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ACTIVITY OF ENZYME CATALASE IN ALFALFA (*Medicago sativa* L.) AS AN INDICATOR FOR ABIOTIC STRESS

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Abstract

To understand the adaptability of alfalfa (*Medicago sativa* L.) to environmental stress, the activity of antioxidant enzyme catalase in alfalfa shoots were analyzed at three slopes, subjected to drought stress during vegetation. The presence of the enzyme catalase is a signal that changes occur in plants, due to certain environmental abiotic stress factors. Catalase is a ferment that intensely catalyzes the decomposition of hydrogen peroxide into water and oxygen, which is produced under stressful conditions. Owing to the toxic nature of H_2O_2 to living plant cell, the importance of catalase is of great essence for the plants to adapt to stressful environmental conditions. Therefore, increased antioxidant activity of the enzyme catalase is considered as an indicator of abiotic stress conditions in plants.

In this paper, the activity of catalase enzyme at alfalfa (*Medicago sativa* L.) was investigated, depending on the climatic conditions in three regions of Tetovo, Skopje and Ovche Pole, in 19 different locations in the Republic of North Macedonia. The results showed that in the Ovche Pole region, where the arid period is the longest, the activity of the catalase enzyme is highest in all three slopes. The enzyme catalase is an indicator of abiotic stress in the Ovche Pole region.

Key words: *antioxidant activity, Walter climate diagram, water deficit, hydrogen peroxide*

INTRODUCTION

Catalase activity is one of the most important indicators of antioxidant activity of plants. Results of numerous studies indicate increased catalase activity in drought conditions and increased soil salinity, i.e. vegetation of culture on halophyte soil. Hydrogen peroxide occurs in stressful conditions, as do other reactive oxygen radicals, and it occurs at heat shock, metallic stress, at pathogenic infections in plants, at photooxidative processes in plants induced by abiotic stress conditions such as cold, drought, salinity and ozone stress.

The three main mechanisms that reduce alfalfa yield due to water deficiency are: (1) reduction of absorption in photosynthetic radiation, (2) reduction of radiation efficiency, and (3) reduction of harvest index (Earl and Davis, 2003). The results of Clarke and Siddique's research in 2004 showed that low temperatures, wind and water erosion also had a negative impact, contributing to oxidative stress in alfalfa and increased catalase activity.

Wang et al., (2009) performed an analysis of antioxidant enzyme activity during alfalfa germination in drought conditions and increased salinity. The results indicated that tolerance of varieties in saline and dry stress conditions, during germination, was associated with increased activity of antioxidant enzymes. The alfalfa response, due to water deficit, depends immensely on the severity of stress, growth phase and the physiological state. This results in a 49% reduction in biomass and an 18% increase in leaf-to-tree ratio (Bouizgaren et al., 2013). Water deficiency caused in the alfalfa, as in other legumes, to decrease leaf area, to reduce leaf number, to close the stoma, so that thereby limit the CO_2 assimilation, the photosynthetic activity and the growth (Butleska Gjoroska et al., 2016; Yousfi et al., 2016; Tardieu et al., 2014).

The research in this paper aims to determine the activity of enzyme catalase in alfalfa (Medicago sativa L.) from three slopes, on 19 investigated locations in three regions of Tetovo, Skopje and Ovche Pole in the Republic of North Macedonia. The climatic conditions in the studied regions are determined with climate diagrams by Walter, and the results clearly indicate that the arid period, observed with water deficit and soil salinity in the Ovche Pole region, confirms the increased activity of the catalase enzyme.

MATERIAL AND METHODS

Plant material

Alfalfa plant material (Medicago sativa L.) was collected from three different regions in the Republic of North Macedonia: the Skopje region, the Ovche Pole region and the Tetovo region, from 19 different locations in three slopes (Table 1). The material was collected during the vegetative cycle (from June to August) in 2013. In the first, second and third slope, plants were collected from 15 to 17 June, from 16 to18 July and from 17 to 19 August, respectively.

Table 1. Description of the locations of the examined locations altitude (m), latitude (^{0}N) and longitude (^{0}E) with the dates of first, second and third slope.

Nr	Location	Region	Altitude (m)	Latitude (°N)	Longitude (°E)	Firs slope (date)	Second slope (date)	Third slope (date)
1	Bogovinje	Tetovo	531,50	41,9236809	20,9168772	15.06.2013	16.07.2013	17.08.2013
2	Vrutok	Tetovo	682,41	41,7665300	20,8381550	15.06.2013	16.07.2013	17.08.2013
3	Dzepchiste	Tetovo	474,48	42,0331690	21,0001650	15.06.2013	16.07.2013	17.08.2013
4	Galate	Tetovo	600,73	41,8381370	20,8813700	15.06.2013	16.07.2013	17.08.2013
5	Zelino	Tetovo	1605,94	41,9006530	21,1175770	15.06.2013	16.07.2013	17.08.2013
6	Peckovo	Tetovo	991,87	41,7843700	20,8311530	15.06.2013	16.07.2013	17.08.2013
7	Jegunovce	Tetovo	658,34	42,1245655	21,0875064	15.06.2013	16.07.2013	17.08.2013
8	Avtokomanda	Skopje	246,68	42,0006868	21,4536642	16.06.2013	17.07.2013	18.08.2013
9	Sopiste	Skopje	1017,16	41,8638490	21,3083500	16.06.2013	17.07.2013	18.08.2013
10	Dracevo	Skopje	264,41	41,9352675	21,5098515	16.06.2013	17.07.2013	18.08.2013
11	Saraj	Skopje	424,88	42,0017493	21,2815977	16.06.2013	17.07.2013	18.08.2013
12	Radishani	Skopje	392,32	42,0732769	21,4479917	16.06.2013	17.07.2013	18.08.2013
13	Vlae	Skopje	256,07	42,0072938	21,3801924	16.06.2013	17.07.2013	18.08.2013
14	Glumovo	Skopje	274,74	41,9817742	21,3103747	16.06.2013	17.07.2013	18.08.2013
15	Cheshinovo	Ovche Pole	294,00	41,8735350	22,2905610	17.06.2013	18.07.2013	19.08.2013
16	Karbinci	Ovche Pole	342,98	41,7882100	22,2622460	17.06.2013	18.07.2013	19.08.2013
17	Oblesevo	Ovche Pole	297,63	41,8639320	22,2622460	17.06.2013	18.07.2013	19.08.2013
18	Lozovo	Ovche Pole	277,86	41,7806752	21,8995629	17.06.2013	18.07.2013	19.08.2013
19	Mustafino	Ovche Pole	289,18	41,8407190	22,0789350	17.06.2013	18.07.2013	19.08.2013

Determination of climate conditions according to climate diagrams by Walter

During the examination, the meteorological data for the investigated regions were monitored at the National Hydrometeorological Department of the Republic of North Macedonia. The data for Tetovo region were obtained from Tetovo meteorological station, the data for Skopje region from meteorological station at Zajchev Rid, and for Ovche Pole region the meteorological data were obtained from meteorological station in Stip.

From the mean monthly temperatures and the sums of rainfall presented in the climate diagrams by Walter (1957), the humid and arid nature of the climate during the vegetation period of the study in the three regions examined is determined (Figure 1, 2 and 3).

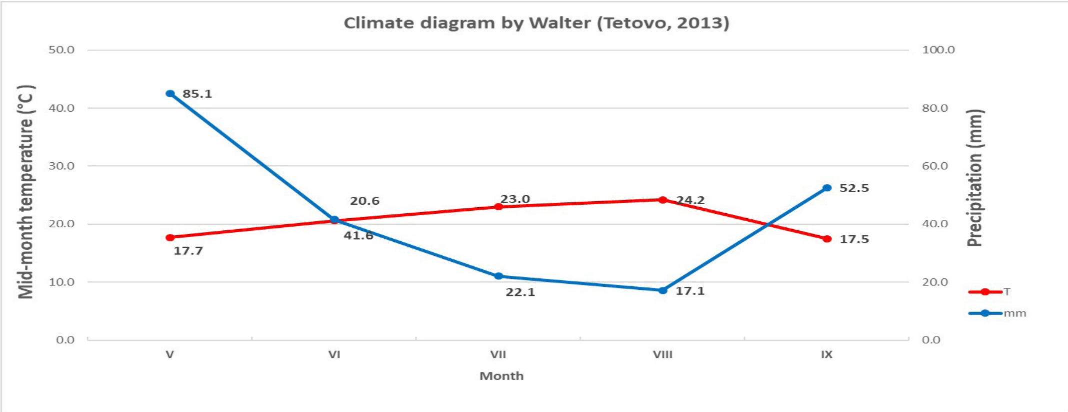

Figure 1. Climate Diagram by Walter for the Tetovo region, 2013.

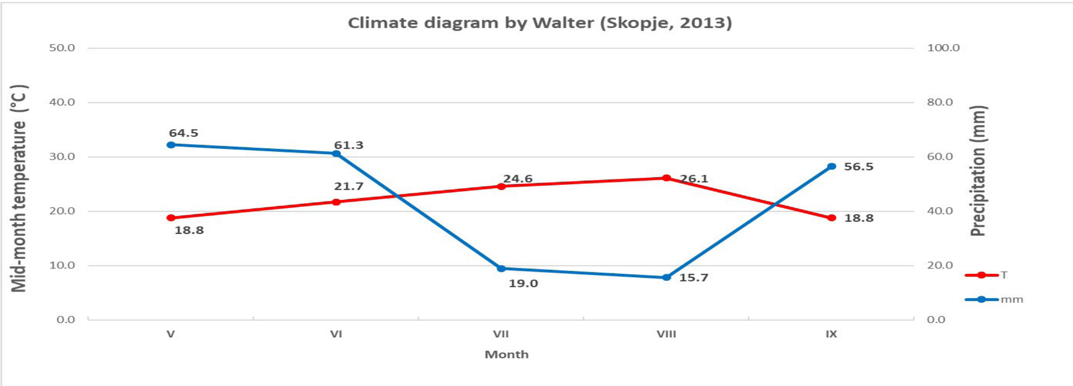

Figure 2. Climate Diagram by Walter for the Skopje region, 2013.

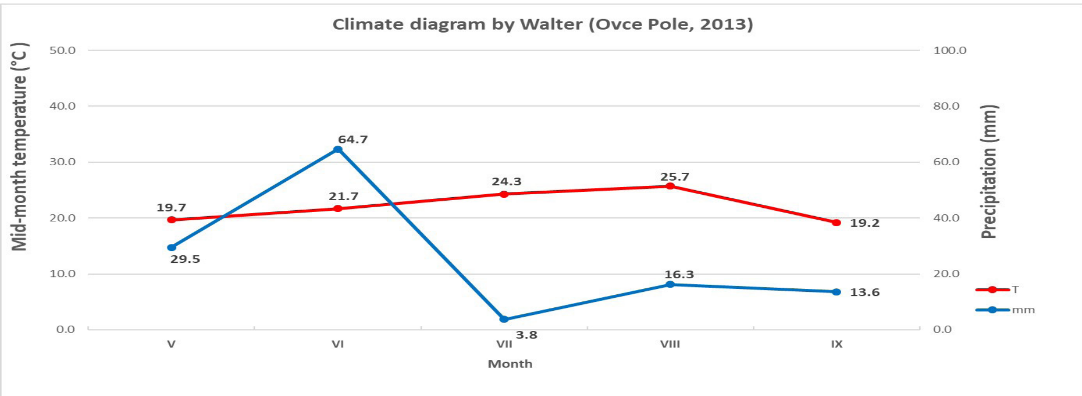

Figure 3. Climate Diagram by Walter for the Ovche Pole region, 2013.

Determination of antioxidant activity of the enzyme catalase

The catalase determination was titrimetric done by the method of Bach and Oparin (1923). The quantitative determination of catalase is based on the properties of H_2O_2, which remains dissolved after the catalase effect, to react with $KMnO_4$ to form free oxygen. The equation resulting this reaction is as follows:

$$2KMnO_4 + 5H_2O_2 + 4H_2SO_4 \longrightarrow 2MnSO_4 + 2KHSO_4 + 8H_2O + 5O_2$$

Based on the difference in milliliters of $KMnO_4$ solution used for the titration of the control and the test sample, the amount of H_2O_2 is obtained, which is dissolved by the fermenter. In addition to $KMnO_4$, that serves as a means of decomposing the extra H_2O_2 after incubation, J_2 is used. The reaction that occurs is presented in the following equation:

$$H_2O_2 + 2KJ + H_2SO_4 \longrightarrow K_2SO_4 + J_2 + 2H_2O$$

The excess of iodine is determined with $Na_2S_2O_3$.

To determine the content of the catalase enzyme, 2 g of dry plant material was weighed on analytical scales. The material in the mortar was macerated with a small amount of quartz sand and distilled water. The mixture was poured into a 100 ml flask and stirred well. Then 2-3 drops of toluene were added and the flask was filled up with distilled water up to the indicated mark, and the mixture was left at room temperature for 2 hours. After extraction, the content was filtered and the filtrate was used as the catalase extract.

A volume of 20 ml of the filtrate was placed (divided) into four flasks. Then, two flasks (1 and 2) were heated until boiling and boiled for 5 minutes to inactivate the enzyme. After boiling, the flasks were left to cooled at room temperature. Flasks 3 and 4 were not heated since they were used as a control. After heating and cooling, in the test samples (1 and 2), as well as in control samples (3 and 4), a volume of 20 ml of distilled water and 5 ml of H_2O_2 solution (1%, v/v), previously neutralized with NaOH solution, were added and left at room temperature for 30 minutes, followed by addition of 5 ml of H_2SO_4 (10%, v/v) and titration with 0.02 M $KMnO_4$ solution. Catalase activity (Ac) was calculated by the amount of H_2O_2 in mg, dissolved over 30 minutes, using catalase containing 1 g of test material (1 ml of 0.02 M $KMnO_4$ is equivalent to 1.7 mg of H_2O_2)

$$Ac = \frac{(a - b) \times 1.7}{g}$$

where:

a – 0.02 M $KMnO_4$ spent for the control test (ml),

b – 0.02 M $KMnO_4$ spent for the test trial (ml),

g - quantity of the test plant material in grams (g).

Statistical data processing

One-way analysis of variance (ANOVA) was used to statistically analyze the results in order to determine significant differences ($p < 0.05$ and $p < 0.01$) between the arithmetic mean of the samples. All analyzes were performed in three repetitions, presented as mean value.

To determine the significance of the difference between the investigated parameters and their rankings at the level of 0.05 and 0.01, the results were post-hoc analyzed using Duncan's multiple range test. The statistical package for the Social Sciences software (IBM SPSS Statistics Software v. 23) was used for statistical result processing.

RESULTS AND DISCUSSION

According to the climatic conditions during the investigated period, it was noticed that they were moderately favorable for cultivation of alfalfa in certain different locations in the Tetovo and Skopje regions, but in the Ovche Pole region the climate condition was with extremely low annual precipitation, which reflects the yield of the culture studied. During the survey in the Tetovo region, the Walter's climate diagram in figure 1, shows that the highest average monthly air temperatures were recorded in July and August. The highest amount of monthly precipitation was in May (85.1 mm) and September (52.5 mm). The surface that is located by both curves when the precipitation curve is below the temperature curve at the climate diagram by Walter, gives the arid (dry) period. The arid period of the investigated period is June, July and August.

The climatic conditions for the Skopje region are shown on the Walter climate diagram, in figure 2. The highest average monthly air temperature was measured in July and August, and the highest amount of monthly precipitation was measured in May and June. The arid period is from July to September.

During the research period in the Ovche Pole region, from Walter's climate diagram in figure 3, it can be noticed that the highest average monthly air temperature was measured in July and August, and the mean monthly maximum temperatures were also in July and August, and the minimum were in May and September. The arid period is from June to September, and is still growing to the following month of October. The arid period is dominant in this region, as evidenced by lower rainfall, especially in July (3.8 mm precipitation).

In table 2, the mean value of the content of the enzyme catalase, in the first, second and third percentiles, at 19 locations in the three regions studied, are shown. The content of enzyme catalase (%) deployed by the regions, in the three slopes, in dry plant material from alfalfa is presented in the table 3.

Table 2. Content of enzyme catalase (%) in the examined locations, in the three slopes, in dry plant material from alfalfa.

Location	First slope		Second slope		Third slope		All slopes together	
	Catalase %	*p<0.05 **p<0.01	Catalase %	*p<0.05 **p<0.01	Catalase %	*p<0.05 **p<0.01	Catalase %	*p<0.05 **p<0.01
Bogovinje	33.3±1.7	*g **g	37.3±1.7	*k **h	35.5±2.4	*k **j	35.4±2.4	*i **l
Vrutok	13.8±0.4	*a **ab	13.3±0.1	*a **a	14.5±1	*ab **ab	13.8±0.8	*a **a
Dzepciste	12.9±0.5	*a **a	14.1±1.7	*ab **a	15.9±2.8	*abcd **abcd	14.3±2.1	*a **ab
Galate	21.2±0.3	*cde **cde	19±1.4	*cdef **abcd	15.8±2	*abcd **abcd	18.7±2.7	*bc **cdef
Zelino	20.9±0.7	*cd **cde	24±1.5	*ghi **def	22.9±1	*efgh **defgh	22.6±1.7	*def **efgh
Peckovo	16.2 ±1.5	*abc **abcd	17.1±0.5	*abcd **abc	17.8±2.3	*abcde **abcde	17±1.6	*ab **abcd
Jegunovce	22.2±2	*def **cde	23±2.9	*fghi **cdef	20.7±0.4	*cdefg **bcdefg	22±2	*de **efgh
Avtokomanda	15.2±1.1	*ab **abc	15.2±2.1	*abc **ab	12.8±2.4	*a **a	14.4±2.1	*a **ab
Sopiste	32.2±3	*g **fg	32.1±3.3	*j **gh	25.9±2.9	*ghi **fghi	30.1±4.1	*h **k
Dracevo	20.1±2.5	*bcd **bcde	22.1±1	*efgh **cdef	21.2±0.4	*defg **bcdefg	21.1±1.6	*cd **defg
Saraj	22.5±9.7	*def **de	18.1±1.9	*bcde **abcd	14.6±8.8	*ab **abc	18.4±7.4	*bc **bcde
Radisani	13.2±2.4	*a **ab	17.2±2.4	*abcd **abc	15.4±1	*abc **abcd	15.3±2.5	*a **abc
Vlae	26.8±2.2	*f **ef	22.9±0.4	*fghi **cdef	19.1±4.5	*bcdef **abcdef	22.9±4.2	*def **fghi
Glumovo	22.6±0.6	*def **de	26.4±6.2	*hi **ef	28.1±2.3	*hij **ghi	25.7±4.1	*fg **hij
Cesinovo	26.3±0.9	*ef **ef	26.9±1.9	*i **fg	28.7±1	*ij **hij	27.3±1,6	*gh **jk
Karbinci	9.6±2	*bcd **abcde	20.6±0.7	*defg **bcde	19±3.4	*bcdef **abcdef	19,7±2.1	*bcd **def
Oblesevo	24.8±1.2	*def **e	25.7±2.7	*hi **ef	23.8±3.3	*fghi **efgh	24.7±2.4	*efg **ghij
Lozovo	20±3	*bcd **bcde	20.5±3.1	*defg **bcde	22.1±1	*efg **cdefgh	20.9±2.4	*cd ** defg
Mustafino	24.7±1.6	*def **e	23.9±3	*ghi **def	32.1±1.8	*jk **ij	26.9±4.4	*g ** ijk

*The mean value in each column marked with the same letter does not differ significantly after the Duncan test for p <0.05

**The mean value in each column marked with the same number does not differ significantly after the Duncan test for p<0.01

Table 3. Content of enzyme catalase (%) by regions, in the three slopes, in dry plant material from alfalfa

Region	Slopes			
	First *p<0.05, **p<0.01	Second *p<0.05, **p<0.01	Third *p<0.05, **p<0.01	All slopes together *p<0.05, **p<0.01
Tetovo	20.1, *a, **a	21.1, *a, **a	20.4, *a, **a	20.5, *a, **a
Skopje	21.8, *a, **a	22.0, *a, **a	19.6, *a, **a	21.1, *a, **ab
Ovche Pole	23.1, *a, **a	23.5, *a, **a	25.2, *b, **a	23.9, *b, **b

*The mean value in each column marked with the same letter does not differ significantly after the Duncan test for p <0.05

**The mean value in each column marked with the same number does not differ significantly after the Duncan test for p<0.01

On location level, the highest content of the catalase enzyme, in all three slopes separately and in all three slopes together, is at the location Bogovinje, at the Tetovo region. For Bogovinje, the value of 33.3±1.7% for the first slope was measured, 37.3±1.7% for the second slope, 35.5±2.4% for the third slope, and 34.5±2.4% for all slopes together (Table 2).

Analysis by regions showed that in the first and second slope, there was no significant difference in the mean value of catalase according to Duncan's test for both p<0.05

and $p<0.01$. In the third slope, the Duncan test showed that the Ovche Pole region was significantly different from the mean value of catalase enzymes of the Tetovo and Skopje regions for $p<0.05$, but for the $p<0.01$ Duncan test showed no significant difference in mean value at the level of regions (Table 3).

When the mean value of catalase was analyzed by regions for all three slopes together by Duncan for $p<0.05$, the Ovche Pole region is significantly different from the mean value of catalase at the Tetovo and Skopje regions, while the Tetovo and Skopje regions do not differ significantly from each other. The same analyzes with Duncan's test for $p<0.01$ showed that only Tetovo and Ovche Pole differ significantly.

The significant difference between regions is primarily due to environmental factors. This suggests that certain factors (temperature shock, agrochemical factors, air pollution, metal stress, high soil salinity, pathogenic infections and other biotic and abiotic stress factors) could be the cause of the increased production of H_2O_2. For these reasons there is an increased activity of catalase, measured by the amount of dissolved H_2O_2, in alfalfa at Ovche Pole region. The presence of enzyme catalase is a signal that changes occur, as a result of certain abiotic environmental stressors.

Increased activity of enzyme catalase also indicates for higher resistance of alfalfa, which means that the Ovche Pole region, which has the highest catalase content, has the highest resistance, and is the result of adaptive alfalfa ability in this region. Tetovo region has the lowest resistance, and thus the lowest value of the catalase enzyme.

Results of numerous studies indicate increased catalase activity under drought and increased salinity (Antolínlara et al., 2010; Rubio et al., 2001). Low temperatures, wind and water erosion also have a negative impact, which contributes to the occurrence of oxidative stress in alfalfa and increased catalase activity (Huang et al., 2007).

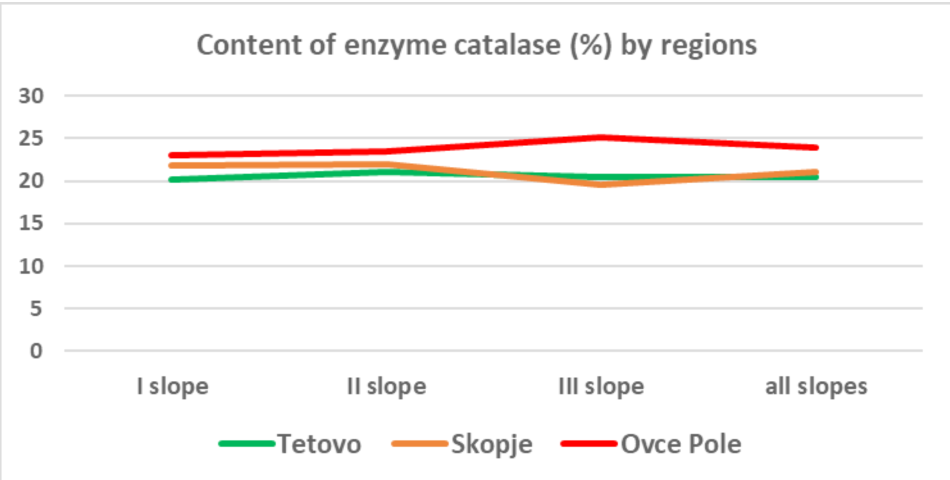

Figure 4. Content of enzyme catalase (%) by regions in first, second and third slope, and in all slopes together.

Drought stress conditions in the Ovche Pole region are also confirmed in this research, where the highest catalase content was recorded in all three slopes in this region. The highest content of catalase enzymes by regions was recorded in the third slope in the Ovche Pole region, which is undeniable evidence that drought and stress conditions were most obvious in the third slope in August (Figure 4).

CONCLUDING REMARKS

Based on the results obtained from this research paper, the following explicit conclusions can be ascertained:

- At all 19 investigated locations, the highest content of catalase in all slopes separately and in all slopes together, is in the location Bogovinje, in the Tetovo region, the highest measured value is in the second slope (37.3 ± 1.7%);
- In the first slope the lowest content of the catalase is measured at the location Karbinci, Ovche Pole region, in the second

slope at the location Vrutok, Tetovo region, in the third slope at the location Avtokomanda, Skopje region, and for all three slopes the lowest value is registered again in the Tetovo region at location Vrutok;

- The existence of drought stress conditions and high soil salinity in the Ovche Pole region lead to the highest catalase content recorded in all three slopes in this region. In the Ovche Pole region the highest content of the enzyme catalase was registered in the third slope 25.2%, which is certain evidence that drought, as abiotic stress, was most pronounced in August;
- According to the measured values for the activity of the enzyme catalase, the most favorable location for alfalfa cultivation in the Tetovo region is Dzepciste, in the Skopje region is the location Avtokomanda and in the Ovche Pole region is the location Karbinci;
- The most favorable region for growing alfalfa is Tetovo region, followed by Skopje region and Ovche Pole region, as a region in which unfavorable abiotic stress conditions are registered;
- The results of the influence of climate factors on catalase activity in alfalfa allow us to conclude that catalase may serve as an indicator of abiotic stress.

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АКТИВНОСТА НА ЕНЗИМОТ КАТАЛАЗА ВО ЛУЦЕРКА (*Medicago sativa* L.) КАКО ИНДИКАТОР ЗА АБИОТСКИ СТРЕС

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Резиме

За да ја разбереме способноста за адаптација на луцерката (*Medicago sativa* L.) на стрес во животната средина предизвикан од суша ја анализиравме активноста на антиоксидативниот ензим каталаза во три откоси за време на вегетацијата. Присуството на ензимот каталаза е сигнал дека се случуваат промени во растенијата, кои се резултат на одредени абиотски стрес фактори на средината. Каталазата е фермент кој интензивно го катализира разложувањето на водородниот пероксид на вода и кислород, кој се создава во стресни услови. Со оглед на токсичниот карактер на H_2O_2 за живата клетка, значењето на каталазата е многу големо за растенијата за адаптација на стресните услови на животната средина. Затоа, зголемена антиоксидативна активност на ензимот каталаза се смета како индикатор за абиотски стресни услови кај растенијата.

Во овој труд е испитувана активноста на ензимот каталаза кај луцерка (*Medicago sativa* L.) во зависност од климатските услови во три региони Тетово, Скопје и Овче Поле, на 19 различни локации во Република Северна Македонија. Резултатите покажаа дека во Овчеполскиот регион, каде што аридниот период е најдолг, активноста на ензимот каталаза е највисока во сите три откоси. Ензимот каталаза е индикатор за абиотскиот стрес во Овчеполскиот регион.

Клучни зборови: *антиоксидативна активност, клима-дијаграми по Walter, воден дефицит, водород пероксид.*