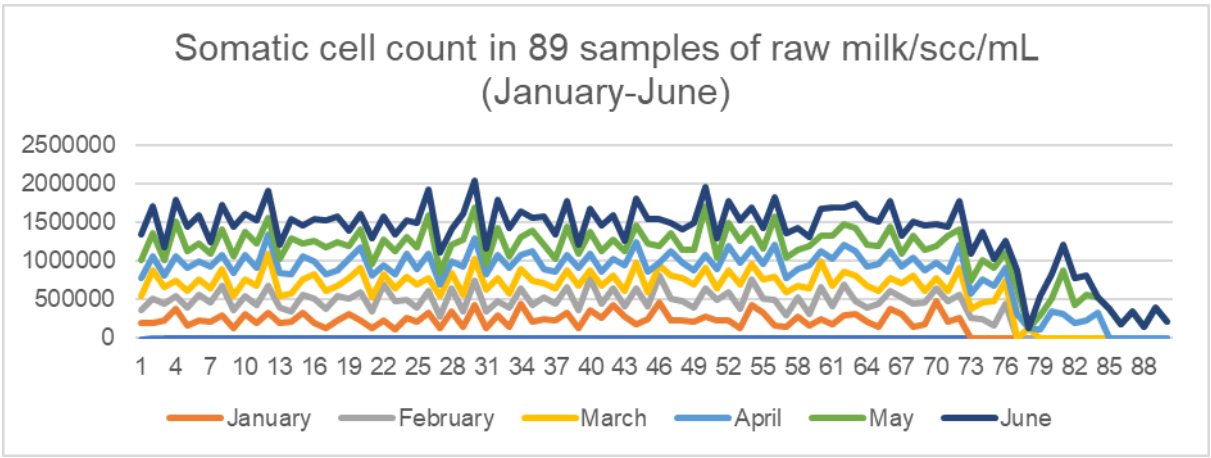

Figure 2. Somatic cell count in 89 samples of raw milk from the "Ovče Pole" region for period January-June, 2018.

Total bacterial count

According to the analysed 1320 samples that were taken twice in the first and second half of the month (analysis 1- 667 samples and analysis 2-653 samples) only 138 samples that meet the national standards while according to the EU (Council Directive 92/46 EEC). Presented by months as the average value of the bacteria were determined in raw milk it is obvious that the average value in January is the lowest with 326069.44 cfu/mL while in May it is the highest with 623395.6 cfu/mL.

Table 1. Total bacterial count (average value from January-June, 2018).

Total bacteria count in samples of raw milk for the period (January-June 2018 year)				
Month	**Number of samples from Analyst 1**	**Number of samples from Analyst 2**	**Mean value 1**	**Mean value 2**
January	80	72	348860,75[d]	326069,44[d]
February	87	81	392069,76[d]	332160,49[d]
March	102	102	464715,68[c]	454764,70[c]
April	124	124	581274,19[b]	538637,09[b]
May	137	137	623395,6[a]	605548,18[a]
June	137	137	538208,82[b]	552102,9[b]

Note: Mean values were calculated of two replicates and two analytical measurements. The different superscript letters (a–d) mean significant differences ($p < 0.05$) among the results in the same column in decreasing order.

The results of our research on the samples selected from the Ovče Pole region showed that only 10.45% meet the criteria according to the Rulebook on amending the rulebook on special requirements for safety and hygiene and the manner of the procedure for performing official controls of milk and dairy products (Official Gazette of the Republic of Macedonia, No. 197 of 28.10.2016) where the limit for the allowed number of bacteria is 400 000 cfu/mL, while none of the samples meet the criteria of the European legislation. The highest statistical significance had samples collected in May, while the lowest significant values indicated samples collected from January which can be linked by the temperature fluctuation. According to the findings of O' Connell et his research group, the bulk tank milk can be stored at 2°C or 4°C for up to 96 h with minimal deterioration of quality as long as the milk entering the tank has minimal bacterial contamination (O' Connell et al., 2016).

Many of microorganisms gain entry to the milk from equipment and/or personnel, zoonotic pathogens can also be introduced into milk from unhealthy animals. As a consequence of this risk, posterization or other treatments are employed to remove disease-causing microorganisms.

CONCLUDING REMARKS

The results presented in this research showed that the quality of raw milk in relation to the somatic cell counts and the presence of aflatoxin is at a satisfactory level. Monitoring of somatic cell numbers has been simplified by automated cell counters that allow large numbers of milk samples to be evaluated quickly. Somatic cells tend to be higher in afternoon milking's, which undoubtedly occurs because of the shorter milking interval and lesser fluid milk dilution of sloughed epithelial cells. Therefore, increased frequency of milking (three or four times/day) may slightly elevate.

The previous Aflatoxin crisis due to high AFM1 contamination of maize in 2013 has increased the awareness of the food safety risk managers; induced regulatory measures, research, and innovation activities; and reinforced the consciousness of the food business operators. Consequently, they have implemented strict monitoring and regular control along the feed and food chain utilizing the availability of rapid and less expensive detection kits. This self-control and corrective measures at dairy farms resulted in the slow decrease of AFM1 contamination. In the text, the references should be cited as the following examples: Novakov (2001) or (Dumas et al., 2006, 1999).

The paper remarks the importance among the milk production and food safety, closely related to the assurance of the milk quality and the prevention of milk spoilage. The dairy industry management programmes as for food safety, the milk quality and the dairy products are preventing the microbial contamination.

Actually, dairy farms are to reduce the milk contamination source from udder and the dairy cow herd health status and the production environment, by hygiene practices in the cow herd management and good milk conserving in the raw milk bulk tank. The food hygiene protocols are fundament for to reduce the microbial contamination of the raw milk and pasteurized milk, regarding the health risk by the microbial pathogens in the food borne diseases and bacterial spoilage, source of deteriorating dairy products and milk.

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ВКУПЕН БРОЈ НА БАКТЕРИИ, СОМАТСКИ КЛЕТКИ И ПРИСУСТВО НА АФЛАТОКСИН М1 ВО СУРОВО МЛЕКО ОД РЕГИОНОТ НА ОВЧЕ ПОЛЕ, РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

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Резиме

Мониторингот на квалитетот на млекото и млечните производи започнува од фарма, а завршува во рацете на консументите. Суровото млеко мора да ги задоволи критериумите за квалитет, што подразбира да нема остатоци од лекови, додадена вода, да нема појава на талог, загадувачи или други непожелни контаминенти. Во нашето истражување определивме вкупен број на бактерии, соматски клетки и присуство на афлатоксин М1 во примероци од сурово млеко од Овчеполскиот регион селектирани во период јануари-јуни 2018 година. За потребите на оваа истражување, во 1320 примероци беше определен вкупен број на бактерии, бројот на соматски клетки беше определен во 478 примероци, додека идентификација и квантификација на афлатоксин М1 беше определен во 60 примероци на сурово млеко.

Резултатите од ова истражување покажаа дека 95,5 % од анализираните примероци го задоволуваат стандардот за бројот на соматски клетки, додека во 2 примерока од сурово млеко имаше детектирано зголемено присуство на афлатоксин М1 со максимално количество од 0,58 mg/kg свежо млеко. Во согласност со Европските стандарди за квалитет на сурово млеко, најголем дел од примероците на сурово млеко од Овчеполски регион во кои се анализираше вкупен број на бактерии (89,55%) не го задоволуваа стандардот. Резултатите добиени од анализите на сурово млеко индицираа дека фармерите не се придржувале кон добра земјоделска пракса и нивото на контаминација беше високо поради лоша хигиена, несоодветно чување на млекото по молзењето и недоволна едукација на фармерите за хигиена при производство на млеко.

Клучни зборови: *сурово млеко, вкупен број на бактерии, вкупен број на соматски клетки, афлатоксин М1*

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 19, No. 2, 2021

Manuscript received: 01.10.2021
Accepted: 16.10.2021

In print: ISSN 2545-4447
On line: ISSN 2545-4455
doi: https://doi.org/10.46763/JAPS21192027k
Original scientific paper

ONE FACTORIAL ANOVA IN ASSESSMENT OF GROUNDWATER QUALITY IN VULNERABLE AREA OF AGRICULTURE POLLUTION

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Abstract

Arsenic polluted groundwater was found in the Strumica region located in the south-east part of the Republic of North Macedonia where an intensive agriculture production is concentrated on the area of around 1000 km^2. Out of 185 samples collected from boreholes, 64 samples have arsenic in their concentrations greater than 10 µg/L, from which 30 samples have a concentration greater than 50 µg/L with a maximum concentration of 176.56 µg/L. Pollution mostly occurs in the groundwater located in the central part of the valley characterized by alluvial plains and young aquifer. Around 57% of the polluted samples have origin from deep groundwater with a depth greater than 40 m. Reductive environment, high Fe, Mn, HCO_3^- as well as low SO_4^{2-} and NO_3^- content in polluted samples suggests that reductive dissolution is a major mechanism by which arsenic is released into the groundwater. Highly polluted samples are characterized with high concentrations of Mn and Fe. Other investigated ions are presented in low concentrations. One factorial ANOVA showed significant differences between the As concentrations in shallow and deep groundwater. Multivariate factor analysis was performed to identify the covariance structure between the investigated variables. Arsenic was positively correlated with HCO_3^- and Mn in shallow groundwater and with HCO_3^-, Ca, and Mn in deep groundwater suggesting that arsenic is mobilized in groundwater by reductive dissolution of Mn oxides from the bedrock.

Keywords: *arsenic, shallow groundwater, deep groundwater, reductive dissolution*

INTRODUCTION

Groundwater is a major source of irrigation in the world. If arsenic polluted groundwater is used for irrigation, serious problems may occur in agriculture production. Permanent irrigation of soil with arsenic polluted water may contribute to the accumulation of this toxic element in the topsoil or subsoil and after some time render the soil unfit for agriculture production (Bhattacharya et al., 2002). According to Jiang and Singh (1994), agricultural inputs like pesticides and fertilizers may also increase the concentration of arsenic in topsoil while environmental and climate conditions may contribute to its leaching into the groundwater. Plants can accumulate some amount from the soil or the contaminated irrigation water. The quantity of the accumulated As depends on plant variety and the contamination level. Arsenic is a phytotoxic element which may cause chlorosis, yield decrease, and stunt of the plant growth. Plants usually accumulate arsenic in roots and shoots, but some plants like rice, lettuce, carrot, and potatoes are capable to accumulate As in the edible parts of the plant making it unsuitable for human consumption or other intended use (Kabata-Pendias A. and Mukherjee B. A., 2007). The pollution of groundwater with arsenic has become a global concern problem. Polluted groundwater has been found in many parts of the world in different hydrogeological and geochemical conditions. Literature data show that majority of the arsenic polluted groundwater provinces are in young unconsolidated sediments, usually from

Quaternary or Holocene age in arid or semiarid settings (Rosas et al., 1999; Smedley et al., 2002), or in large alluvial deltaic plains (Berg et al., 2001; Smedley et al., 2005; Polya et al., 2005). This, heavy metalloid and oxyanion-forming element can reach the groundwater from natural sources like mineralization and geothermal activity or human activities like mining, industry, and the use of arsenical pesticides in agriculture and forest preservation. Arsenic pollution of groundwater which is related to mineralization and mining activities are localized in recognized regions and have been reported worldwide like the USA (Twarakawi N.K.C. and Kaluarachchi J.J., 2006) Canada (Bernard D.W., 1983; Grantham D.A. and Jones J.F., 1977), Africa (Smedley et al., 2007), Greece (Komnitsas K. et al., 1995), India (Chakraborti D. et al., 1999; Pandey P.K. et al., 2002), Mexico (Armienta M.A. et al., 2005), Thailand (Williams M., 1996), England (Thornton I., 1994), etc. Arsenic polluted groundwater associated with geothermal fluids has been reported in the USA (Wilkie J.A. and Hering J.G., 1998; Welch A.H. et al., 2000), Japan (Swedlund P.J. and Webster J.G., 1998), and New Zeland (Robinson B. et al., 1995). Data related to arsenic contamination of groundwater associated with pesticide applications are limited and until now have been reported only in the United States (Bednar AJ., 2002; Cai Y. et al., 2002 and Wiegand GE., 1999). In the Republic of Macedonia arsenic polluted groundwater has been found in Pelagonia valley the region of Prilep (max. 75 μg/L) (Mirčovski V. et al., 2014), and the region of Strumica (max. 117.8 75 μg/L) (Ivanova Š. and Ambarkova V., 2015). According to Ravenscroft et al. (2009), there are four mobilization mechanisms of arsenic in nature: reductive dissolution (RD), alkali desorption (AD), sulphide oxidation (SO), and geothermal (GT). The purpose of this study was to make an assessment of arsenic pollution origin in groundwater which is used for irrigation and situated under intensive agriculture activities using chemometric methods like single factorial analysis of variance and multivariant factor analysis.

MATERIAL AND METHODS

Investigated area

The investigation was conducted in the Strumica valley, located in the south-eastern part of North Macedonia, approximately 15 km to the west of the border with Bulgaria. The groundwater of the investigated area belongs to the transboundary Petrich valley aquifer shared by the Republic of North Macedonia and Bulgaria and it is hydraulically linked with the surface water of the Struma/Strymonas river basin (Fig.1). The Aquifer is made up of Pliocene, predominantly, and Quaternary Lake sediments, alluvial sands, gravels, clays, and sandy clays (UNECE 2011). The Strumica river is a transboundary tributary to the Struma/ Strymonas river which source is in western Bulgaria (Vitosha Mountain, south of Sofia) and ends in the Aegean Sea (Strymonikos Gulf – Greece) (Fig. 1). The discovered thickness of the basal lithozone ranges from 20-50 m (Rakicevik et al., 1973). The region is characterized by an intensive agriculture production since the 1950's when cotton was the main cultivated crop for the existence of domestic growers. The construction of the irrigation systems Turia and Vodocha in 1979, contribute to the replacement of cotton production with early vegetable production which contributes to the development of the food cane industry. The region is reached in hydro geothermal water which according to Gorgieva et al. (2000) belongs in the hydrothermal systems in the fractured granites of Paleozoic or Mesozoic age. Springs and boreholes with different temperatures are present within small distances in the village of Bansko. The maximum measured temperature is 73°C and the predicted maximum temperature is 120°C (Gorgieva, 1989). The reservoir in the granites lies under thick Tertiary sediments. An abundant mine with copper and gold deposits is present in the village of Ilovitca located in a northwest-southeast striking Tertiary magmatic arc, that covers large areas of Macedonia, Serbia, Central Romania, Southern Bulgaria, Northern Greece, and Eastern Turkey (Carter S., 2008).

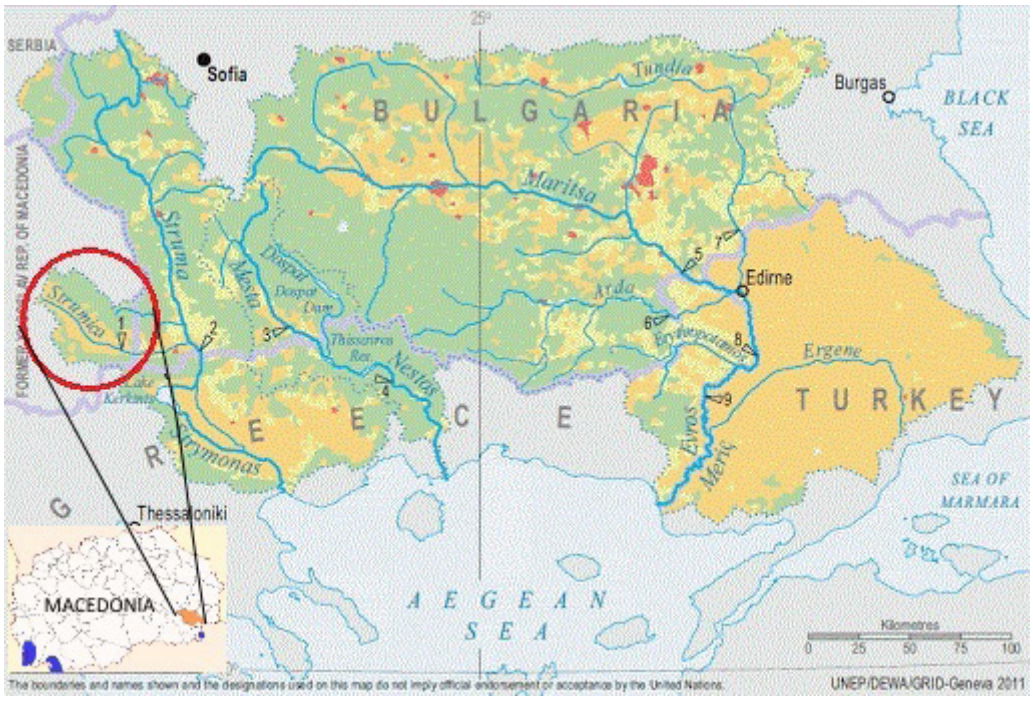

Figure 1. Geographic location of the investigated region, the Strumica valley.

Chemical analysis

Each sample was collected from a borehole located in the field of agricultural production, according to EPA guidelines (Johnston, 2007) and analysed for the quantity of major cations, anions, heavy metals, and trace elements. Anions like chlorine, carbonate, and bicarbonate were analysed by volumetric methods. Sulphate (SO_4^{2-}), nitrate (NO_3^-), nitrite (NO_2^-), and ammonia (NH_4^+) were determined by the colorimetric method using spectrophotometer type JENWAY 6715, UV Vis (EPA 375.4; EPA 352.1; EPA 354.1; EPA 350.2). pH is measured by pH meter HANNA HI 2211-01 and electrical conductivity is measured with conductometer JENWAY 4520, *in situ*. The total oxidation state of arsenic (As), magnesium (Mg), sodium (Na), potassium (K), calcium (Ca), phosphorus (P), manganese (Mn), copper (Cu), nickel (Ni), cobalt (Co), iron (Fe), zinc (Zn) and lead (Pb) were analysed by inductively coupled plasma-mass spectrometry (ICP-MS), (Agilent 7500 CX). The equipment was linearly calibrated from 1 to 100 µg /L, using a certified standard solution (Sigma ICP Multielement Standard Solution). Linearity was checked after every 10 samples. Accuracy has been tested by analysing a certified reference material, NIST SRM 1643 c "Trace elements in water." Bias ranged from 2 to 7%. Precision expressed as intermediate precision was better than 10% for all analysed elements.

Statistical analysis

Descriptive statistics analysis was used to perform analysis of data, including mean, median, maximum and minimums, standard deviation, and variance. One factorial ANOVA in excel was performed to see if there are significant differences of As concentrations in shallow and deep groundwater. Groundwater composition was subject to a factor analysis to understand the covariance structure between As and other variables. Varimax normalized type of rotation and multiple R – square methods were used for the extraction of the loadings. Descriptive and factor analysis are performed in the statistical program Statistica version 10 (StatSoft Inc., 2011).

RESULTS AND DISCUSSION

Chemical and physical characteristics of groundwater

A total number of 185 groundwater samples have been analysed for their quality and heavy metal content (Tab. 1). The depth of the investigated boreholes ranged between 4.5 and 130 m with a median of 21 m. The analytical results show that the pH values of groundwater samples varied between 6.84 and 8.67 with a median of 7.83, which indicates that waters are generally slightly alkaline. The electrical conductivity of groundwater varies between 1.22 and 17.49 dS/m at 25^{0}C with a median value of 4.74 dS/m. According to Sawyer N.G and Mc Cartly D.L. (1967), the total hardness expressed as mass of $CaCO_3$ ranged between 0.03 – 915.07 mg/L. Around 32% of groundwater samples belong in the very hard category, 25% in the hard category, 28% belong in the moderately hard category and 25% were characterized in soft category. All heavy metals and trace elements except As, Mn, and Fe are found below the national MCL (Maximum Concentration Limits).

Almost 35% of investigated samples have total arsenic content greater than 10 µg/l, from which 16% have concentrations greater than 50 µg/L with a maximum concentration of 176.56 µg/L. The most polluted were samples from the village of Robovo (eight out of nine investigated samples) with a concentration range from 65.23 – 176.56 µg/L, then samples from the village of Sachevo where seventeen out of nineteen investigated boreholes exceeded the level of 10 µg/l, with the concentration range from 23.31 to 172.42 µg/L. In the village of Ednokukjevo, thirteen out of eighteen samples (range 10.37 – 109.46 µg/L) and in the village of Borievo eleven out of twelve investigated samples were polluted (11.54 – 80.42 µg/L) with a concentration greater than 10 µg/l. Arsenic polluted groundwater was sampled mostly from the boreholes located in the central part of the valley characterized by alluvial plains and young aquifer.

Table 1. Statistic summary of concentrations of chemical variables resulting from the descriptive analysis of investigated samples

	Min	Max	Mean	Median	SD	CV
d (*m*)	4.50	130	40.2	21.00	34.32	85.36
pH	6.84	8.67	7.85	7.83	0.45	5.67
ECw (*dS/m*)	1.22	17.49	4.88	4.74	2.46	50.41
HCO_3^- (*mg/L*)	0.04	750.97	269.65	265.25	156.61	58.08
Cl^- (*mg/L*)	4.19	614.31	39.59	25.13	55.77	140.88
NO_3^- (*mg/L*)	0.14	284.44	23.30	2.98	45.50	195.27
NO_2^- (*mg/L*)	<LOD	35.85	0.73	0.025	3.99	546.69
NH_4^+ (*mg/L*)	<LOD	55.89	1.12	0.09	5.01	448.86
SO_4^{2-} (*mg/L*)	<LOD	300.45	24.97	17.57	37.73	151.06
Na (*mg/L*)	1.4	36.71	7.06	5.95	5.07	71.84
PO_4^{3-} (*mg/L*)	<LOD	7.8	0.54	0.19	1.1	202.62
K (**µg/L**)	1.15	354.44	12.06	5.38	2.35	16.58
Ca (*mg/L*)	7.43	411.18	51.10	39.84	39.61	77.52
Mg (*mg/L*)	1.07	96.14	13.55	9.77	12.51	92.33
As (**µg/L**)	0.08	176.56	21.58	2.60	38.51	178.45
Mn (**µg/L**)	<LOD	3328.88	465.10	288.55	606.78	130.46
Fe (**µg/L**)	<LOD	3165.71	212.29	71.69	386.89	182.25
Ni (**µg/L**)	0.32	21.58	3.36	2.59	2.67	79.49
Cu (**µg/L**)	<LOD	21.55	1.35	1.04	1.74	128.66
Zn (**µg/L**)	2.34	1371.41	49.79	14.22	160.16	321.67
Pb (**µg/L**)	0.06	16.35	0.92	0.47	1.66	181.78
Co (**µg/L**)	0.25	2.1	0.39	0.25	0.36	91.62

Most of the arsenic polluted samples (42 samples) have depth between 21-100 m. Only fifteen samples have depth not greater than 20 m and 7 samples have a depth between 100 - 125 m. The contaminated groundwater is mostly alkaline (pH between 7.5 – 8.53), with a high concentration of bicarbonate (HCO_3^- 177.06 – 511.87) and moderate conductivity (ECw 2.48 – 7.2) (Tab. 2). Arsenic bearing rocks like calcite forms of limestone, iron oxide minerals, and sodium feldspars are common for the investigated region (Rakicevik and Pendzerkoski 1973). Groundwater from the boreholes in the village of Bansko, an area rich in geothermal springs, shows no significant content of arsenic in groundwater. The most important geothermal spring in this region has an arsenic concentration of 22.52 µg/L, suggesting that arsenic presence in groundwater in the region have no geothermal origin. Reducing environment present in the groundwater of the investigated area, high Fe, Mg, HCO_3 as well as low SO_4 and NO_3 content suggests that reductive dissolution is a major mechanism by which arsenic is released into the groundwater.

Table 2. Statistic summary of concentrations of chemical variables resulting from the descriptive analysis of arsenic polluted samples

	Min	Max	Mean	Median	SD	CV
d (m)	17.00	125.0	64.77	76.50	38.38	59.26
pH	7.50	8.53	8.02	8.035	0.34	4.22
ECw (*dS/m*)	2.48	7.20	4.95	4.98	1.17	23.65
HCO_3^- (*mg/L*)	177.06	511.87	359.23	385.45	92.34	25.70
Cl^- (*mg/L*)	6.28	49.53	15.89	11.09	11.67	73.45
NO_3^- (*mg/L*)	0.65	19.81	3.77	1.60	5.33	141.27
NO_2^- (*mg/L*)	< LOD	0.120	0.03	0.03	0.02	63.23
NH_4^+ (*mg/L*)	< LOD	17.930	1.51	0.39	3.39	224.34
SO_4^{2-} (*mg/L*)	0.77	25.760	7.06	2.50	7.91	112.01
Na^+ (*mg/L*)	1.66	18.350	8.39	7.45	3.61	42.98
PO_4^{3-} (*mg/L*)	< LOD	7.80	1.09	0.23	1.87	172.32
K (*mg/L*)	1.23	10.26	4.83	4.84	2.59	53.59
Ca (*mg/L*)	12.71	70.97	41.40	37.44	16.22	39.18
Mg (mg/L)	3.39	42.33	9.30	6.51	9.26	99.63
As (**µg/L**)	50.04	176.56	101.93	90.60	38.61	37.88
Mn (**µg/L**)	68.42	2175.17	692.13	592.86	498.72	72.06
Fe (**µg/L**)	28.01	1048.61	258.33	112.52	270.58	104.74
Ni (**µg/L**)	0.54	8.99	2.64	1.70	2.49	94.17
Cu (**µg/L**)	< LOD	4.25	0.86	0.38	1.00	115.7
Zn (**µg/L**)	2.90	88.73	21.73	12.1	22.04	101.43
Pb (**µg/L**)	< LOD	16.35	1.16	0.25	3.04	261.92
Co (**µg/L**)	< LOD	0.70	0.30	0.25	0.12	40.24

Highly polluted samples with arsenic concentration greater than 50 µg/L are characterized with low content of sulphate (0.77 – 25.76 µg/L), phosphate (0.025 – 7.8 µg/L), potassium (1.23 – 10.48 µg/L), calcium (12.71 – 75.48 µg/L), magnesium (3.39 – 42.33 µg/L), nickel (0.54 – 8.99 µg/L), cuprum (0.25 – 4.25 µg/L), zinc (2.91 – 88.73 µg/L), lead (0.25 – 16.35 µg/L) and cobalt (0.25 – 0.7 µg/L). Concentrations of iron (28.01 – 3165.71 µg/L) and manganese (68.42 – 2175.17 µg/L) showed higher values than in unpolluted samples.

Statistical analysis

One factorial ANOVA

Single-factor ANOVA was performed to investigate if there are significant differences between As concentrations in shallow groundwater with depth up to 40 m and deep groundwater with a depth greater than 40 m. For that purpose, the obtained values for As concentrations were previously normalized using Box-Cox transformation. The analysis showed that F (37.97) is higher than F critical (3.89) and p-value (4.17 E^{-9}) is much lower than 0.05 which indicates that there is a significant

difference between shallow groundwater and deep groundwater regarding As concentration. The mean values of As concentrations 15.86 ppm and 34.05 ppm for shallow and deep groundwater respectively, indicate that higher concentrations are present in deep groundwater suggesting its natural origin.

Table 3. Single factor ANOVA for As concentrations in shallow and deep groundwater

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	93.83	1	93.83	37.97	4.17E-09	3.89
Within Groups	472	191	2.47			
Total	565.83	192				

Factor analysis

Factor analysis performed for all groundwater samples revealed two factors with eigenvalues greater than one which explains only 29.96% of the total variance and 44.35% of communalities leaving too many residuals (unexplained sums of squares) (Table 4). Due to the information from this analysis, it was not possible to give a logical explanation of the association of variables. It is assumed that this outcome due to different chemical processes dominated in shallow and deep groundwater. The composition of shallow groundwater is more prone to the processes of sorption and desorption as a result of clay and organic matter content in the topsoil and subsoil. The composition of deeper groundwater is more prone to the processes related with the aquifer composition. To lower the percent of residuals, and to obtain more clear associations, factor analysis was performed separately for shallow and deep groundwater. Factor analysis for shallow groundwater revealed four factors with eigenvalues greater than one which explain 43.64% of the total variance and 50.19% of communalities (Table 5). The analysis positively associates As with HCO3- and Mn suggesting that arsenic is mobilized in groundwater by reductive dissolution of Mn oxides from the bedrock. Factor analysis for deep groundwater revealed five factors with eigenvalues greater than one. Arsenic was positively associated with HCO3-, Ca, and Mn in the third factor which explains 10% of the total variance (Table 6). The obtained result is in accordance with the association obtained from the analysis of shallow groundwater, which is difficult to conclude based on the analysis when shallow and deep groundwater were statistically processed together.

Table 4. Factor analysis for all investigated samples

	F1	**F2**	**Comm**
HCO_3^-	-0.15	**0.76**	70.34
Cl^-	**0.68**	**0.55**	85.64
NO_3^-	**0.56**	-0.01	47.48
SO_4^{2-}	**0.67**	0.27	64.97
Na	0.11	0.04	29.39
PO_4^{3-}	-0.16	-0.06	17.88
K	0.29	0.10	45.74
Ca	**0.61**	**0.65**	88.76
Mg	0.45	**0.50**	67.71
As	**-**0.49	030	36.04
Mn	-0.16	**0.60**	44.44
Fe	-0.30	0.12	15.59
Ni	0.23	0.32	37.36
Cu	0.19	0.02	18.74
Zn	0.01	0.10	16.67
Pb	0.06	-0.00	10.53
Co	0.43	**0.55**	56.67
E-value	3.69	1.41	**44.35%**
Total variance %	21.68	8.28	

Table 5. Factor analysis of investigated variables for shallow groundwater of the Strumica region

	F1	**F2**	**F3**	**F4**	**Comm**
HCO_3^-	0.38	**0.67**	0.04	0.01	71.03
Cl^-	**0.87**	-0.01	-0.07	0.14	86.85
NO_3^-	0.23	-0.27	0.07	**0.66**	55.16
SO_4^{2-}	**0.69**	-0.21	0.03	0.15	67.04
Na	0.15	0.05	-0.27	0.28	29.28
PO_4^{3-}	-0.22	-0.05	0.05	**0.54**	31.44
K	0.18	0.01	-0.21	0.47	53.15
Ca	**0.84**	0.11	0.18	0.24	90.28
Mg	**0.71**	0.04	-0.12	-0.12	65.83
As	-0.18	**0.65**	-0.04	0.02	43.37
Mn	0.28	**0.58**	0.07	-0.09	54.30
Fe	-0.09	0.34	-0.00	-0.05	13.41
Ni	0.24	0.24	0.36	0.35	39.22
Cu	0.09	-0.08	**0.59**	-0.03	35.09
Zn	0.13	0.02	**0.61**	-0.11	37.46
Pb	-0.06	0.03	0.25	0.04	13.36
Co	**0.74**	0.12	0.12	0.05	66.92
E-value	3.71	1.63	1.09	1.01	**50.19%**
TV %	21.85	9.58	6.40	5.95	

Table 6. Factor analysis for investigated variables for deep groundwater of the Strumica region

	F1	**F2**	**F3**	**F4**	**F5**	**Comm**
HCO_3^-	-0.12	0.24	**0.86**	-0.01	-0.13	86.33
Cl^-	**0.84**	0.15	-0.06	0.19	0.12	84.43
NO_3^-	**0.58**	-0.32	-0.11	0.13	-0.26	71.20
SO_4^{2-}	0.41	0.10	0.06	0.05	**0.71**	69.35
Na	0.13	0.06	0.09	0.09	**0.77**	79.76
PO_4^{3-}	-0.04	0.05	0.09	**-0.75**	-0.07	57.72
K	**0.84**	0.04	0.05	-0.07	0.31	84.14
Ca	0.40	-0.01	**0.80**	0.15	0.12	85.21
Mg	0.22	0.14	0.16	-0.03	-0.45	54.66
As	-0.23	0.14	**0.59**	-0.07	0.03	53.85
Mn	-0.04	-0.39	**0.50**	-0.14	-0.21	63.87
Fe	-0.07	0.01	-0.05	**-0.65**	-0.08	48.10
Ni	0.21	**-0.79**	-0.00	0.11	-0.09	74.67
Cu	0.36	-0.37	-0.19	0.23	-0.01	60.74
Zn	-0.04	-0.14	0.07	0.09	-0.09	32.65
Pb	-0.10	**-0.63**	-0.25	-0.03	0.13	68.88
Co	-0.06	**-0.75**	-0.05	0.00	0.03	65.85
E-value	3.00	2.56	1.83	1.44	1.24	**67.14%**
TV %	16.66	14.22	10.19	7.98	6.91	

CONCLUDING REMARKS

The assessment of arsenic pollution of groundwater situated under the intensive agriculture activities was investigated in this study. The investigation was performed on the Macedonian part of the Petrich valley aquifer, located in the central part of the Strumica valley. Although, the region has potential for agrochemical, industrial and geothermal pollution, the investigation shows that groundwater is naturally contaminated from arsenic reach geological formations.

The mechanism of reductive-dissolution from Mangan oxides are recognized as the main process that contributes to groundwater pollution. The obtained concentration levels of pollution show that groundwater from these contaminated boreholes could be hazardous for humans and animals and should not be considered as a potential source for drinking water. Regarding the agricultural production no significant symptoms of plant toxicity were observed in the field (unpublished data). Even though, there should be an awareness for the possible threat of As contamination in the critical points for agriculture production in the future. The investigation of soil pollution in these critical points should be priority in order to determine the impact of polluted irrigation water in the region.

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ЕДНОФАКТОРИЈАЛНА АНОВА ВО ПРОЦЕНКА НА КВАЛИТЕТОТ НА ПОДЗЕМНИТЕ ВОДИ ВО КРИТИЧНИ ОБЛАСТИ НА ЗАГАДУВАЊЕ КОЕ ПОТЕКНУВА ОД ЗЕМЈОДЕЛСКИ АКТИВНОСТИ

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Резиме

Зголемени концентрации на арсен се детектирани во подземните води на Струмичкиот регион, лоциран во југоисточниот дел на Македонија, каде што интензивно земјоделско производство е концентрирано на површина од околу 1000 km^2. Од вкупно 185 примероци на подземна вода собрани од различни бушотини, 64 покажаа концентрации повисоки од 10 µg/l. Од нив, 30 примероци имаа концентрации повисоки од 50 µg/L со максимална концентрација од 176,56 µg/L. Ваквите зголемени концентрации на арсен се забележани кај примероците од подземна вода собрани од бушотини лоцирани главно во централниот дел на Струмичката Котлина кој се карактеризира со алувијални почви и млади подземни базени. Околу 57% од загадените примероци се собрани од бушотини со длабочина поголема од 40 m. Редуцирачката средина, високите вредности за Fe, Mn, HCO^{3-}, како и ниските вредности добиени за SO_4^{2-} и NO_3^- сугерираат дека редуктивната дисолуција е главниот механизам на ослободување на арсенот во подземните води. Примероците каде што беа забележани повисоки концентрации на As се карактеризираат со високи концентрации на Mn и Fe. Другите испитувани јони беа присутни во ниски концентрации. Анализата со еднофакторијалната АНОВА покажа значителна разлика помеѓу концентрациите на As во плитките (< 40 m) и длабоките (> 40 m) примероци на подземна вода. Мултиваријантната факторна анализа покажа позитивна корелација помеѓу As, HCO^{3-} и Mn во плитките примероци и As, HCO_3^-, Ca и Mn во длабоките примероци на подземна вода. Ваквиот резултат оди во прилог на заклучокот дека As е ослободен во подземните води со редуктивна дисолуција на оксидите на Mn кои влегуваат во составот на карпите од подземните базени.

Клучни зборови: *арсен, плитка подземна вода, длабока подземна вода, редуктивна дисолуција.*

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 19, No. 2, 2021

Manuscript received: 07.12.2021
Accepted: 13.12.2021

In print: ISSN 2545-4447
On line: ISSN 2545-4455
doi: https://doi.org/10.46763/JAPS21192037k
Original scientific paper

REAL TIME PCR METHOD FOR PPV DIAGNOSTIC ON PLUMS AND APRICOT IN THE REPUBLIC OF NORTH MACEDONIA

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Abstract

Real-time PCR (Polymerase Chain Reaction) or qPCR is a method by which the amount of the PCR product can be determined in real-time, and is very useful for investigating gene expression. The main advantages of qPCR are that it provides fast and high-throughput detection and quantification of target DNA sequences in different matrices. The lower time of amplification is facilitated by the simultaneous amplification and visualization of newly formed DNA amplicons. The development and application of molecular methods for the detection of pathogens has significantly changed the diagnosis and control of plant diseases, various environmental samples, including hosts tissues, soil, water and air. With real-time PCR method, it is possible not only to identify and detect the presence or absence of the target pathogen, but it is also possible to quantify the amount present in the sample allowing the quantitative assessment of the number of the pathogen in the sample. Detection and accurate identification of plant pathogens is one of the most important strategies for controlling plant diseases to initiate preventive or curative measures.

Plum pox virus (PPV), the agent of sharka, is the most devastating virus infecting stone fruits. The PPV control is mainly based on prevention, and its quick and reliable detection is considered crucial in this strategy. In this study DAS-ELISA and real-time PCR were compared for evaluating their potentialities and limits for large scale surveys. Plum (*Prunus domestica* L.*)* hosts and apricot (*Prunus armeniaca* L.) samples from several different locations were included in laboratory test analyzes, plant organs (phloem, buds, flowers, leaves and fruits) and parts of them, different seasons (spring, summer and winter period 2017/20), presence or absence of symptoms were considered for comparison. Using DAS-ELISA tests and a universal set of antibodies (BIOREBA), have proved the presence of virus of *Plum pox* in all examined samples, especially from samples collected in summer, but also in virus status examination in winter and early spring season. The examination found high concentrations of viral antigens in plant samples (OD 2.912-2.752, for 30 min / 405 nm). Real-time PCR show amplification plot for positive PPV samples on plums and apricot.

Keywords: *DAS-ELISA tests, real-time PCR, molecular methods, plant disease detection, target pathogen, PPV*

INRODUCTION

Plum pox potyvirus (PPV), the causal agent of sharka disease, is most economically destructive virus diseases affecting stone fruits in Europe and Mediterranean region. The virus is very detrimental because it reduces the quality of fruits and cause a premature dropping (Dunez & Sutic, 1988; Nemeth, 1994). PPV is considered one of the significant limiting factors for a profitable plum growing, predominately peaches, nectarines, apricots, plums and prunes (Plesko et al., 2011).

Since its discovery, sharka has been considered as a calamity in plum orchards. In highly susceptible plum varieties present in North Macedonia, such as Požegača and Stenlej, PPV causes a premature fruit drop and reduces fruit quality, which leads to total yield loss. The same symptoms and loses are obviously in the peach and cherry orchards. Eight PPV strains (PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec, PPV-W, PPV-T and PPV-CR) have been recognized so far. Three major strains (PPV-M, PPV-D and PPV-

Rec) are the most widely dispersed and occur frequently in many European countries (Bagi et al., 2016, Jevremović, 2012).

DAS-ELISA test (Double antibody sandwich enzyme – linked immunosorbent assay) using BIOREBA kits and One Step Real Time PCR using Applied Biosystems 7500 Real-time PCR System, are one of the most effective molecular diagnostic tools for PPV.

The method of choice for quantification of nucleic acid (DNA, RNA) in all areas of molecular biology is the polymerase chain reaction in real-time or the quantitative PCR (qPCR) method. Quantitative PCR is the "gold standard" technology for quantifying nucleic acids, and since its first report describing real-time PCR detection in 1993, its use has grown exponentially. Newer technological advances expand the range of applications, from high-resolution melting detection to digital PCR. Nowadays, it is a very affordable technique to achieve robust and reliable analysis. Real-time PCR (Polymerase Chain Reaction) or qPCR is a method by which the amount of the PCR product can be determined, in real time, and is very useful for investigating gene expression. The main advantages of qPCR are that it provides fast and high-throughput detection and quantification of target DNA sequences in different matrices. The lower time of amplification is facilitated by the simultaneous amplification and visualization of newly formed DNA amplicons (EPPO 2004, 2006).

The most obvious is the use of qPCR in molecular diagnostics, where it is slowly replacing conventional methods. It is used to detect, identify and quantify disease-causing microorganisms (bacteria, viruses and fungi). With qPCR the metro decreases, contamination and erroneous results. It also allows large quantities of samples to be processed in less time (up to 384 or even 1536 reactions) and has thus proven to be an indispensable method in diagnostic laboratories. However, it should be noted that the method detects only the presence of DNA or RNA of a microorganism and does not report its viability (Varga & James, 2005). As a result, conventional microbiological techniques are sometimes still needed along with qPCR, is also used to detect and quantify genetically modified organisms or to perform genotyping. This means that different alleles of the same gene or individual nucleotide polymorphisms (SNPs) can be detected that can be used as genetic diagnostic or prognostic markers for certain diseases (Klarik & Ricchi, 2017).

MATERIAL AND METHODS

Plant material

One of the most important strategies for controlling plant diseases is accurate, early detection and identification of plant pathogens. In fact, this is the basis of plant disease management. Improved disease control with appropriate phytosanitary monitoring for identification and diagnosis of viruses, bacteria, fungi, phytoplasmas, nematodes and insects as well as the introduction of new diagnostic methods for detection of pathogenic plants are necessary in order to respond to emerging environmental challenges in agriculture. Detection and accurate identification of harmful plant pathogens is essential to improve plant disease control strategies. Early detection and identification of plant pathogens and viruses provide a basis for understanding their biology and appropriate strategies for controlling that particular pathogen.

Laboratory analyses

The laboratory analyses of the collected material were completely performed in the UNILAB laboratory at the Department of Plant and Environmental Protection, Faculty of Agriculture.

For direct PPV detection using DAS ELISA Technique, 0.5 g of fresh leaves were homogenized with tissue homogenizer (BIOREBA, by using Bioreba extraction bags) with 5 ml plant extraction buffer from commercial kit. Different laboratory analyses and methods are used to identify PPV (*Plum pox potyvirus*), ELISA serological method, and the greatest emphasis is placed on laboratory analysis using state-of-the-art molecular real-time PCR analysis. DAS ELISA "ready to read plates" were read after 30 and 60 min of incubation at 25°C and samples were considered positive if absorbance (A_{405nm}) was greater than or equal to two times that of

negative control.

Real-time PCR (quantitative PCR, qPCR) is now a well-established method for the detection, quantification, and typing of different microbial agents in the areas of clinical, veterinary diagnostics, environmental, food safety, plant disease its. Although the concept of PCR is relatively simple, there are specific issues in qPCR. These include the use of correct terminology and definitions, understanding of the principle of PCR, difficulties with interpretation and presentation of data, the limitations of qPCR in different areas of microbial and environmental diagnostics, parameters important for the description of qPCR performance (Fig. 1).

With the increasing amount of sequencing data available, it is literally possible to design qPCR assays for every microorganism (groups and subgroups of microorganisms, etc.) of interest. The main advantages of qPCR are that it provides fast and high-throughput detection and quantification of target DNA sequences in different matrices. The lower time of amplification is facilitated by the simultaneous amplification and visualization of newly formed DNA amplicons. Moreover, qPCR is safer in terms of avoiding cross contaminations because no further manipulation with samples is required after the amplification. Other advantages of qPCR include a wide dynamic range for quantification (7–8 Log10) and the multiplexing of amplification of several targets into a single reaction. The multiplexing option is essential for detection and quantification in diagnostic qPCR assays that rely on the inclusion of internal amplification controls. The principle of real-time PCR relies on the use of fluorescent dye. In general, the principle of the present method is stated below. The amount of the nucleic acid present into the sample is quantified using the fluorescent dye or using the fluorescent-labelled oligos. When a dye or probe binds with the target template, it releases a fluorochrome which resultantly emits fluorescence for the detector to detect. The detector captures a signal as a positive template amplification. Two types of chemistry are available for the real-time quantitative PCR:

- DNA binding dye (Intercalating dye-based method)
- Sequence-specific probe (Hydrolysis Probe-based detection method)

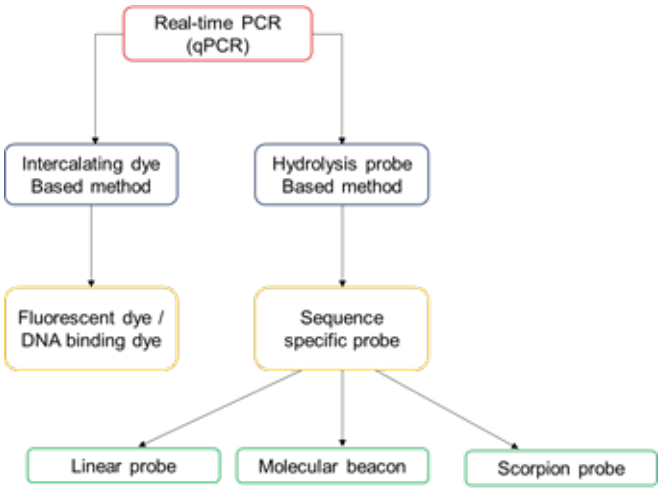


Figure 1. Principle of real-time PCR (qPCR)

If DNA is present in the sample in a higher quantity, amplification and quantification start at the early stage of the reaction; otherwise, the amplification starts in the late stage. As like the conventional PCR, there are three main steps in real-time PCR:

- Denaturation
- Annealing
- Extension

Denaturation occurs at 94°C where the double-stranded DNA is denatured and two single-stranded DNA is generated. The DNA is melted. This single-stranded DNA is the sight of the annealing for the primers in the later step of the amplification.

Annealing occurs at 55°C to 66°C in which the sequence-specific primer binds to the single-stranded DNA. Along with it, the fluorescent dye or the probe bind to the DNA sequence too. Extension occurs at 72°C at which the Taq DNA polymerase activated highest. In this step, the Taq adds dNTPs to the growing DNA strand. The real-time quantitative PCR is more sensitive and accurate than the endpoint PCR. Because, the amplification is measured in real-time, during the reaction. After each reaction, the fluorescence is emitted and it is reported by the detector.

The real-time PCR method is undoubtedly more accurate and reliable than other methods (Kralik & Ricchi, 2017).

It is used for the quantification of DNA, RNA and gene expression. The sample source for the real-time quantification is gDNA, cDNA, RNA, Gene of interest, synthetic oligos, total RNA or plasmid DNA. The real-time or quantitative analysis is divided into two other methods:

- Standard curve analysis
- Relative quantification

Advantages of Real-time PCR:

1. The method is cost-effective.
2. It is time-efficient
3. More sensitivity and specificity
4. Fewer templates required

DNA binding dye method is the best technique for real-time detection.

The dye has its own fluorescence. Once the dye binds to the double-stranded DNA the fluorescence emitted by the dye increases 100 to 1000-fold than the original signal. However, the original dye fluorescence is taken as the baseline (as a reference) for the detection.

The method is rapid, quick, reliable and cost-effective. Also, the chance of error in the experiments is less and the reaction setup is simple & easy to use.

The result of the experiment depends on the specificity of the primers used in the PCR reaction. Because even though the primers remain bound non-specifically, the DNA binding dye binds to the non-specific sequence and gives the fluorescent signals. As the dye detects the double-stranded DNA to bind, even if the dsDNA is non-specific, the dye binds to it. Therefore, the chance of the non-specific detection is high in the SYBR green dye-based method. The SYBR green is one of the most popular dyes used in real-time PCR.

A melting curve analysis helps to identify non-specific bindings during the reaction. After completion of the amplification reaction and capturing fluorescence signals, melting the template (again) determines non-specific bindings if any. During melting, at a high temperature, the template starts denaturing which consequence dye dissociation and reduce fluorescence.

The quantification is achieved by amplifying and monitoring the DNA or RNA present in the sample. For the quantification of the gene expression, the RNA is quantified into the real-time PCR.

Eight PPV strains (PPV-M, PPV-D, PPV-EA, PPV-C, PPV-Rec, PPV-W, PPV-T and PPV-CR) have been recognized so far. Three major strains (PPV-M, PPV-D and PPV-Rec) are the most widely dispersed and occur frequently in many European countries. DAS-ELISA test using BIOREBA kits and ONE Step Real Time PCR using Applied Biosystems 7500 Real-Time Laboratory analyses were performed on a 7500 real time PCR instrument with appropriate kits for detection and amplification of Plum pox virus - Plum pox virus One-Step Real-Time PCR with Taq-Man® technology.

The collected material or sample is first RNA extruded by manual method with a suitable plant RNA extraction kit - PureLink ™ RNA Mini Kit which provides a simple, reliable and fast column method for isolating high quality total RNA from a wide range of samples without the need for hazardous reagents such as phenol (RNeasy Plant Mini kit (Qiagene, USA).

The resulting RNA is then used for further laboratory analysis. The used kit for detection and amplification of Plum pox virus - Plum pox virus One-Step Real-Time PCR contains: prepared master mix, enzyme for reverse transcription, positive and negative control.

The protocol is entered into the real-time polymerase chain reaction instrument with the following conditions given in the appropriate detection kit.

The interpretation of the results is as follows:

The sample is positive:

- When the Ct value of the gain curve is below or equal to 35.

The sample is negative:

- When there is no amplification curve

- When the Ct value of the gain curve is higher than 35.

The cDNA od each sample was used in separate real-time PCR reactions for detection of PPV. The 20µl real-time PCR reactions for PPV were performed in 1H TaqMan Universal Master Mix (combine 19,375 µl of the PPV Master Mix with 0.625 µl of the RT-Enzyme per reaction, and 1/10 diluted cDNA as a template.

Real-time PCR reactions were run in duplicates for each undiluted or dilute cDNA on Applied Biosystems 7500 Real-time PCR Systems, using cycling conditions for the One-step Real-time RT-PCR kit (55°C for 15 min., 95°C for 10 min., 95°C for 15 sec. (denaturation) and 60°C for 60 sec. (annealing and elongation). Data were acquired and analysed using the 7500 Real-time PCR System Sequence Detection System Software v2.3.

RESULTS AND DISCUSSION

In our paper, plum (*Prunus domestica* L.*)* hosts and apricot (*Prunus armeniaca* L.) samples from several different locations were included in laboratory test analyses, plant organs (phloem, buds, flowers, leaves and fruits) and parts of them, in different seasons (spring, summer and winter period 2017/20), presence or absence of symptoms were considered for comparison (Fig. 2 & 3). DAS-ELISA and One Step Real Time PCR techniques are included to confirm the presence and concentration of PPV in different plant material (leaves, stem, flower and fruits). Using DAS-ELISA tests and universal set of antibodies (BIOREBA), has proved the presence of virus of Plum pox in all examined samples, especially in samples collected in winter and early spring season. The examination found high concentrations of viral antigens in leaves samples (OD 2.912-2.752, for 30 min, 405 nm) (Fig. 4).

Total RNA extraction and quality of extracted tot RNA was different between plant samples. The quality of totRNA from plum samples was high and uniform while quality of totRNA from apricot samples was much lower (data not shown).

Real-time PCR show amplification plot for positive samples (Fig. 5). For Real-time diagnostic we used One-Step Real Time RT-PCR kit for Detection of *Plum pox potyvirus*, by running method using the following conditions and pre-heat the thermal cycler block to 45°C.

Real-time positive sample is:

Ct value of the gain curve is below or equal to 35 (Ct=30 for our PPV positive samples)

The sample is negative:

When there is no amplification curve (negative control)

When the Ct value of the gain curve is higher than 35 (some of our plum and apricot samples) (Fig. 5b)

As expected, results have proved presence of *Plum pox potyvirus* (PPV) in plums and apricot in Eastern part of our country. The results were supported by application of state-of-the-art molecular methods for rapid, accurate detection and quantification of pathogenic viruses.

The results from laboratory testing and quantification of PPV from plum and apricot samples, showed that the highest concentrations of viral antigens were found in leaves, followed by flowers and stem, but the samples from fruits did not showed presence of PPV. The latest might be due to very low titter of antigen and they cannot be identified by DAS-ELISA.

Figure 2. Symptoms caused by PPV on plums (season 2019) – small and morphology deformation.

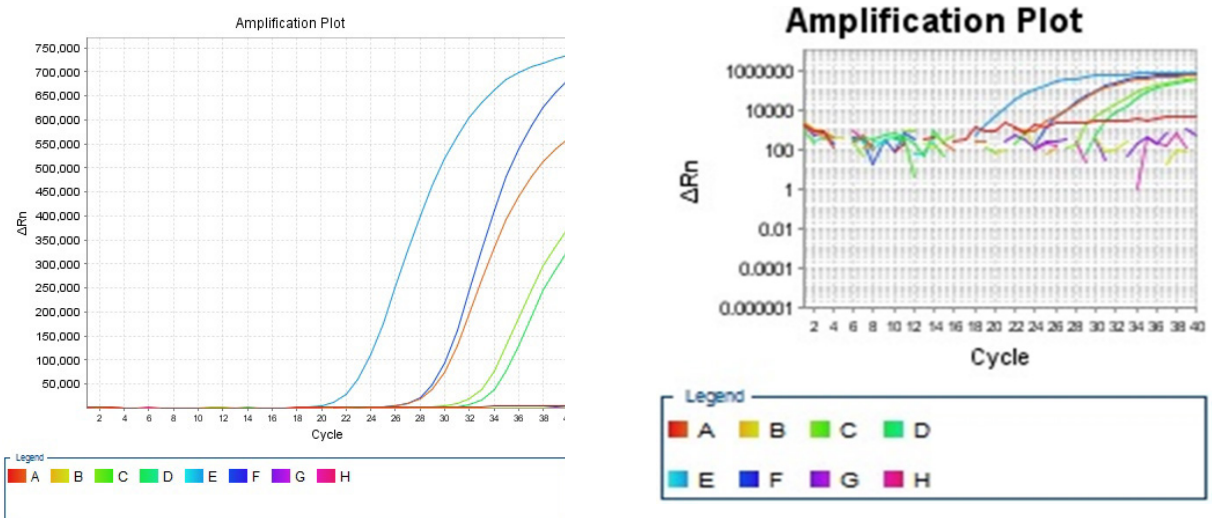
a) b)

Figure 3. PPV symptoms on apricot (season 2020)
a) Chlorotic rings on apricot leaves.
b) Chlorosis on leaves and morphological deformation on fruits.

Measurement count: 1 Filter: 405				30 min								
	1	2	3	4	5	6	7	8	9	10	11	12
A	2,912	2,752	2,728	2,866	0,096	0,1	0,093	0,097	0,092	0,099	0,096	0,094
B	0,094	0,082	0,09	0,083	0,787	0,836	0,866	0,869	0,085	0,083	0,085	0,081
C	0,18	0,161	0,448	0,437	0,444	0,384	1,201	1,211	1,247	1,38	1,243	1,196
D	1,584	1,119	1,003	0,967	1,276	1,673	0,686	0,654	1,016	0,999	1,118	1,212
E	0,22	0,15	0,979	0,878	0,987	0,972	0,822	0,664	0,076	0,083		

Measurement count: 1 Filter: 405				60 min								
	1	2	3	4	5	6	7	8	9	10	11	12
A	3,428	3,323	3,265	3,39	0,101	0,106	0,103	0,108	0,104	0,119	0,114	0,103
B	0,102	0,085	0,095	0,089	1,618	1,663	1,733	1,61	0,093	0,086	0,084	0,083
C	0,269	0,245	0,77	0,777	0,835	0,762	2,297	2,274	2,346	2,803	2,118	2,188
D	2,659	2,361	2,297	2,186	3,063	3,047	1,494	1,484	1,928	1,934	2,124	2,124
E	0,364	0,235	2,189	2,11	2,128	2,112	1,635	1,647	0,077	0,084		

Figure 4. Measurement count on ELISA reader, OD 405 nm, after 30- and 60-min. Green number is referring positive control and the last number from the plate is negative reference control. Yellow coloring indicates all our positive samples from plums in row A, B, C and D and E indicates apricot positive samples.



a) b)

Figure 5. One step Real Time PCR results from plum and apricot samples 2018/19 (using One-Step Real-time RT-PCR Kit, Taq-Man® technology).
a) Amplification curves associated to *Prunus* (blue curves for plum and green for apricot) and red line for negative control (healthy plant)
b) Amplification plot for positive *Prunus* samples, negative samples and negative control.

CONCLUDING REMARKS

One of the most important strategies for controlling plant diseases is accurate, early detection and identification of plant pathogens. In fact, this is the basis of plant disease management. Improved disease control with appropriate phytosanitary monitoring for identification and diagnosis of viruses, bacteria, fungi, phytoplasmas, nematodes and insects as well as the introduction of new diagnostic methods for detection of pathogenic plants are necessary in order to respond to emerging environmental challenges in agriculture.

Plant pathogens and viruses infect a wide range of plant species and cause high losses of yields crop quality. Detection and accurate identification of harmful plant pathogens is essential to improve plant disease control strategies. Early detection and identification of plant pathogens and viruses provides a basis for understanding their biology and appropriate strategies for controlling that particular pathogen.

PCR based methods, including real-time PCR are widely used for the detection of plant viruses. In the viral status detection, the most important method is choice of nucleic acid extraction procedure which can greatly influence the reliability of detection and

quantification of target sample. It is important to validate the extraction procedure for different sample matrixes and the ability of the extraction method to provide suitable nucleic acid from each matrix.

Utilization of DAS-ELISA tests and universal set of antibodies (BIOREBA), has proved the presence of virus status of *Plum pox* in all examined samples from plum and apricot, especially in samples collected during winter and early spring season. The results showed high concentrations of viral antigens in leaves samples (OD 2.912-2.752, for 30 min, 405 nm). The universal reagents in the DAS-ELISA format are detecting the '*full spectrum*' of PPV isolates (D, M, EA, C, W and Rec strains). In our study, we have much more samples detected by DAS-ELISA as positive, but we didn't confirm all of them by real-time PCR.

For real-time diagnostic we used One-Step Real Time RT-PCR kit and real time PCR, and pre-method of total RNA extraction by using Plant Mini Kit (Qiagene, USA). The results showed amplification plot for positive plum and apricot samples, randomly chosen ELISA positive samples were used for totRNA quantification. According to our results we would recommend utilization of different method and commercial kit for totRNA extraction and afterwards usage of cDNA dilute 1/10 in real-time.

Quantitative real-time PCR is an accurate, fast, sensitive, cheap and adequate method in genomic research. Real-time PCR has provided a significant value during pandemics or epidemics for sensitive, real-time and rapid detection of pathogens to reduce the mortality and morbidity rate. Real time PCR method is methodology that is used very often today in our country nowadays in human medicine diagnostics, but previously it was intensively exploited in plant pathology. qPCR technology represents a powerful tool in microbial, environmental, food, plant diagnostics.

ACKNOWLEDGEMENT

All laboratory work presented in this paper was carried at the Department of Plant and Environmental Protection, UNILAB laboratory (https://unilab.ugd.edu.mk/), at Faculty of Agriculture, Goce Delcev University, Stip.

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УПОТРЕБА НА МЕТОД ПОЛИМЕРАЗНО ВЕРИЖНА РЕАКЦИЈА ВО РЕАЛНО ВРЕМЕ ЗА ДИЈАГНОСТИКА НА ШАРКА КАЈ СЛИВИ И КАЈСИИ ВО РЕПУБЛИКА СЕВЕРНА МАКЕДОНИЈА

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Резиме

Полимеразна верижна реакција во реално време real time PCR (Real time Polymerase Chain Reaction) или qPCR е метод со кој количината на PCR производот може да се одреди во реално време и е многу корисен за истражување на генетската експресија. Главните предности на qPCR се тоа што обезбедува брза и високопропусна детекција и квантификација на целните ДНК секвенци во различни матрици. Краткото време на амплификација е олеснето со истовремено засилување и визуелизација на новоформираните ДНК ампликони. Развојот и примената на молекуларните методи за откривање на патогени значително ја промени дијагнозата и контролата на болестите на растенијата, различни примероци од животната средина, вклучувајќи ги ткивата на домаќините, почвата, водата и воздухот. Со методот на PCR во реално време, можно е не само да се идентификува и открие присуството или отсуството на целниот патоген, туку исто така е можно да се измери количината присутна во примерокот што овозможува квантитативна проценка на бројот на патогенот во примерокот. Откривањето и точната идентификација на растителните патогени е една од најважните стратегии за контрола на растителните болести за да се иницираат превентивни или куративни мерки.

Вирусот на шарка кај сливата (PPV) е најопасниот вирус што го инфицира коскестото овошје. Контролата на PPV главно се заснова на превенција, а нејзиното брзо и сигурно откривање се смета за клучно во оваа стратегија. Во оваа студија, DAS-ELISA и PCR во реално време беа споредени за евалуација на нивните потенцијали и граници за истражувања од големи размери. Домаќините на PPV сливата (*Prunus domestica* L.) и примероците од кајсијата (*Prunus armeniaca* L.) од неколку различни места беа вклучени за лабораториски тест анализи, со колекционирање на растителни органи (флоем, пупки, цвеќиња, лисја и плодови) и делови од нив во различни периоди од годината (пролет, летен и зимски период 2017-2020) и присуството или отсуството на симптоми беа земени како важен фактор за споредба. Со користење на DAS-ELISA тестови и употреба на универзален сет на антитела (BIOREBA) е докажано присуството на вирусот на шарка кај сливата во сите испитани примероци од слива и кајсија, особено од примероците собрани во лето, но и при проверка на статусот на вирусот во зима и рана пролетна сезона. Тестирањето откри високи концентрации на вирусни антигени во растителните примероци (OD 2,912-2,752 за 30 мин. / 405 nm). PCR во реално време прикажува амплификација за позитивни примероци PPV на сливи и кајсија.

Клучни зборови: *DAS-ELISA tests, real-time PCR, молекуларни методи, патоген, PPV.*

JOURNAL OF AGRICULTURE AND PLANT SCIENCES, JAPS, Vol 19, No. 2, 2021

Manuscript received: 18.08.2021
Accepted: 30.08.2021

In print: ISSN 2545-4447
On line: ISSN 2545-4455
doi: https://doi.org/10.46763/JAPS21192047t
Original scientific paper

THE ROLE AND IMPORTANCE OF AGROBIODIVERSITY FOR AGRICULTURE

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Abstract

Biodiversity and agriculture have an inseparable relationship, with interdependent interactions between their constituent components. Agriculture as a basic anthropogenic activity is one of the main factors in the directions of development in biodiversity. Agrobiodiversity is the basis of the existence of food production processes and provides and secures many essential aspects of modern living. Many of these processes are completely unknown, and some of them we are not even aware that they are a consequence of the biological activity of various organisms. Thus, biodiversity provides food, clean drinking water, energy, raw materials for industry, tourism and recreational opportunities, scientific research, and medicine. If agricultural practices that promote biodiversity are used, such as: crop rotation, cover crops, buffer zones, use of biopesticides, beneficial insects and intercrops, then natural processes in soil, plants and environment are significantly intensified for the benefit of farmers, plants and the overall environment. On the other hand, if techniques and methods that reduce biodiversity are applied in the agroecosystem and its environment, such as fertilizers, chemical pesticides, hormones, intensive processing, monoculture and others, processes of reduction, disappearance and pollution of the environment and the overall biodiversity occur. Therefore, careful selection of agricultural practices is needed that would not jeopardize the survival of the species, and at the same time high yields with good quality will be achieved.

The aim of this review paper is to present up to date relationship between biodiversity and agriculture and to highlight current issues of biodiversity loss and methods for its conservation.

Keywords: *genetic resources, indigenous varieties, agrobiodiversity, organic production, sustainable agriculture, protection, conservation*

INTRODUCTION

There are many different definitions of agrobiodiversity, but they all describe the variability of many plants, animals and microorganisms that are directly or indirectly involved in the production of food, textiles, fuels, pharmaceuticals, fodder, forestry, aquaculture and livestock, but also microorganisms, insects or animals involved in an agroecosystem such as predators, pollinators or soil microorganisms that have a beneficial effect on crops growth (Brookfield & Stocking, 1999).

Agrobiodiversity can be defined as an interaction between genetic resources, the environment and the practices undertaken by a group of different people, so managing water and soil as resources are quite different. Therefore, agrobiodiversity covers a wide range of animals, plants and microorganisms that are essential for sustainable agroecosystems (FAO, 2005).

Agrobiodiversity is a result of natural selection, but also many years of careful inadvertent selection of good and healthy plants and animals by farmers and ranchers (FAO, 2005). Farmers have seen by experience that only the best species survive and each year from their yields they keep only seeds with the best characteristics and highest quality for the next sowing or the healthiest animals for reproduction. Thus, according to FAO (2005) agrogenetic resources include:

1. Post-harvest residues of certain crops, animal species, wild plant species and

wild-caught animals.
2. Species that support the food production process such as soil microorganisms, pollinators, predators and other insects and animals.
3. The wider environment in which agroecosystems are located, that also has an impact on food production.

When we talk about agrobiodiversity, we should distinguish it from the term biodiversity which is a much broader term and includes agrobiodiversity itself. Biodiversity tells us about the overall variability of all living organisms that can be found on planet Earth. While agrobiodiversity includes only those organisms that are involved in agricultural production and food production (Fig. 1).

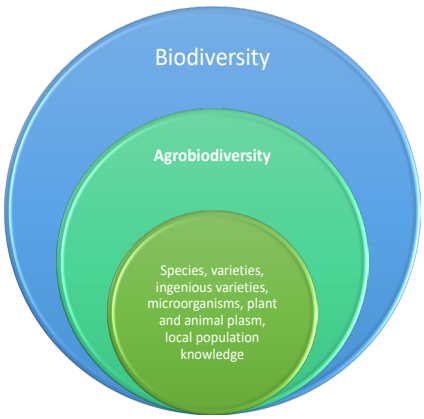

Figure 1. The components of biodiversity.

According to the Ecological Society of America agrobiodiversity could be divided into 3 interrelated levels (Fig. 2):

1. Genetic agrobiodiversity - includes all domesticated species and their wild relatives used in agriculture. It includes all newly created man-made varieties and animals. It includes wild species that are a significant source of genetic variability.
2. Species agrobiodiversity - includes all species whether wild or domesticated that are dependent on agricultural practices.
3. Agroecosystem variability - includes the ecological habitats of plants, animals and microorganisms that fit in time and space, at the level of farm, field or relief.

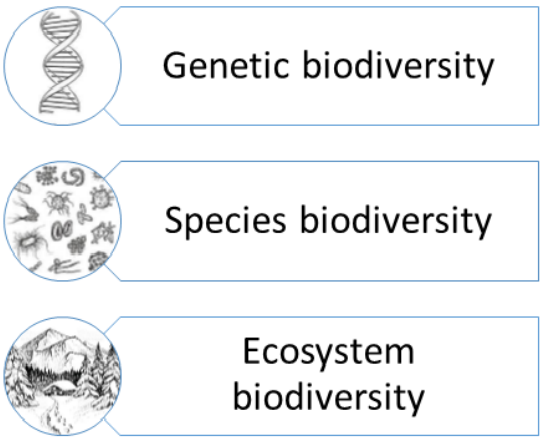

Figure 2. Three different levels of agrobiodiversity division.

Genetic resources as part of agrobiodiversity

All the diversity in the ecosystem is delivered from genetic resources. Genetic resources could be defined as a source of genetic variability. Total genetic material or all alleles from different genes present in cultivars and other wildlife is called plant genetic resources.

Plant genetic resources are once again called plant germplasm, gene pool or genetic reserve. Germplasm is a seed or other plant reproductive material, such as a leaf, stem, root, pollen, cell culture, DNA, from which it can be made as a mature plant. It is any kind of genetic material that can be used to protect the species and the populations. In the genetic sense of germplasm, a substance is felt in the cell nucleus, which determines the following properties of the organism and transmits their characteristics to future generations. Germplasm has not only reproductive value, but also with the selection and breeding can be improved and used to improve other crops and obtain new varieties (Ilieva, 2012). A variety could be defined as group of plants within a species, recognized for some improved features, that are retained after reproduction.

Ilieva (2012) according to origin of cultivated plants found in certain agroecosystem, divides them into four groups: indigenous species, introduced species, wild species, and breeding material (Fig. 3).

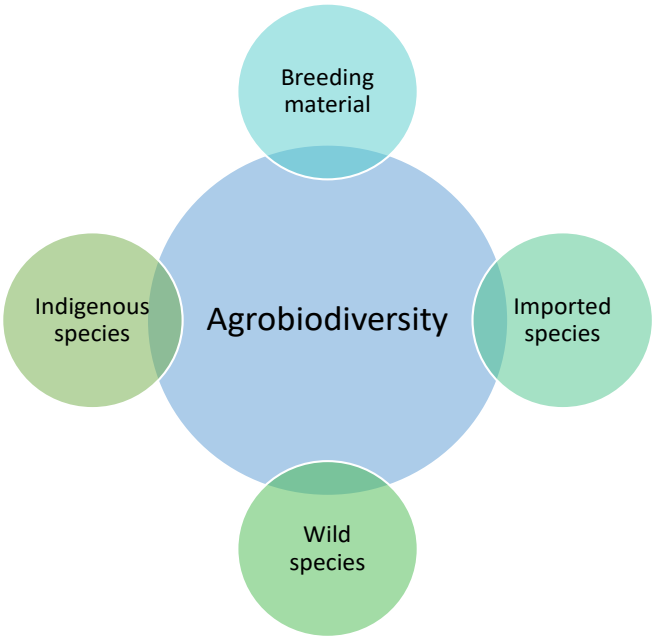

Figure 3. Agrobiodiversity's plant components.

Contribution of the biodiversity to agriculture

Agriculture represents a basic source of food for humans and animals as well as one of the main sources of raw materials for the textile, pharmaceutical and leather industries. The great diversity of our diet would not be possible without biodiversity. Biodiversity is the result of continuous evolution of plants and animals (Dudley & Alexander, 2017). The life we live would not be the same if the evolution of animals and plants did not take place in the direction in which it has taken place to this day. The large selection of crops contributes to the health and well-being of people through the various nutritional values that cultures possess. Biodiversity is the basis of agriculture. Its existence is crucial for the production of food and other agricultural goods, the benefits of which provide food, nutritional value and human well-being. Biodiversity is also the basis for the existence of all plant and animal species and their varieties (Convention on Biological Diversity, 2008).

According to the Convention on Biological Diversity (2008), services and benefits of biodiversity are:

1. Direct services - food, fuels, textile fibres, biochemicals, fresh water and genetic resources.
2. Regulatory services - flood protection, erosion control, pollination, pest control, climate impact and disease.
3. Cultural services - knowledge of farmers, profession, education, recreational services, cultural and religious values, inspiration.

4. Support to other processes - water cycle, nutrient cycle, primary production, provide habitat and atmospheric oxygen.

Apart from offering ecological and nutritional security through biodiversity, agriculture is also an important driver of socio-economic circumstances in society, with many people their main occupation being agriculture. The global agricultural workforce represents 1.3 billion people, agriculture is their main occupation. It represents 22% of the total population of the planet and 46% of the total working-age population (Harris, 2001). According to Bélanger & Pilling (2019) 7,000 out of approximately 24,000 known higher plants are directly involved in agriculture. Nevertheless, this distribution is not equal as out of these 7,000 plants only 30 plant species account for 90% of total agricultural production. Corn, wheat, rice, potatoes provide half of the total necessary nutrition of mankind. The situation is no different in the animal world, where out of 15,000 known mammals and birds, only 30-40 animals are domesticated, and 14 of them including chickens, cattle, pigs, sheep and goats occupy 90% of all domestic animals. That is the reason for huge genetic erosion in the last decades (Bélanger & Pilling, 2019).

Agriculture influence on biodiversity reduction

According to the Convention on Biological Diversity (2008), agriculture reduces biodiversity through the following practices:

1. Crop production

Intensive agricultural practices contribute to the reduction of biodiversity (Benton et al., 2003). Lack of crop rotation and cultivation in monoculture is one of those reasons. The use of pesticides and mineral fertilizers reduces soil biodiversity, destroying some important microorganisms and pollinators. Drainage systems and intensive tillage homogenize the fields and lead to a lack of diversity in ecosystems. Encouraging hybrids and superior varieties neglects the indigenous varieties, while the destruction of forests, swamps and meadows contributes to the loss of local flora and fauna.

2. Livestock production

Modern livestock production emphasizes animal yields rather than animal welfare. Nowadays intensive livestock farming is in stables where the animals are provided with constant access to water and selected food that stimulates them to produce more meat, milk or eggs. Increasingly, high-yielding breeds are being bred to replace indigenous and local domestic animals.

Biodiversity increases the resistance of plants to stressful conditions, provides an opportunity to adapt to adverse challenges and it is a key factor in increasing intake and reducing output in sustainable agricultural production (Isbell et al., 2015). The trend of increasing world population puts additional pressure on agricultural production to obtain higher yields, but at the same time restrictions on the use of chemical pesticides, fertilizers and lack of land appear as another limiting factor (Pimentel et al., 1997). All these facts pose a series of questions about how food and other ecosystem services would be provided without disrupting natural flows in nature. Therefore, biodiversity proves to be extremely important in terms of contributing genetic resources, pollinators, predators and microorganisms, if intensively involved in production, could replace some of currently used raw materials.

Biodiversity and soil fertility

All microorganisms such as bacteria, protozoa, worms, mice, insects, larvae, algae and fungi are components of biodiversity. Soil is a living substance that is home to thousands of micro- and macroorganisms, as well it is a basis for plant production (Giller et al., 1997). The number of micro and macroorganisms in the soil depends on soil type, location, presence of organic matter and climatic conditions (Petersen & Luxen, 1982; Koleva Gudeva et al., 2012). Most microorganisms are present in soils with high content of organic matter. The pedosphere is one of the richest with organisms, often up to 2-3 billion microorganisms can be found in 1 gram of soil. The number of microorganisms is referred to as soil biogenicity. Soil fertility and biogenicity are interrelated. Soils with higher biogenicity are also characterized by higher fertility (Hasan, 2000). The application of appropriate agro-technical and land management measures can increase the number of organisms in the soil, but also harm them.

Soil microorganisms are also crucial in the chemical change of soil and the transformation

of minerals, with the secretion of various organic acids that act on the soil minerals. For example, certain bacteria attack the highly resistant silicates, such as kaolinite, and break them down. They also act on some hard-to-dissolve fertilizers (Rodrıiguez & Fraga, 1999). Microorganisms play an important role in the oxidation and reduction of nitrogen, sulfur, iron and manganese minerals. With the help of nitrifying bacteria, NH_4^+ turns into NO^{2-} and then NO^{3-} a process called nitrification.

Biodiversity and plant pollination

Pollination of flowers can be done by wind or by animals such as insects, birds and bats. Insect pollination is essential for many plants (Fig. 4). Crops of the *Rosaceae* family are pollinated by insects. The most common insect pollinators in agricultural systems are honeybees represented with 20,000 species (Delaplane et al., 2000). Wasps, moths, butterflies, and beetles can also serve as pollinators (Rader et al., 2016). In addition to honeybees, there are other types of pollinators, such as beetles, which are bred and sold for successful pollination. Vertebrate pollinators include bats, monkeys, rodents, squirrels, lemurs, etc.

Pollination by insects and other animals provides us healthy and safe food, increases yield, enriches biodiversity, maintains ecosystems and ensures the survival of over 75% of plant species.

Figure 4. Insect pollinator (photo: Saso Arsov).

Biodiversity and the fight against insects and diseases

Modern agricultural production is almost unimaginable without the use of chemicals that help farmers in fight with diseases and pests (Mellon et al., 2001). But nature itself has its own mechanisms of elimination of the most common pathogens. The existence of biodiversity in agro-ecosystems optimizes the fight against weeds, diseases and pests. There are beneficial insects that feed on certain insects, such as ladybugs that feed with aphids (Francis et al., 2001). Insects are one of the most common and diverse organisms in the environment. More than a million different species of insects are described worldwide, and about 10,000 new species are described each year. The vast majority of insects are beneficial or neutral to crop production - less than 1% of known insect species are considered pests (Stork, 2018).

Some plant-eating insects reach harmful levels only under special conditions, while others are well-adapted to tolerate or exploit certain crops or crop production systems and can regularly cause economic losses. Understanding the environmental principles underlying insect population dynamics and community population interactions can help organic producers manage their farm insects, both pests and beneficial species, to prevent or reduce crop losses (Pal & McSpadden, 2006). The insect populations are dynamic, the number of individuals in a population can change on daily, seasonal and yearly basis as a result of interactions with the environment. The environment itself is changeable and can provide different availability of the resources that insects need to survive. The number of resources available can affect the size that an insect population can achieve (Khaliq et al., 2014). This concept is sometimes referred to

as the carrying capacity of the environment (Schowalter, 2019). Growing unwanted crops by rotation or resistant varieties and preserving natural enemies combines factors to reduce pest populations or promote the attraction of beneficial insects.

Other benefits of agrobiodiversity

According to Koleva Gudeva et al. (2012), other benefits of agrobiodiversity are: provision of medicines, wood products, diversity of the nature, protection of resources, climate impact, provision of genetic resources, tourist opportunities and scientific research.

In addition to the numerous benefits of agriculture, biodiversity is an essential component in many other industries and elements of human life (Institute of Biodiversity Conservation, 2005). The variety and abundance of medicinal plants open a new door for the pharmaceutical industry where most of the medicines are derived from plants. Biodiversity affects the microclimate of an environment, but also the macroclimate. The presence of forest strips makes the air pleasant and protects us from soil erosion. Forests are also the primary resource for the wood industry from which are made products such as furniture, firewood, building materials, art, etc. Ecosystem biodiversity has been used for several scientific studies that contribute to a better understanding of processes in nature and gives us answers for some long-standing questions. Biodiversity is a unique opportunity to attract tourists and visitors who admire the scenic views of nature and the diversity of plants, animals, and relief formations. However, all these industries harm biodiversity if resources are not used sustainably.

Agrobiodiversity and the organic production

Unlike the conventional farming systems, organic agroecosystems can use pesticides only as a last option and only limited non-synthetic or synthetic materials. Organic producers need to adapt practices which "maintain or enforce natural resource of work, including soil and water quality" (USDA, 2000). Environmental consideration of insects, especially their biology and interactions with plants, other organisms, and the environment, is essential to design a successful organic farm. The environmental understanding of the insects helps an organic farmer can develop a farm plan that can be used to reduce the risk of insects to reach the economic level of damage, identify them, and also use them as beneficial for different kind of agricultural services. Successful organic farm management has used the environmental knowledge of insects to recruit them as allies in terms of maintaining and preventing their natural resource base and reducing the chances of them becoming harmful (Culliney & Pimentel, 1986).

Each type of soil organism occupies a different sequence in the food chains and favours a different source of substrate and nutrients. Many organisms in the soil rely on the organic matter. A rich supply of diverse source of organic matter generally supports presence of wider spectre of organisms (Gomiero et al., 2011). It is highly recommended mixing and change of spatial-temporal distribution of plant species and varieties for creation of various resources that will stimulate soil biodiversity. Different habitats support complex mixtures of the organisms and through crop rotation or intercropping, different organisms may be present in the soil and their presence can support different processes: improvement of nutrient transport, natural processes for control of harmful organisms, improvement of soil fertility etc. (Watson et al., 2002). Soil biodiversity activity can be stimulated by improving the living conditions in soil, such as improving the aeration, suitable temperature, and quantity and quality of nutrients. In this regard, care should be taken to reduce tillage, minimize soil compaction and reduce chemical utilization.

Organic production is a great example of the symbiosis between modern agricultural production and the use of biodiversity. Organic production is based on the principle that integrates biodiversity and cares about the environment. It has a holistic approach to the overall ecosystem and respects natural flows in nature (Underwood et al., 2011). Thus, organic production requires a variety of techniques that promote and maintain biodiversity, such as intercropping, use of animal and green manure, use of biopesticides and biological control, companion plants, reduced soil treatment, buffer strips, etc.

Agrobiodiversity as an opportunity to save the yield

Agricultural production today is reduced to growing new high-yielding varieties that are in demand in the market (Frankel & Soule, 1981). Therefore, in one region can happen only one or two varieties of a certain crop to be grown. This increases the risk of losing yields, because that variety, despite all the good features, probably has a weakness in certain environmental conditions. Thus, the loss of the yield of that dominant variety in a certain vegetation year as a consequence of various factors would mean the loss of the full yield. Hence, the existence of divergence would somehow ensure farmers' work. Possession of indigenous crop populations on fields would mean possession of varieties resistant and adapted to the microclimate (Vasić et al., 2013). They are product of natural long-term selection that has taken place continuously in the same area (Cleveland et al., 1994). Thus, these varieties have the best resistance to climatic factors that occur in that region, but also to sporadic pathogens. On the other hand, their feature is the poor- and low-quality yield that does not meet the needs and tastes of the modern consumer. It is important to note that yields in local populations are low but quite stable, especially in critical environmental conditions.

Local varieties are characterized by specificity for only one specific region, and they have high heterogeneity. Their heterogeneity is often used by breeders to enhance an existing variety that lacks some quality. They are characterized by high plasticity and tolerance. They are resistant to lodging, low and high temperatures, pathogens, etc., but the main reason for their avoidance in commercial production are the low yields. Consequently, many species have been either completely lost or lost for local utilization (Jarvis et al., 2011).

Agricultural practices to promote biodiversity

Promoting agricultural practices that include greater biodiversity in agroecosystems would require radical changes in current agricultural production. Intensive agricultural production is reduced to great homogeneity in terms of assortment, relief landscape, uniform and depleted soil, dependence on fertilizer application, and mandatory application of pesticides (Dudley & Alexander, 2017). The education of farmers shall play a key role in transformation of this situation, as well as their financing. Application of the following practices significantly contributes to increasing biodiversity in agroecosystems:

- crop rotation
- sideration
- biocontrol
- selection of suitable variety
- animal manure application
- cover plants
- buffer strips

1. Crop rotation represents the rotation of crops in time and place, as opposed to monoculture. This would mean that the same crop should not be sown on the same plot for several years in a row. The benefits of crop rotation are well known, certain weeds, diseases, and insects are eliminated, yields are increased, plants make optimal use of soil nutrients, and cultivation of certain crops such as plants of the family *Fabaceae* increases the content of readily available soil nitrogen (Mihajlov, 2013). Crop rotation significantly affects not only the yield but also the agrobiodiversity in the soil and the attraction of various insects.
2. Sideration or green manure is the cultivation of legumes or other crops that enrich the soil with organic matter and necessary nutrients. These plants are grown until the moment of their flowering and then incorporated in the soil. Their presence not only increases the content of organic matter and nutrients in the soil but also encourages microbial interaction in the soil (Underwood & Tucker, 2016). Soil processes associated with plant used for green manure are related to the decomposition of plant residues into organic matter and the nitrogen-fixing processes in legumes. Growing plants as green manure also attracts a variety of beneficial insects.
3. Phytopathology and entomology use the term biocontrol to refer to the use of antagonistic microorganisms or insects to reduce the number of suitable hosts such as weeds, insects, or pathogens. In a broader sense, the term also refers to the secretions obtained from certain organisms which, if

applied to a suitable undesirable organism in a certain plot, have an antagonistic relationship. Throughout their life cycle, plants and pathogens interact with a wide range of organisms. These interactions can significantly affect plant health in a variety of ways. To understand the mechanisms of biological control, it is useful to evaluate the different ways in which organisms interact. Organisms must have some form of direct or indirect contact. Odum & Kuenzler (1955) proposed that the interactions of two populations can be defined according to the results obtained for each. The types of interactions are named reciprocity, commensalism, neutralism, competition, amensalism, parasitism, and predation. While the terminology has been developed for macroecology, examples of all these types of interactions can be found in the nature at both the macroscopic and microscopic levels. Because the development of plant diseases involves both plants and microbes, the interactions that lead to biological control take place on multiple levels. From a plant point of view, biological control can be considered as a net positive result resulting from various specific and non-specific interactions. Using the spectrum of Odom concepts, we can begin to classify and functionally differentiate the various components of ecosystems that contribute to biocontrol. Mutualism is an association between two or more species where both species benefit. Sometimes, it is a mandatory lifelong interaction that involves close physical and biochemical contact, such as those between plants and mycorrhizal fungi. However, they are generally optional and opportunistic. For example, bacteria of the genus *Rhizobium* can reproduce either in the soil or, to a much greater extent, by their interconnection with leguminous plants. These types of interdependence can contribute to biological control by strengthening the plant with improved nutrition and/or by stimulating the host's defences. Protocooperation is a form of interdependence, but the organisms involved do not depend solely on each other to survive. Other examples of biological control are the cover crops used to attract natural predators to pests by providing elements of their habitat. This is a form of biological control known as habitat enhancement but achieved using cover crops. Findings on the relationship between crop presence and predator/pest population dynamics are mixed, indicating the need for detailed information on specific crop types and management practices to best complement the given integrated pest management strategy.

4. Selection of suitable plant varieties and animal breeds increases and promotes biodiversity. According to Mazid & Khan (2015), the use of a suitable variety increases the yield by 10-35%. If cultivated varieties are appropriate for the region of cultivation, there are economic and environmental benefits. Cultivation of suitable varieties and crops reduces costs for pesticides, fertilizers, and soil treatment, thus contributes to a cleaner environment. A clean environment is one of the preconditions for better biodiversity.
5. The use of animal manure contributes to the enrichment of the soil with various nutrients that are important for plant growth and development (Fig. 5). Another more important feature of manure is that they influence the texture of the soil and enrich it with organic matter (Darwish et al., 1995). In intensive agricultural production, it is recommended to apply manure once in three years, while in organic farming it is applied almost every year due to the limited access to synthetic fertilizers. Animal manure is added to encourage the living organisms in the soil and to perform various interactions and decomposition processes. Microorganisms transform the elements in manure and make them easily available for uptake by plants through the root system (Henis, 1986). The indirect value of animal manure for biodiversity is the reduction of applied synthetic fertilizers which have negative impact on soil biodiversity, pollute groundwater and have a potentially harmful effect on plants and humans.

Figure 5. Animal manure (photo: Sasho Arov).

6. Cover crops are commonly used agricultural practice. These are plants that are sown on the agricultural fields before, after, or together with the main crop. These crops have many beneficial effects for both farmers and the environment such as erosion control, soil fertility conservation, water quality, pest and disease control, biodiversity, and wildlife. Most often, cover plants are not crops of economic importance and their management is not intensive, so they create favourable conditions for biodiversity development (Snapp et al., 2005). Combining cover plants with a suitable crop rotation creates ideal conditions for the development of very complex biodiversity in agricultural fields. In a study by Price et al. (2008), researchers compared the composition of insect species and sparrows between conventional and cotton fields, where cover crops had previously been used in the southern United States. Cotton fields and cotton fields with clover as cover crop, left to grow between the cotton rows during the early cotton growing season were sown for experimental purpose. During the migration season, they found that insect densities were 7 to 20 times higher in cotton fields with clover cover culture than in conventional cotton fields. Other examples of cover crops are nematode-resistant white mustard (*Sinapis alba* L.) and radish (*Raphanus sativus* L.) where the nematode population is significantly reduced, by 70-99%, depending on the species and time of cultivation (Hossain et al., 2015).
7. Buffer belts are primarily used for protection against soil erosion (Barling & Moore, 1994). These are usually long strips of perennial trees planted in large valleys where wind erosion often occurs. Trees can be of different types and varieties, but they are usually deciduous. In addition to erosion protection, buffer belts are also recommended in organic farming to protect crops from the adverse effects of chemical reagents used nearby. With the help of the wind, pesticides are easily transferred to wider distances. Buffer belts are home to several micro and macro-organisms with beneficial effects on the agroecosystems (Ma et al., 2002). In our country, tree protection zones are used in Ovce Pole, as protection of the soil from wind erosion and damage to cereal crops.

Loss of biodiversity

Biodiversity loss can be defined as a decrease in the number of individuals of a particular species or the permanent extinction of a particular species. The loss of biodiversity does not directly affect only the organisms that inhabit a certain area, but also certain habitats, landscapes, and loss of genetic variability.

When we talk about the loss of genetic variability, we come to the term genetic erosion, which refers to the disappearance of a certain gene or allele, and sometimes this

term describes the complete loss of a certain species. Genetic erosion occurs as a result of the disappearance or inability of one parent to mate in a population of rare endemic species. In such small, almost homogeneous populations of organisms, genetic variability is reduced to minimal genetic differences and inbreeding depression is very common. Inbreeding is known to reduce biological viability and may lead to extinction. In agriculture, genetic erosion refers to the loss of certain alleles and complexes of genes such as those found in indigenous populations that are associated with high adaptability to the conditions of the environment from which they originate (Maxted & Guarino, 2006). The main factor for the loss of these varieties is their replacement with more productive varieties, legal decisions, change of the agricultural system, overuse, degradation of the environment. Cultivation of introduced varieties has the greatest implications in the process of extinction. In the case of loss of domestic breeds of animals, usual reasons are use of exotic crossings, weak government conservation laws, neglect of certain breeds due to low profitability, intensive production, lack of proper animal disease management, lack of sufficient pastures, and other elements required by the environment, lack of control of inbreeding and carelessness in selection processes with rare breeds. Another increasingly common term in this context is "genetic pollution" and it refers to the transmission of pollen, and consequently the genes from genetically engineered plants to their wild relatives (Meilink et al., 2015).

The main causes of biodiversity loss are pollution, habitat loss, overuse, climate change, natural disasters, hunting, and introduction of invasive species (Fig. 6).

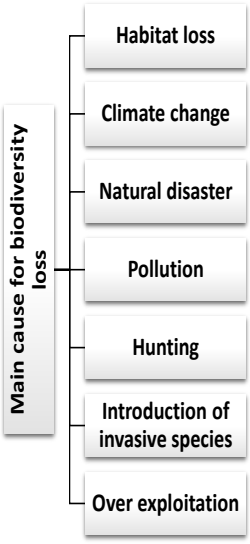

Figure 6. The main causes of biodiversity loss (adapted from Singh et. al., 2021).

According to Singh et al. (2021), the biggest causes of biodiversity loss are:

1. Habitat loss - A major threat to biodiversity and species survival is habitat loss. This is a serious issue for both wildlife and humans. Habitat and wildlife are interconnected. Habitat destruction, degradation, and fragmentation are the three dominant categories of habitat loss. Habitat destruction is the mass extinction of a species of their natural habitat, making it incapable of supporting domestic ecosystems and species. The development of agricultural practices, reduced resources such as food, water, air quality, mining, pollution, logging, catastrophic fishing activities, activities related to urbanization, and interruption of ecosystem-related processes are the dominant elements of habitat degradation. Habitat degradation affects both habitat species and humans. Erosion, nutrient depletion, and desertification cause further degraded soil loss. Habitat fragmentation is another gigantic issue that arises because of human development activities. These fragmented

areas disrupt the habitats of animal and plant species, isolate animal communities, and compress genetic diversity.

2. Climate change - Biodiversity and climate change are strongly linked. Although the climate has changed constantly throughout Earth's history with the development and extinction of ecosystems and species, accelerated climate change is disrupting ecosystems and the species' ability to acclimatize, increasing biodiversity loss. Rapid climate change stimulates the loss of biodiversity, endangering human interests and the safety of clean water, air, medicine, and additional natural resources on which we depend. Climate change, among other components, such as habitat loss, land degradation, hunting, over-exploitation of certain species, etc., is becoming a major threat to the biodiversity. Allegations of sudden climate change are often corroborated by a variety of extinction statistics on species that until recently were part of biodiversity.
3. Natural disasters - Natural disasters, such as volcanoes, fires, floods, hurricanes, drafts, epidemics, tsunamis, etc. cause great loss of biodiversity. In humid tropical areas such as central Africa, eastern and northern Australia, some areas in South America, floods are common. Tropical areas have a lot of vegetation, and a huge number of animals survive in the vegetation. Due to flooding, large amounts of nutrients are leached from the soil. Drought has also led to dry soil and decrease of the water levels. Both animals and plants suffer in this situation.
4. Pollution - Air pollution affects the respiratory system of animals and adversely affects their well-being, including the ability to lay eggs and changes in behaviour. Air pollution is also known to affect the reproductive ability of animals and hence breeding failure. The indirect impact of air pollution on animals is difficult to assess because it is difficult to investigate in a controlled environment. Water pollution has a detrimental effect on biodiversity. Synthetic fertilizers usually contain nitrogen and phosphorus and they are added to soil to increase crop productivity. Nitrogen and phosphorus are removed from the soil to water bodies or groundwater. The presence of these nutrients in the water leads to eutrophication or the overgrowth of plants. Eutrophication causes a decrease in oxygen levels in waters which is harmful to biodiversity. Fish and other aquatic animals die due to a lack of dissolved oxygen in the water. Just like fertilizers, pesticides can also accumulate in water bodies. Pesticides adversely affect running water bodies such as lakes and ponds since fertilizers are not washed off and aquatic animals have difficulty in reproduction. Environmental microplastics also affect larval survival, reduced food intake, and gradual weight loss in aquatic animals, which eventually leads to their extinction. Soil pollution is another factor that negatively affects biodiversity. Heavy metal contaminated soil greatly affects the well-being of microorganisms necessary to sustain the life of soil organisms. Excess heavy metals present in the soil do not decompose easily and accumulate from plants (Singh et al., 2021). Immense use of fertilizers, pesticides, and antibiotics used in agriculture is very harmful to biodiversity. The increased presence of nutrients in the soil causes a vigorous growth of grass species, which leads to the suppression of the growth of wildflowers, necessary for bees and other pollinating insects.
5. Hunting - Hunting is the leading cause of the extinction of many animals in the food chain. Because of this, different species in certain regions are adversely affected as they face food shortages or complete inaccessibility of food compared to the normal state. Hunting is a major operator for biodiversity loss. Hunting activities carry a significant burden on wildlife, causing a huge decline in wildlife and leading to disrupted and inefficient ecosystems. Unsustainable collection of aromatic and medicinal herbs and mushrooms also harms biodiversity. The mushrooms used for consumption are present in only certain regions and their excessive and uncontrolled collection may lead to their non-appearance in the next season. There are many examples where whole forests are devastated by wild collectors of

medicinal plants.

6. Excessive exploitation - Excessive exploitation of species from their natural habitat in higher numbers than they can reproduce new species. Currently, almost one-third of the Earth's vertebrates that are facing extinction are vulnerable to overexploitation. Extreme fishing and hunting are examples of over-exploitation. Similarly, various animals and plants are collected for use as pets, trophies, or souvenirs.

Biodiversity conservation

The loss of flora and fauna due to human activities has been going on for millennia, but only recently have we begun to understand the consequences of this loss for structure and function of ecological systems at the biome scale and the Earth system (Murray, 2017). Although relevant biodiversity conservation factors increasingly recognize the need to restore and conserve entire systems, their priorities and interventions remain focused on scales that are too small to address the functions of the biome or the system as a whole. It is constantly argued that a new global initiative is needed to address the past and current loss of flora and fauna and its functional units.

According to Brütting et al. (2013) there are generally two conservation approaches of existing biodiversity: 1. *in situ* and 2. *ex situ conservation*:

1. *In situ* - an approach that includes methods and tools that protect species, genetic varieties, and habitats in their natural habitats. It is a favourable approach among environmentalists for the protection of habitats and ecosystems.
2. *Ex situ* - an approach that includes methods that remove plants, animals and microbiological species, and genetic varieties from their natural habitat. These methods are popular with agronomists and biologists and help maintain species populations.

Restoration and rehabilitation approaches include methods that rely on *in situ* and *ex situ* tools to re-establish species, genetic varieties, communities, populations, habitats, and ecological processes. Ecological regeneration usually involves the reconstruction of natural and semi-natural ecosystems on degraded lands. This includes the reintroduction of most indigenous species, while ecological rehabilitation involves repairing ecosystem processes.

***In situ* conservation**

It is a dynamic way of preserving germplasm compared to the static nature of *ex situ* conservation. Allows continuous evolution of cultures by allowing natural selection to act on it. Lately, *in situ* conservation has been attracting a lot of attention and efforts are being made to preserve genetic resources under its natural environment. It is important to preserve species that are difficult to preserve in an extra situation. *In situ* conservation involves maintenance of genetic variation in the location where it occurs, either in the wild or in traditional farming systems (Fig. 7).

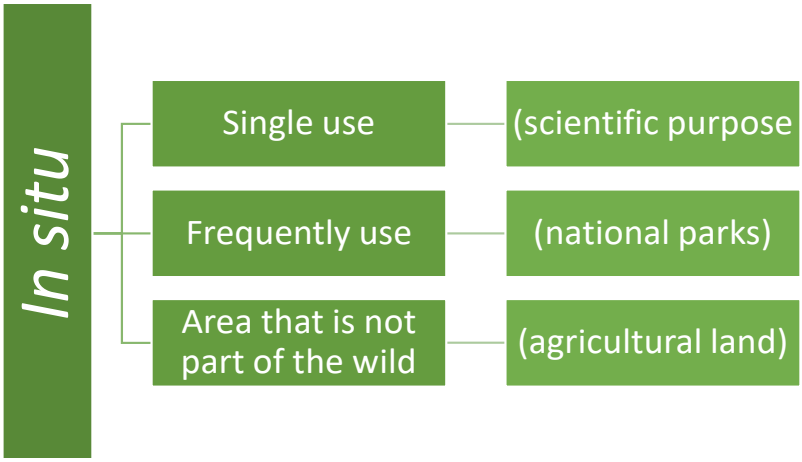

Figure 7. *In situ* methods of biodiversity conservation (adapted from Zegeye, 2016).

Wildlife conservation includes the location, designation, management, and monitoring of genetic diversity at a specific, natural site. This technique is most suitable for most wildlife species, as it can be relatively inexpensive when management is minimal. Genetic variation present in wild plant populations is a necessary condition for evolutionary adaptation to the changing environment and hence the survival of the species. Genetic variation is therefore essential for maintaining a sustainable population. To establish a genetic reserve for the target taxon or group, the effective population size must be assessed. Genetic reserves are suitable for orthodox seeds, i.e. seed types that can be maintained in long-term storage with a combination of moisture content reduction and low-temperature storage and unorthodox seeds, i.e. seed types that cannot be stored for a long time. Genetic reserves also allow multiple storages of the taxon in a single reserve and allow continuous evaluation of the species. However, the disadvantages are that the stored material is not immediately available for human exploitation, and if the management mode is minimal, little characterization or assessment data may be available.

Modern breeding methods have largely taken the place of traditional farming methods, leading to the loss of many old varieties. It is necessary to preserve the diversity found in both wild relatives and old varieties of domestic species. Farm preservation involves maintaining traditional cropping systems or growing by farmers within traditional farming systems. The advantage of preserving the farm is that it provides landscaping and those wild species dependent on traditional agriculture. However, yields may be lower than their contemporary successors and may be less popular within farmers. Therefore, some form of motivation, even subsidies, associated with regular monitoring may be needed to ensure sustainability. Home garden conservation is a variant of farm preservation. It involves growing a variety of materials in homes, backyards, or gardens and focuses on growing food crops, medicinal plants, herbs, and spices grown primarily for home consumption. Although the individual sizes of such gardens are likely to be small, genetic safety can be achieved by agglomerating neighbouring households. Home gardens are often the source of traditional varieties lost from larger agricultural systems. It should be noted that modern economic forces will tend to act against the continuous maintenance of old varieties, and they undoubtedly currently suffer from rapid genetic erosion, therefore, a developed system of *ex situ* conservation is necessary.

Ex situ conservation

Ex situ conservation is a technique of conserving all levels of biodiversity outside their natural habitats through various techniques such as zoos, aquariums, botanical gardens, and gene banks (Borokini et al., 2010). It plays a key role in communicating problems, raising awareness, and gaining broad public and political support for conservation and breeding activities of endangered species (Fig. 8).

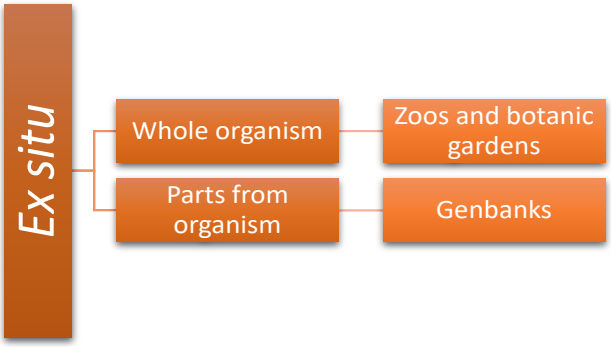

Figure 8. *Ex situ* types of conservation (adapted from Zegeye, 2016).

Limitations of *ex situ* conservation include keeping organisms in artificial habitats, deteriorating genetic diversity, inbreeding depression, captivity adjustments, and accumulation of harmful alleles. Currently, several stakeholders are actively working on biodiversity conservation through *ex situ* conservation strategies through the establishment of gene banks, botanical gardens, and zoos (Zegeye, 2016).

Zoos

Zoos are places where animals are confined to cages or semi-natural and open areas, displayed to the public and in which they can also breed. They are considered important means of preserving biodiversity. Zoos attract as many as 450 million visitors each year and are therefore uniquely positioned to have very high educational and economic value (Carrizo et al., 2013). Zoos not only act as places for entertainment and observation of animal behaviour but also institutions, museums, research laboratories, and information banks for rare animals, as well as domestic animals. Over the last few decades, zoos have made significant progress in the cooperative management of *ex situ* populations, both of wild and indigenous animal.

Botanical gardens

Botanical gardens consist of plants, grown outdoors or in greenhouses. They are used to grow and display plants primarily for scientific and educational purposes (Waylen, 2006). They also include herbariums, laboratories, libraries, museums, and experimental or research plantations. They can contain a certain family, genus, or group of varieties, natural plants, wild relatives, medicinal, aromatic, or textile plants. There are over 2,000 botanical gardens that have 80,000 plant species in their collections and receive hundreds of millions of visitors a year. Furthermore, they have a valuable and distinctive mix of staff dedicated to plant research, systematics, conservation education and raising the public awareness. They are extremely well networked with each other and with other professionals, conservation organizations, and NGOs (Blackmore et al., 2011). They provide a variety of services to sectors that use and preserve plant diversity, such as agriculture, forestry, pharmaceutical, and biofuels industry, protected area management and ecotourism, and have a unique opportunity to attract visitors and scientific institutions to document and preserve plant diversity through shaping and mobilizing citizens towards current environmental challenges. Botanical gardens allow cultivated plants to be grown under relatively modified environmental conditions (intensive cultivation, relatively high fertility, and high levels of disturbance).

Genbanks

Genbanks are biological repositories that aim to preserve genetic material. In plants, it can be by freezing parts of plants or storing seeds, and in animals by freezing sperm or eggs in specially designed refrigerators with a certain temperature. The main purpose of the existence of gene banks is to preserve genetic diversity, which would help in future research and the formation of new species (Clarke, 2009). They exist to preserve the wild and cultural species on which humans depend. In addition to the genetic resource, there is information about that organism. They represent the gene pool which is actually the basis of all genes contained in a particular seed. The size of the gene pool directly affects the evolutionary path of that species. Cryobanks are *ex-situ* gene banks. In this way, cultivated plants, plants for medicinal purposes, or endangered species are preserved. Avocados, papaya, coffee, and walnuts have seeds that cannot withstand low temperatures, and all those protocols are needed for conventional seed gene banks and therefore are stored cryogenically. The Russian Academy of Sciences preserves 7 varieties of strawberries and raspberries that must also be cryogenically preserved, 250 endangered species from Russian territory, and 20 plants of pharmaceutical importance. In this way, fruit crops, tropical and subtropical fruits are preserved. Their seeds are placed in bottles with liquid nitrogen and they are frozen and their vital functions are stopped until they are thawed. In humans and animals, this is applied by freezing sperm in special ampoules. *In vitro* tissue and organ culture techniques gives possibilities whole organs or part of plant tissues (buds, rhizomes, meristematic tissue) to be multiplied in identical plants (clones) with the same characteristics as the parental plant.

CONCLUDING REMARKS

Agrobiodiversity is a basic resource in agricultural production and offers a wide selection of species, varieties, populations and genotypes. All processes that take place in the process of food production are directly or indirectly related to agrobiodiversity. Biodiversity, in addition to the numerous benefits to agriculture, is part of many industries and provides raw materials. Apart from the direct benefits, it offers us many regulatory, cultural and social benefits in all spheres of life. Taking in consideration all above presented, we can drove certain conclusions related to importance and role of agrobiodiversity in sustainable agriculture:

Agriculture is one of the main drivers of positive and negative changes in biodiversity, both in plants and animals. Agroecosystems can be the biggest promoters of biodiversity, but also the biggest destructive force.

The principles and techniques applied in agricultural production determine the direction of movement of changes in plant and animal populations.

Soil fertility is a reflection of soil biodiversity, which is composed of thousands of micro- and macroorganisms. On the other hand, soil fertility is directly related to the yield and health of plants.

A key moment in agriculture is pollination and many crops are dependent on entomophile pollination. The biodiversity of insect pollinators provides security and stability in the agroecosystem.

Population of beneficial insects in and near agroecosystems optimizes the control of weeds and economically harmful insects. A proper management of beneficial insects and their attraction brings numerous benefits for an agroecosystem.

Organic food production is based on the use of biodiversity and maximization of natural processes in the environment, as well as achieving high yields. The symbiosis of agrobiodiversity and human activities offers the organic farming as an example for rational use of land resources without experiencing negative consequences for any of the parties involved in the process.

Agrobiodiversity offers practical applicable solutions for management with extremely rapid manifestations of nature, which negatively affect the growth and development of crops. Agrobiodiversity ensures yields at specific times.

Agriculture shall promote and balance biodiversity at the same time together with application of certain agricultural practices. Proper application of cultivation techniques as crop rotation, sideration, buffer belts, animal manure utilization, biological control and cover crops provides higher yields in the current cultivation season. Moreover, it contributes to balanced ecosystems with long-term positive effect on cultivation. Application of those practices increases the biological activity in the soil, improves organic matter content, raises the populations of beneficial insects, and reduces the need for application of chemical inputs.

The loss of biodiversity is a direct consequence of human activities on Earth. The loss refers to reduction in the number of individuals in a population or the complete extinction of a species. The extinction of the species affects the stability of the food chains and negatively affects the agricultural production, particularly when certain species or varieties with potential to cope extreme conditions are lost.

Biodiversity conservation is key to successfully tackling environmental change. The principles of biodiversity conservation are commonly known as *in situ* and *ex situ*. It is particularly important all measures for prevention of loss of some species to be undertaken. The local farmers who grow indigenous varieties in their gardens also contribute to this preservation, as well as different institutions that intentionally conserve the genetic resources with various approaches.

Overall, the biodiversity, in addition of being able to intensify agricultural production, is an inseparable integral element of any agroecosystem. It is important to emphasize that the methods and principles applied in agriculture should be moderate, balanced and appropriate to the current situation in the systems. Techniques that invoke modern intensive production, including the use of any synthetic inputs, genetically modified plants, or overuse of resources, can have a negative long-term effect on biodiversity. Of utmost importance are all efforts to conserve and preserve biodiversity from its extinction.

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УЛОГАТА И ЗНАЧАЊЕТО НА АГРОБИОДИВЕРЗИТЕТОТ ЗА ЗЕМЈОДЕЛСТВОТО

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Резиме

Биодиверзитетот и земјоделството имаат нераскинлив однос со меѓусебно зависни интеракции меѓу нивните составни компоненти. Земјоделството како основна антропогена активност е еден од главните фактори во насоките на развој во биодиверзитетот. Агробиодиверзитетот е основата на постоењето на процесите на производство на храна и обезбедува и осигурува многу есенцијални елементи од современото живеење. Многу од тие процеси се целосно непознати, а за некои од нив не сме свесни дека се последица на биолошката активност на разните организми. Па така, биодиверзитетот обезбедува храна, чиста вода за пиење, енергија, суровини за индустријата, туристички и рекреативни можности, научни истражувања и лекови. Доколку се користат земјоделски практики кои го промовираат биодиверзитетот, како на пример: плодоред, покривни растенија, буферни појаси, употреба на биопестициди, корисни инсекти и меѓупосеви, тогаш природните процеси во почвата, растенијата и животната средина значително се интензивираат во корист на земјоделците, растенијата и целокупната животна средина. Од другата страна, доколку се применуваат техники и методи кои го редуцираат биодиверзитетот во агроекосистемот и неговата околина како вештачки ѓубрива, хемиски пестициди, хормони, интензивна обработка, монокултурно одгледување и други, настапуваат процеси на редуцирање, исчезнување и загадување на животната средина и целокупниот биодиверзитет. Затоа е потребен внимателен избор на земјоделски практики кои не би го загрозиле опстанокот на видовите, а истовремено ќе се постигнат високи приноси со добар квалитет.

Целта на овој прегледен труд е да се презентираат односите меѓу биодиверзитетот и земјоделството и да се истакнат актуелните прашања за губењето на биодиверзитетот и методите за негова конзервација.

Клучни зборови: *генетски ресурси, автохтони сорти, агробиодиверзитет, органско производство, одржливо земјоделство, заштита, конзервација.*

Journal of Agriculture and Plant Sciences, JAPS, Vol 19, No. 2

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ISSN 2545-4447 print
ISSN 2545-4455 on line
Vol. 19, No. 2, Year 2021