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USING HUMIC ACIDS AND *VITIS VINIFERA* SEED EXTRACT IN DECREASING THE THERMAL STRESS ON *PRUNUS AVIUM*

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Abstract

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In this paper, the low temperature resistance of some cherry varieties (Kordia, Simone, Regina and Summit) is discussed with a particular emphasis on the method of measuring the electrolytes leakage, the total content of free ions and the peroxydase determined enzyme activity. The aim was to eliminate the negative effects that generate the oxidative stress in cherry by administering a fertilizer based on humic acids and polyphenolic extract from *Vitis vinifera* seeds. The cherry crop fertilization was done through the "drip" and the foliar system using 10 L ha^{-1} fertilizer with the "drip" system and 5 L ha^{-1} with the foliar fertilization. Once the average temperature drops below 0°C, the ion leakage in fertilized plants showed values of 1.06–2.12 times lower than in unfertilized plants. The permeability index value was of 0.14–0.27 in fertilized plants and of 0.15–0.40 in unfertilized plants and the total content of free ions in all varieties of fertilized cherries was smaller. During negative temperatures each fertilized variety responded differently with an increase in the enzyme activity and, at the start of the vegetative activity when the average temperature recorded positive values it was 1.23–1.47 times higher in fertilized plants.

Key words: Resistance to cold, sweet cherry, humic acids, *Vitis vinifera*

Introduction

The cherry is grown with results more or less satisfactory depending on the local climatic conditions. The adaptation to seasonal temperature changes is a prerequisite for the culture of many cherry species. The annual acclimatization process involves structural and metabolic adjustments that occur in the plants (Gomez et al., 2005).

The low temperature is the main environmental factor limiting the productivity and geographical distribution of the horticultural plants. This decreases the biochemical activities in the plants, disturbs the normal functioning of the physiological processes with the formation of some permanent damages with the reduction of the ions flow in the plant. An important cause of the damages caused by the low temperature is associated with the production of active forms of oxygen (hydrogen peroxide, singlet oxygen, superoxide and hydroxyl radical). Reactive oxygen species (ROS) are produced continuously as byproducts of different metabolic pathways which are located in different cellular compartments such as chloroplast, mitochondria and paroxysms (Rio et al., 2006; Navrot et al., 2007). Through a variety of reactions, $O_2^{\bullet-}$ leads to the formation of H_2O_2, $OH^{\bullet}$ and other ROS. The ROS comprising $O_2^{\bullet-}$, H_2O_2, $_1O^2$, $HO_2^{\bullet-}$, $OH^{\bullet}$, ROOH, ROO^{+} and RO^{+} are highly reactive and toxic and cause damage to proteins, lipids, carbohydrates and DNA which ultimately results in cellular death (Mittler, 2002, Apel and Hirt, 2004; Mahajan and Tuteja, 2005; Tuteja, 2007; Tuteja, 2010; Khan and Singh, 2008; Gill et al., 2010; Waraich et al., 2012).

The most important feature of the reactive oxygen species is the instability, which makes them highly reactive because of their tendency to regain their missing link electron from another simpler molecule or from the stable organic macromolecules.

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They have a high oxidative capacity, being able to alter the biochemical features of the cells and their conformation, process called cellular oxidation or oxidative stress.

It is considered that the plants that are resistant to oxidative stress, caused by oxygen free radicals, are resistant to low temperatures, drought and salinity (Akram and AL-Qurainy, 2006; Petcu et al., 2007).

In order to increase the plant resistance to low temperatures there were initiated a series of biochemical researches on the dynamics of the water-soluble substances, free amino acids (proline), phytohormones (abscise acid), cellular enzymatic processes (concentration of sugars, ascorbic acid, catalase activity, peroxydase, etc.).

In this paper, the low temperature resistance of some cherry varieties is discussed with particular emphasis on the method of measuring the electrolytes leakage, the total content of free ions and the peroxydase determined enzyme activity. The aim was to eliminate the negative effects that generate the oxidative stress in cherry by administering a fertilizer based on humic acids and polyphenolic extract from *Vitis vinifera* seeds.

The humic substances in the coal and the polyphenols extracted from the *Vitis vinifera* seeds (gallic acid, monomers of flavan 3-ol: catechin, epicatechin, galocatechin, epicatechin 3-O gallate, dimers, trimers and polymers of the procyanidins having an antioxidant activity 20 times stronger than vitamin C and 50 times stronger than vitamin E) contained in the obtained biofertilizant will help to eliminate the effects of oxidative stress and to agro-chemically optimize the soil.

The formation of free radicals in the soil and plants from the oxidative stress occurs in three stages (Bita - Dumitru and Preda, 2005; Dumitru and Grecu, 2010):

Initiere

$$RH \longrightarrow R^{\bullet} + H^{\bullet} \quad (1)$$

Propagare

$$R^{\bullet} + O_2 \longrightarrow ROO^{\bullet} \quad (2)$$

$$ROO^{\bullet} + RH \longrightarrow ROOH + R^{\bullet} \quad (3)$$

Intrerupere

$$ROO^{\bullet} + ROO^{\bullet} \longrightarrow ROOR + O_2 \quad (4)$$

$$ROO^{\bullet} + R^{\bullet} \longrightarrow ROOR \quad (5)$$

$$R^{\bullet} + R^{\bullet} \longrightarrow 2\,R \quad (6)$$

The polyphenolic antioxidants in the *Vitis vinifera* seeds intervene in the radical mechanism, which they interrupt by transfer of H (Apak, 2008) by the following scheme:

$$R^{\bullet} + AH \longrightarrow RH + A^{\bullet}$$

$$RO^{\bullet} + AH \longrightarrow ROH + A^{\bullet}$$

$$ROO^{\bullet} + AH \longrightarrow ROOH + A^{\bullet}$$

$$R^{\bullet} + A^{\bullet} \longrightarrow RA$$

$$RO^{\bullet} + A^{\bullet} \longrightarrow ROA$$

$$ROO^{\bullet} + A^{\bullet} \longrightarrow ROOA$$

The antioxidants are the first line of defense against the free radicals that are generated in plants with an important role in maintaining the health of the plants (Coulson et al., 2006).

The humic acids cause changes occurring in the plant metabolism due to their absorption (once these compounds enter plant cells occur biochemical changes in the membranes and the cytoplasm compounds in the cells) (Akram and Al-Qurainy, 2006; Popov, 2008). The humic acid molecules affect the cellular membrane permeability resulting in the intensification of the electronic transport and the exchange of minerals needed in specific metabolic processes. Regulating the osmotic pressure results in their increased permeability (Laila and Elbording, 2009).

Materials and Methods

The study on the behavior in culture at low temperatures in the Valcea area – Romania of some cherry varieties on low vigour rootstocks was executed in the locality Copaceni, located at the latitude 45, longitude 23.983 45º0′0″ North, 23º58′59″.

The plant material used included cherry branches taken directly from the field from 4 varieties (Kordia, Simone, Regina and Summit) in late November and December 2013 and January-March 2014 fertilized with humic acids and extract obtained from *Vitis vinifera* seeds. The cherry crop fertilization was executed through the "drip" system and the foliar system during April-October 2013 by using 10 L ha^{-1} fertilizer for the "drip" and 5 L ha^{-1} for the foliar fertilization.

For the harvested plant material was determined the electrolyte leakage, the total free ions content, the permeability index and the peroxydase enzymatic activity.

All the chemicals and reagents used were of analytical grade and were purchased from Fluka and Merck.

Determining electrolytes leakage

Cherry branch segments of length 10 cm and diameter 5 mm were introduced into tubes containing 50 mL of distilled water. The samples were incubated at room temperature for 24 hours after which the conductivity of the solution was measured with a conductivity meter type Hanna HI 8633. After measuring the

conductivity the samples were re-incubated for 15 minutes at a temperature of 120°C. The final conductivity was determined after 24 hours from the samples re-incubation (Linden, 2002).

For determining the total content in free ions was milled 1 g of plant material in 20 mL of distilled water. The electrolyte solution conductivity was measured with the conductivity meter and the results were expressed in μS g^{-1} fresh substance.

The permeability index was obtained by making the ratio between the membrane permeability and the total content value of free ions. The results are shown in Tables 1-4.

Determining the peroxydase activity EC 1.11.1.7

5 g of ground plant tissue is weighed, is transferred to a measuring flask with 100 mL distilled water and is brought to the mark; it is mixed well and left 15 minutes for extraction, the mixture is stirred several times; is filtered through a fluted filter into a clean and dry flask. Of the filtrate 10 mL is taken into another clean and dry flask, 1 mL of hydrogen peroxide 0.5 M is added, 1 mL 1% guaiacol, is mixed and left at room temperature for 30 minutes. A brown color is obtained more or less intense according to how active is the enzyme.

It is determined the solution extinction to a photometer Cary 50, at the wavelength of 420 nm, by 1 cm vat, using distilled water as a blank. If the solution is highly colored, an appropriate dilution is made (Dumitru and Marinescu, 2010). Depending on the extinction determined and the dilutions performed, the peroxydase activity in extinction per 1g of product is expressed:

$$A = \frac{E \times d}{G},$$

where: E – determined extinction; G – quantity of enzymatic substance or enzyme source, g or mL; D – dilution.

Table 1

Experimental data obtained for the cherry samples in December 2013

Average temperature - 4,57°C						
Sample	Control			Fertilized		
	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index
Kordia	427	2010	0.21	251	1293	0.19
Simone	265	1764	0.15	188	1260	0.14
Regina	560	2180	0.25	280	1320	0.21
Summit	400	2309	0.17	215.5	1275	0.16

Table 2

Experimental data obtained for the cherry samples in January 2014

Average temperature – 2.1°C						
	Control			Fertilized		
Sample	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index
Kordia	1444	4.375	0.33	540	2510	0.21
Simone	760	2.714	0.28	466	2490	0.18
Regina	1620	4.378	0.37	645	2575	0.25
Summit	1368	4.560	0.3	502	2508	0.2

Table 3

Experimental data obtained for the cherry samples in February 2014

Average temperature – 1.98°C						
Sample	Control			Fertilized		
	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index
Kordia	2010	5222	0.38	1056	4010	0.27
Simone	1530	3825	0.4	890	3850	0.23
Regina	2412	7500	0.32	1056	4010	0.26
Summit	1720	6164	0.27	1160	4610	0.25

Table 4
Experimental data obtained for the cherry samples in March 2014

Average temperature +3,88°C						
Sample	Control			Fertilized		
	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index	Conductibility, μS g^{-1}	Total content in free ions, μS g^{-1}	Permeability index
Kordia	1102	3353	0.32	410	1801	0.22
Simone	910	3334	0.27	350	1670	0.2
Regina	1321	4002	0.33	412	1780	0.23
Summit	925	3667	0.25	460	2000	0.23

Statistical analysis

Three replicates of each sample were used for statistical analysis. Analysis of the data was performed on the original data by one-way analysis of variance (ANOVA) or regression analysis. Differences at $P < 0.05$ were considered significant.

Results and Discussions

The plants exposed to low temperatures develop physiological and biochemical reactions that determine their tolerance to freezing. Low temperature and photoperiod shortage are two major factors that determine cold acclimation in plants (Gomez et al., 2005). During the cold acclimation the cells and tissues of the plants suffer a variety of modifications which allow their operation at lower temperatures allowing for survival. The occurrence of a stress due to low temperature in plants triggers a wide range of responses, from the modification of gene expression and cellular metabolism to changes in the growth and production rate.

● The appearance of oxygen reactive species due to thermal stress requires the intervention of some exogenous antioxidants that, with those present in the plant tissues, help reduce its negative effects.

● The humic substances in coal and the polyphenols extracted from the *Vitis vinifera* seeds (gallic acid, monomers of flavan 3-ol: catechin, epicatechin, galocatechin, epicatechin 3-O gallate, dimers, trimers and procyanidins polymers having an antioxidant activity 20 times stronger than vitamin C and 50 times stronger than vitamin E) were used to increase the antioxidant activity of the four cherry varieties studied (Kordia, Simone, Regina and Summit) and to agro-chemically optimize the soil.

● The fertilizer effect was highlighted by determining the physiological state of the plants (measuring the electrolyte leakage, the total content of free ions and the permeability index) and measuring the enzyme activity (peroxydase), Table 5.

Therefore, the ability to estimate the degree of cold resistance of the plants is important because on it depends the proper functioning of the physiological and biosynthetic processes during the growing season.

The electrolyte leakage test and the enzyme activity in plants are often used to determine the plants behavior to low temperatures.

By analyzing the experimental data obtained from the measurements executed, we find the following:

With the decrease of the average temperature in December 2013 to –4.38°C, in January 2014 to –2.18°C and in February 2013 to –1.98°C, the ions leakage for all varieties of fertilized and unfertilized cherry registered an increase. Thus, the unfertilized plants of the Kordia variety present a value of the ion leakage increase 14.5 times higher than the beginning of the plant exit period from the vegetative pause, the variety Simon 17.19 times, the variety Regina 12.36 times and the variety Summit 14.95 times.

In March, with the registration of the positive average temperature (+3.88°C) and the beginning of the plant exit period from the vegetative pause, the ion leakage in fertilized plants

Table 5
Peroxide enzyme values (POD) in cherry samples

Peroxydase activity, UE/g						
Sample	Decembrie 2013		Ianuarie 2014		Februarie 2014	
	Control	Fertilized	Control	Fertilized	Control	Fertilized
Kordia	0.92	1.853	2.118	2.985	1.01	2.397
Simone	0.785	1.378	1.873	2.314	0.832	1.634
Regina	1.35	2.18	2.51	3.102	1.512	2.943
Summit	0.89	1.663	2.02	2.984	0.959	1.9

decreased being 1.82 times less for the variety Kordia, 1.68 times for the variety Simon, 1.82 times for the variety Regina and 1.85 times for the variety Summit than the values obtained at the end of February 2014.

The fertilized samples presented during December 2013-February 2014 lower ion leakages than the unfertilized samples 1.90 times for the variety Kordia, 1.71 times for the variety Simon, 2.12 times for the variety Regina and 1.06 times for the variety Summit.

In March 2014, the fertilized samples registered an ions leakage to the unfertilized samples 2.68 times lower for the variety Kordia, 2.6 for the variety Simon, 3.2 for the variety Regina and 2.01 for the variety Summit.

The permeability index presents values for the unfertilized samples in December 2013 between 0.15–0.25 to the fertilized samples of 0.14–0.21, in January 2014 of 0.18–0.25 to the 0.28–0.37 and in February of 0.27–0.4 to 0.23–0.27.

The values obtained after using the electrolyte leakage test (especially potassium), which is based on the cellular membranes damage principle, reveals the tissue damage estimate for the four varieties of cherry. It is noted that the 4 varieties of cherry have a different negative temperatures reaction due to the fact that the temperatures below 0°C lead in most cases to extracellular freezing. The plasma membrane forms a barrier against the growing ice crystals, causing the movement of water outside the cell, due to lower chemical potential of the ice in comparison with that of the water. The stress generated by this cellular dehydration caused by the freezing is extremely severe and the plant cells will lose most of the osmotically active water. There is a reduction in enzyme activity, changes in the metabolism and reduction in the photosynthetic ability in the plant tissues (Dubey, 1997; Barrett et al., 2004). In the plants membranes, these changes are often associated with the permeability increase and the integrity loss (Campos et al., 2003) because the cells are subjected to a stress that causes the loss of tissues electrolytes in the environment (Mattsson, 1996; McNabb and Takahashi, 2000).

The appearance of oxygen species which are highly reactive, affecting the major cellular components following the metabolic changes during negative temperatures, causes plants to create their own defense mechanism. The main role in the oxidative stress defense have the antioxidant enzymes, dismutase superoxide (SOD), then the peroxydase ascorbate (APX), peroxydase (POD), catalase and antioxidant molecules with low molecular weight (ascorbic acid, glutathione).

The determining of the enzymatic peroxydase activity (POD) is considered a measure of the current stress level, or a measure of evaluating the plant ability to adequately respond to this biochemical factor induced by the physiological state of the plant (Bradford, 1976; Mittler, 2002). Table 5 presents the peroxydase enzyme values corresponding to the cherry samples in November 2013.

From the data obtained it appears that the peroxydase value in early November 2013 is 2.01 times bigger for the fertilized Kordia variety than for the unfertilized, 1.76 times for the Simone variety, 1.6 times for the Regina variety and 1.86 times for the Summit variety March 2014.

During the negative temperatures each variety responded with an increase in the enzyme activity, response which was different among the varieties. The fertilized Kordia cherry variety showed a peroxydase activity 1.41 times bigger than the unfertilized variety, the variety Simone 1.24 times, the variety Regina 1.23 and the variety Summit 1.47 times.

The beginning of the vegetation activity with the temperature increase in March 2014 is followed by a decrease of the peroxydase values between 1.66–2.25 times for the unfertilized samples and 1.05–1.57 for the fertilized samples.

The increase in the peroxydase enzymatic activity in the biotic stress conditions according to the determinations executed shows the importance of detecting and quantifying it along with other antioxidant enzymes for the deeper knowledge of the plants pathophysiology and the creation of plant protection solutions and techniques in crop stress conditions.

Corroborating the data obtained from determining executed on the 4 varieties of cherry emphasizes the role of the fertilizer based on humic acids and polyphenolic extract of *Vitis vinifera* seeds in increasing resistance to the stress caused by low temperatures. The membrane degradation is reduced to the fertilized plants as a result of the humic acids which have multiple actions on the plants, including: increase of the ARN messenger concentration essential for the biochemical processes; increase of the protein content and the enzymatic synthesis by increasing their concentrations (catalase, peroxydase, diphenoloxydase, poliphenoloxydase, invertase (Albayrak and Camas, 2005; Akram and Al-Qurainy, 2006); increase of the energy production through the formation of triphosphate adenosine (ATP) in plants and the hormone synthesis (Popov, 2008; Popova et al., 2008). The humic substances enhance the plant metabolism through the increased availability of various minerals present in the humic molecules and enhance the electronic transport.

From the data obtained we remark the varieties Regina and Kordia as being the most responsive to the applied treatment. Not to be neglected the varieties Summit and Simone that also have a good response to the treatment.

Conclusions

The regeneration or growth capacity of some plants is controlled by certain endogenous substances, not reacting separately but complementing each other. The use of the bio-

fertilizer obtained from humic acids and polyphenolic extract of *Vitis vinifera* seeds constitutes a mixture of endogenous substances that, by their properties, contributes to the protection of the cherry plants from the stress caused by low temperatures. The antioxidants intake brought by the polyphenolic extract with those present in the plant tissues have contributed to the antioxidant activity increase in fertilized plants and the humic acids significantly contributed to reducing the electrolyte leakage for the fertilized samples during the low temperatures period.

The abundance of raw materials and the low cost price recommend the fertilizer use in the plant crop root and extra-root fertilization.

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