The Impact of Trace Elements Applied with PGPB on the *Vitis vinifera L* Seedlings Resistance to the Copper Excess in Soil

Veliksar Sofia[1], Lemanova Natalia,[1] Gladei M.,[1] David Tatiana[1]

[1]*Institute of Genetics, Physiology and Plant Protection, MCER RM, Chisinau, Moldova*

Abstract *- Cupper (Cu) has an effect of nutritive or toxic element depending on its quantity in the soil and plants. A significant amount of Cu is accumulated in the soil and plant organs due to the necessity of multiple using of Cu-containing compounds for combating powdery mildew on perennial plantations. Our studies have shown that the accumulation of a significant amount of Cu in the soil adversely affects on the plant growth, on the rate of transpiration and the stomatal conduction of leaves and on the intensity of photosynthesis of seedlings. Using 0,5 of the recommended dose of the special trace element complex Microcom-VA for foliar application and a suspension of growth-promoting bacteria (PGPB) Az. chroococcum, B. subtilis L and Ps. putida for incorporation into the soil improves the nutritional conditions of grape seedlings and increases its tolerance to an excess of Cu in the soil.*

Keywords: *copper toxicity, grape seedlings, trace elements, Microcom-VA, plant growth promoting bacteria, photosynthesis, proline*.

I. INTRODUCTION

Copper (Cu) is an important element for plant nutrition with a narrow interval of action. It has an effect as nutritive or toxic element depending on its quantity in the soil and plants. Soil pollution with copper (Cu) is caused mostly by the horticulture plantations, which are repeatedly treated by different pesticides along with Cu- and Zn-containing products. The results of the analysis of the soil samples collected by us during the expedition surveys in the Central zone of Moldova have shown that the accumulation of mobile copper in the soil increases with the age of plantations [15]. The accumulation of Cu in the superficial soil strata leads to a dynamic imbalance of minerals in the soil-plant-atmosphere system, to multiple deviations in the nutritive system, decrease of photosynthetic activity, aggravation of population's health [7] [10] [11] [18]. Preventing and reducing the negative impact of Cu-containing compounds on the environment is one of the most predominant problems in the modern agriculture.

There are few ways to reduce the toxicity of copper. One of them is using of plant growth promoting bacteria and trace elements that contribute to the improvement of plant nutrition. The last year's researches demonstrated the efficacy of applying *bacterial strains for the* development of biological control agents for agricultural plants and environment protection [1] [3] [4][8] [9] [14] [16].

The results of the investigation of the effect of some rizobacteria suspension and trace elements for the grape seedlings tolerance enhancement to Co excess in soil are presented in this paper. For this scope suspension of 3 strains of bacteria (*Agrobacterium radiobacter, Pseudomonas putida X, Bacillus subtilis L),* applied apart and together during the planting of rooted vine cuttings, were used.

II. MATERIAL AND METHODS

1. *Place of experiments and material*. The experiments were performed on the growing platform of the Institute of Genetics, Physiology and Plant Protection. Vine cuttings of two local varieties Victoria and Viorica were rooted in water, after that planted in plastic pots with 11 kg of soil. Cu was added to the lower layer of the soil in pots as $CuSO_4 \cdot 5\ H_2O$ during the planting of cuttings (1200mg Cu per 1kg soil). Different treatments were studied: Control (without Cu), Cu alone and Cu together with suspensions of bacteria and Microcom-VA.

2. Methods. Two-day suspensions of 3 strains of plant growth promoting bacteria (PGPR) - *Agrobacterium radiobacter, Pseudomonas putida X, Bacillus subtilis L* with a titre of 10^7 CFU / mL - were applied apart and all 3 together. Bacterial suspensions were applied into the soil during the plantation of rooted cuttings.

The foliar fertilization of plants was conducted three terms with 15 - days' interval by the micro fertilizer Microcom-VA, created especially for grape. Microcom-VA, includes 6 trace elements in ratios that mostly meet the needs of grapes in the critical phases of plant development during the growing season. According to our preliminary data, the effect of 0, 5 dose of this micro fertilizer together with the suspension of the above mentioned bacteria is equal

or exceeds the effect of the full dose and allows reducing the chemical load on the environment.

The leaves for analyses were sampled 6 days after every fertilization, thoroughly rinsed with water, allowed drying and used for analysis.

The content of chlorophyll in the leaves of the seedlings was determined using a portable device CCM-200 plus (Chlorophyll Content Meter), the intensity of photosynthesis - using a portable device LCi Portable Photosynthesis System, and the content of proline in the leaves - according to the Bates method [2]. The degree of development of the aerial part and the root system of the seedlings and the total accumulation of biomass were determined after ripening of the shoots when digging out the seedlings. The relative metal resistance of the grape seedlings was determined by the Tolerance Index D.S. Wilkins [17] and was calculated by the formula $IT = Me/con \times 100\%$, where *Me* is the plant response to an increased concentration of metal in the experiment, *con* is the response of the plant under control conditions. The average growth of the above-ground part of one seedling to the end of the experiment (annual shoots + leaves, g) as a plant response was used. All studies were performed in triplicate. The obtained results were processed using the Statistics 7.

III. RESULTS AND DISCUSSION

1. Growth and development of grapes saplings under conditions of high concentration of Cu in the soil and the use of trace elements and bacterial suspensions.

An important indicator of the quality of planting material is the *growth and maturation of the shoots* and the development of the root system. The results of determining these parameters in Victoria seedlings, grown in pots with an excess of Cu in the soil are presented in Figure 1.

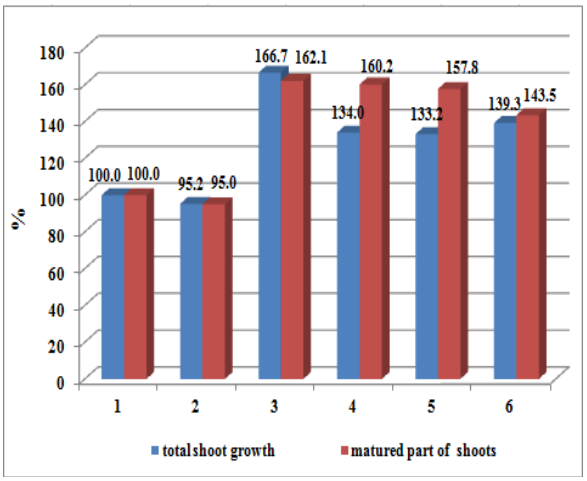

Fig. 1. Effect of bacteria and trace elements (Microcom VA) on the shoots growth and maturation of Victoria grape seedlings under the excess of Cu in soil, 2015, % to the control

Treatments: **1** – Control; **2** - Cu -1200 mg/кg; **3**. Cu -1200 mg/кg + suspension of *Ag. radiobacter* in sol + Microcom VA, foliar; **4** - Cu -1200 mg/кg + suspension of - *Ps. putida* in sol + Microcom VA, foliar; **5** - Cu -1200 mg/кg + suspension of + *B. subtilis L* in sol + Microcom VA, foliar; **6** - Cu -1200 mg/кg + suspension of *Ag. radiobacter* +*B. subtilis L* + *Ps. Putida* in sol + Microcom VA, foliar.

The growth and maturation of shoots of this variety in the presence of a high metal dose are slower compared to the control and were respectively 95,2 and 95,0 %, compared to the control. The introduction of suspension of growth promoting bacteria (PGPR) into the soil during cuttings plantation and foliar application of a solution of the micro fertilizer Microcom-VA had a significant impact on the growth and development of seedlings. The total increase in shoots per plant in these variants was 133, 21 – 166,65 % relative to the plants of the control. The shoots have also ripened significantly better, which is an indicator of the quality of seedlings and their higher survival rate when planted in a permanent place.

Calculations of the *index of seedlings tolerance to an excess of Cu* in the soil showed that this indicator increases with the improvement of the nutritional conditions of the seedlings by the trace elements and bacterial suspensions. In these variants of treatment it was 133,5 – 166,9 % compared with the control, but when Cu was added alone (the second variant) it was 95,4 %.

The *weight of roots* under the Cu surplus in soil was not significantly reduced. At the same time the addition of the bacterial suspension into the soil stimulated the root growth in all variants. It is worth highlighting that an enhanced growth of small roots (roots of 3-4 order), which play a major role in plant nutrition, occurred under these treatments.

The growth of seedlings shoots of another grape variety - Viorica - under similar growing conditions also decreased under the Cu excess in the soil and amounted to 92,37% comparative to the control (fig.2). The degree of shoot maturation has decreased even more – 77, 83% to the control.

Improvement of plant nutritional conditions by the bacterial suspension and trace elements application also contributed to more intensive plant growth, especially when using *Bacillus subtilis L* and Microcom-VA (118.59% to the control). The maturation of the shoots is more intense than in the variant with the introduction of Cu only, however, this indicator does not reach the level of the control.

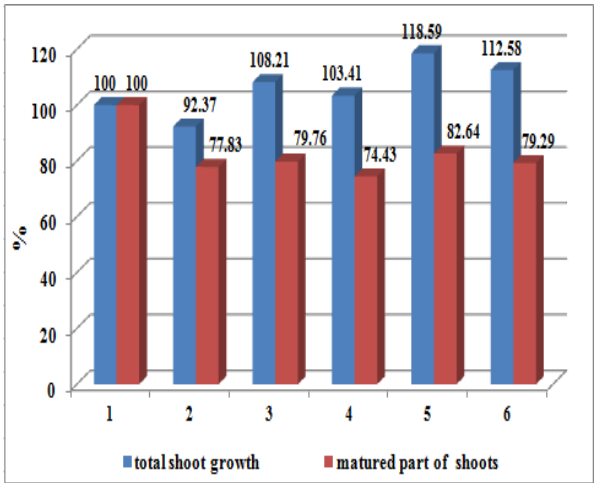

Fig. 2. Effect of bacteria and trace elements on the shoots growth and maturation of Viorica grape seedlings under the excess of Cu in soil, 2016, % to the control.

Treatments: **1** – Control; **2** - Cu -1200 mg/кg; **3**. Cu -1200 mg/кg + suspension of *Ag. radiobacter* in sol + Microcom VA, foliar; **4** - Cu -1200 mg/кg + suspension of - *Ps. putida* in sol + Microcom VA, foliar; **5** - Cu -1200 mg/кg + suspension of + *B. subtilis L* in sol + Microcom VA, foliar; **6** - Cu -1200 mg/кg + suspension of *Ag. radiobacter* +*B. subtilis L* + *Ps. Putida* in sol + Microcom VA, foliar.

2. The effect of trace elements and bacterial suspensions on the photosynthetic activity and the content of proline in the leaves of seedlings under the Cu excess.

The content *of photosynthetic pigments* in the leaves and the intensity of photosynthesis are one of the main indicators of the state of plants during the growing season. These indicators are largely dependent on the environment conditions and plant nutrition. Grape leaves are practically the only one organ capable of synthesizing organic substances from inorganic under the influence of solar energy. Shoots and other organs and tissues of grapes containing a small amount of chlorophyll can also assimilate, but the organic matter synthesized by them is so insignificant, that it matters little for plant nutrition.

Determination of the content of photosynthetic pigments in the seedlings leaves in the dynamics revealed a tendency to decrease under the influence of Cu introduced into the soil by 9-12% as compared to the control (tab.1). It is possible that a decrease in chlorophyll in grape leaves under conditions of an increased amount of copper in the soil is associated with a decrease in other essential elements, in particular in iron. It was demonstrated earlier that a decrease in chlorophyll concentration in the leaves caused by Cu-induced iron deficiency may cause a higher photosensitivity of photosystem II in Cu-treated plants [10] [11] [19].

TABLE 1.
Effect of Cu and nutrients on the content of photosynthetic pigments in the leaves of grape seedlings under the Cu excess, cv. Viorica, mg/g f.w.

Cod of treatments	date of determination		
	05.07.2016	14.07.2016	15.08.2016
1.	5,56±0,25	7,61±0,26	8,8±0,271
2.	5,08±0,36	7,02±0,28	7,76±0,216
3.	6,16±0,38	7,44±0,40	8,69±0,259
4.	6,61±0,44	7,69±0,44	9,14±0,381
5.	6,36±0,32	7,74±0,34	9,89±0,400
6.	6,98±0,36	8,22±0,35	9,09±0,478

Treatments: 1 – Control; 2 - Cu -1200 mg/кg; 3. Cu -1200 mg/кg + suspension of *Ag. radiobacter* in sol + Microcom VA, foliar; 4 - Cu -1200 mg/кg + suspension of - *Ps. putida* in sol + Microcom VA, foliar; 5 - Cu -1200 mg/g + suspension of + *B. subtilis L* in sol + Microcom VA, foliar; 6 -Cu -1200 mg/кg + suspension of *Ag. radiobacter* +*B. subtilis L* + *Ps. Putida* in sol + Microcom VA, foliar.

The addition of a bacterial suspension to the soil and the subsequent foliar treatment with a half dose of a Microcom-VA softens the negative effect of Cu excess. The content of pigments increases in all treatments by the fertilizantes up to the level of control plants and above - by 8-25 % depending on the variant and the term of determination (tab. 1). The high content of photosynthetic pigments in the leaves and their active absorption capacity positively affect the intensity of sugar accumulation - the primary products of photosynthesis, growth and productivity of plants.

The *intensity of photosynthesis* - one of the main indicators of the response of plants to environmental conditions - decreased in conditions of an excess of Cu to 72.85 % comparing with the control (tab. 2). A significant improvement in this indicator occurs when bacterial suspensions and microelements are used, especially under the influence of *Baccilus subtilis* + Microcom VA (10, 35 mg CO^2/dm^2 s).

Under conditions of Cu excess in soil, the *rate of transpiration and stomatal conductance* of leaves noticeably decreases (tab. 2), which reduces the loss of water during transpiration and increases the efficiency of its use. Growth-promoting bacteria and trace elements contributed to the restoration of these indicators to the level of the control and above, which may indicate a decrease in the toxicity of the element.

TABLE 2.
Effect of Cu and nutrients on the photosynthesis intensity and some indicators of water exchange of grapes seedlings under the Cu excess in the soil, cv. Viorica

Cod of treat-ments	photosynthesis intensity mg CO^2/dm^2s	transpiration rate Mmoll/m^2 /s	stomatal conductance mol/ m^{-2} s^{-1}
1.	5,23±0,18	1,27±0,03	0,03±0,01
2.	3,81±0,23	1,06±0,07	0,02±0,01
3.	5,1±0,11	1,57±0,01	0,04±0,01
4.	5,14±0,23	1,24±0,03	0,03±0,01
5.	10,35±0,12	1,81±0,01	0,04±0,01
6.	5,83±0,25	1,64±0,02	0,03±0,01

Treatments: 1 – Control; 2 - Cu -1200 mg/кg; 3. Cu -1200 mg/кg + suspension of *Ag. radiobacter* in sol + Microcom VA, foliar; 4 - Cu -1200 mg/кg + suspension of - *Ps. putida* in sol + Microcom VA, foliar; 5 - Cu -1200 mg/g + suspension of + *B. subtilis L* in sol + Microcom VA, foliar; 6 -Cu -1200 mg/кg + suspension of *Ag. radiobacter +B. subtilis L + Ps. Putida* in sol + Microcom VA, foliar.

The accumulation *of proline* in plant organs is a metabolic response to stress caused by salinization, drought and other stress factors [6] [12]. It is known that proline can protect membranes and proteins from the adverse effects of high concentrations of inorganic ions and extreme temperatures [5] [13].

After three months of seedlings growth, the content of proline notably increased in leaves of plants grown under the surplus of Cu, 136,1 % to the control (tab.3). Application of bacterial suspension into the soil and foliar treatment by Microcom-VA led to decreasing trend of proline content in leaves of plants treated by the excess of Cu.

TABLE 3.
Effect of Cu and nutrients on the proline content in leaves of grapes seedlings under the Cu excess in the soil, cv. Viorica

Cod of treat-ments	29.07.2015		15.09.2015	
	µM/g	% to control	µM/g	% to control
1.	2,59±0,17	100	1,25±0,12	100
2.	3,52±0,17	136,1	1,53±0,13	122,3
3.	2,82±0,17	108,9	0,86±0,03	68,6
4.	2,07±0,13	80,2	1,46±0,03	117,1
5.	2,30±0,09	88,9	1,06±0,05	85,1
6.	2,30± 0,13	88,8	0,97±0,03	77,6

Treatments: 1 – Control; 2 - Cu -1200 mg/кg; 3. Cu -1200 mg/кg + suspension of *Ag. radiobacter* in sol + Microcom VA, foliar; 4 - Cu -1200 mg/кg + suspension of - *Ps. putida* in sol + Microcom VA, foliar; 5 - Cu -1200 mg/g + suspension of + *B. subtilis L* in sol + Microcom VA, foliar; 6 -Cu -1200 mg/кg + suspension of *Ag. radiobacter +B. subtilis L + Ps. putida* in sol + Microcom VA, foliar.

At the beginning of September, when the plant growth process ceased and the shoot maturation began, the proline content in leaves decreased, but comparing to control the same trend, mentioned in July, occurred. Reduction of the proline amount in the leaves in presence of bacteria may be due to bacterial activity in contrasting the oxidative stress, increasing hydration of the leaves, producing biologically active substances, and reduction the amount of a stress-related molecule as ethylene in plant tissues.

D. Rojas-Tapias et al. [13] report a similar fact: inoculation of maize plants with rhizobacteria under conditions of salt stress decreased the concentration of proline in the leaves with a simultaneous increase in plant biomass, and in the absence of stress bacteria increased the level of proline in the leaves. It is possible that PGPR bacteria generate a kind of stress for plants, stimulating their more active growth.

IV. CONCLUSION.

The obtained data indicates the possibility of using a suspension of some strains of bacteria and half dose of Microcom-VA to enhance the resistance of grape seedlings to Cu excess in soil. It has been established that half of the recommended dose of the micronutrient complex Microcom-VA and suspension of plant growth promoting bacteria *(Bacillus subtilis L)* and a consortium of three *strains Az. chroococcum + B. subtilis L + Ps. putida*) improve the nutrition of seedlings, reduce the negative effect of Cu in the soil and increase tolerance of grape seedlings to excess of the Cu content in the soil.

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