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# THE CORRELATION OF THE ACCUMULATION OF COLD UNITS WITH SOME PHYSIOLOGICAL AND BIOCHEMICAL PROCESSES IN THE FLORAL BUDS IN THE CULTIVATION OF NECTARINES (*PRUNUS PERSICA* VAR. NECTARINA) IN NORTH-WESTERN ROMANIA

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## ABSTRACT
The knowledge of physiological and biochemical mechanisms which are at the base of cold resistance for fruit trees cultivated in the temperate climate represent an important aspect for the practice of cultivating trees. The purpose of this study was to determine the relationship between the content of phenolic compounds, proteins and peroxidase in flower buds in 5 varieties of nectarines (Cora, Ark 165, Delta, Romamer II and Crimson Gold) cultivated in N-W Romania and the debut of the stages of ectodormancy, endodormancy and ecodormancy. The dynamic of these compounds was correlated with the calculation of chill units necessary for the phenological stages. The biochemical parameters followed have had a characteristic seasonal dynamic for every cultivation, also influenced by the accumulation of chill units.
**Key words:** *Prunus persica* var. nectarine, phenolic compounds, peroxidase, flower buds, chill units

## INTRODUCTION

For the species of trees inain the temperate climate, the accumulation of chill units during the period of one year represents a very important requirement. The cultivation of a certain species in a certain area and its economical performance are depended upon the fulfilment of these requirements (Lang, 1996; Erez et al., 1979). The situation in which the buds do not receive sufficient refrigeration temperatures during the winter in order for them to completely come out of endodormancy, the tress will develop one or several physiological symptoms associated with chill insufficiency like for example the later appearance of leaves, anomalies in the development of polen and pestle, the development of the buds for the next year could be affected etc. (Byrne et al.,1992). In the case in which the chill requirements are fulfilled early, the forced sleep period (ecodormancy) is prolonged for too long and there is the risk of losing the crop because of late spring frost, a frequent situation in the area (N-W Romania). The problems associated with endodormancy and with what „refrigeration temperatures" represent has not yet been clearly defined. Most researchers agree that temperatures under the point of freezing and higher are not effective for the accumulation of refrigeration units (Fishman et al., 1987 a , Fishman et al., 1987 b ).

No method of characterizing the area under the aspect of the number of refrigeration hours accumulated has yet been validated for N-W Romania which leads to a series of difficulties in establishing the ecological potential for fruit trees in the region. One of the objectives of this study was to characterize the phenological behaviour with the refrigeration requirements for 5 cultivations of nectarines (Cora, Ark 165, Delta, Romamer II, Crimson Gold).

Detailed studies for cultivations of peaches and nectarines, for the purpose of establishing a system of classification the chill requirements for these trees, for a better capitalization of the climactic potential in the studied areas were carried out by Küden et al., 1997 and Pieterse et al., 2006.

The studies carried out by Pérez, (2004) for 33 cultivations of peaches confirm the close relationship that exist between the blooming (moment, duration, bud density, flower density) and the fulfilment of the refrigeration requirements of the cultivations.

The fact that the antioxidant metabolism of plants suffers from modifications in conformity with the season cycle.

Some biochemical markers could indicate the relative value of somnolence in organs, tissues and even cells. Studies have proven that free radicals are formed in buds in the stage of vegetative rest. The plants have specific enzymes, called antioxidant enzymes, for the annihilation of the negative effect of formed free radicals. The antioxidant enzymes from plants are catalase, superoxide dismutase, peroxidase, ascorbate peroxidase, glutation S-transferase. Studies show that it is of great importance to retain a balance between formed free radicals and those which have been annihilated/ discarded. Plants resistant to oxidative stress, generated by free oxygen radicals, are resistant to frost. Protection against free oxygen radicals is done by the action of compounds and antioxidant enzymes. The changes which occured in the activity of some antioxidant enzymes (catalase, peroxidase, ascorbate peroxidase), of the sulfhydryl compounds and the glutation content, were determined during the winter season for two apricot cultivations, with different chill requirements (Bartolini et al., 2006). For both cultivations the enzyme activities and the content of glutation have undergone changes. The smallest antioxidant activities have been detected

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in the variety with the highest chill requirement. These results suggest that even the minimum treshold of the antioxidant activities could be crucial for the elimination of free radicals generated during de winter season.

A second objective of the research has been to determine the dynamic of the phenolic compounds, protein content and peroxidase activity in the flower buds for 5 nectarine cultivations (Cora, Ark 165, Delta, Romamer II, Crimson Gold) in N-W Romania, during the stage of vegetative rest.

## MATERIALS AND METHOD

***Biological material***

The flower buds have been taken at an interval of 12 – 14 days from necatrine tress, which were 5 years old from S.C.P.P. Oradea (Fruit Research Station and Manufacturing Oradea), grafted onto sour cherry trees (*Prunus cerasifera*), cultivated at a distance of 4 x 3 m, belonging to the cultivations: Cora, Ark 165, Delta, Romamer II, Crimson Gold.

***The Determination of Temperatures***

The temperature was made every hour using the logger HANNA 143 HI. The calculation of chill units was made using the UTAH model (Richardson and colab. 1974). The UTAH model allows that different temperatures have different effects on the chill accumulations. The temperatures between 2,5 – 9,1°C are considered to have the biggest contribution to the accumulation of chill units and every hour spent in this interval will have a chill unit attributed to it. Lower and higher temperatures have a negative effect on the accumulation of chill units and they are attributed values accordingly.

***The Preparation of the Vegetal Extract***

Twenty flower buds have been detached from the annual branches, weighed, grinded at low temperatures in the presence of a phosphate buffer pH 7.2 (the ratio between the weight of the buds and the phosphate buffer solution was 1:20), passed through Ependorf tubes and centrifuged at 10000 rpm, at 4° C for 20 minutes. The supernatant was used for the determination of proteins, peroxidase and phenolic compounds. The gathering of was made in the B phases (the swelling of the buds), F (the first open flower), and G (the first fallen petals) after Flekinger, 1945.

***Peroxidase assay***

Enzymatic activity of the peroxidase was measured with a spetrophotometer, the principle of the method being the oxidation of p-phenylenediamine by the peroxidase enzymes present in a vegetal extract, as a result of which a violet-colored solution is obtained. There is a direct proportion between the color intensity of the solution, measured with the spectophotometer at 483 nm wavelenght (UV-VIS Spetrophotometer UVmini-1240, Shimadzu) and the activity of the peroxidase.

***Total Soluble Protein assay***

The protein concentration from the bud extract was determined as described by Bradford (1976), using bovine serum albumin (BSA) as standard. Shortly, for the extraction of proteins we used buds extracted using a 6.7 $\cdot 10^{-3}$M phosphate buffer (pH 6.1). The extract was centrifuged for 20 minutes at 10 000 rpm and $4^0$C. The supernatant was used for the spectrophotometric determination of protein, at wavelength 595 nm (Shimadzu UV-Visible mini-1240).

***Poliphenolic Compounds assay***

Total phenolic content was determined by the Folin-Ciocalteu method. This method combined 100 µl bud extract, 1700 µl distillated water and 200 µl Folin-Ciocalteu reagent; then mixed well using a Vortex. The mixture was allowed to react for 3 minutes, and then 1 ml of 15% $Na_2CO_3$ (Arnous et al., 2001). solution was then added and mixed well. The samples were incubated at room temperature, in the dark for 2 hours. The absorbance was taken at 750 nm using a spectrophotometer (UV-VIS Spetrophotometer UVmini-1240, Shimadzu). The standard curve was linear, between 0.1-0.5 mg/ml gallic acid. The results were expressed in gallic acid equivalents (GAE; mg/g fresh weight). Adequate dilution was needed if the absorbance value measured was over the linear range of the standard curve.

## RESULTS AND DISCUSSIONS

***A). The correlation of cold untis with physiological processes and latency periods***

The calculation of the accumulation of chill units CU (chill units), following the UTAH model (using Wizard Java Script Version 1), over a period of 6 years (2005 - 2010), has discolsed the fact that in the studied area (47°09′13″N 21°55′18″E ) the yearly accumulations of CU have varied between 900 CU in 2005 – 2006, and 1380 CU in 2007 – 2008 (Fig.1).

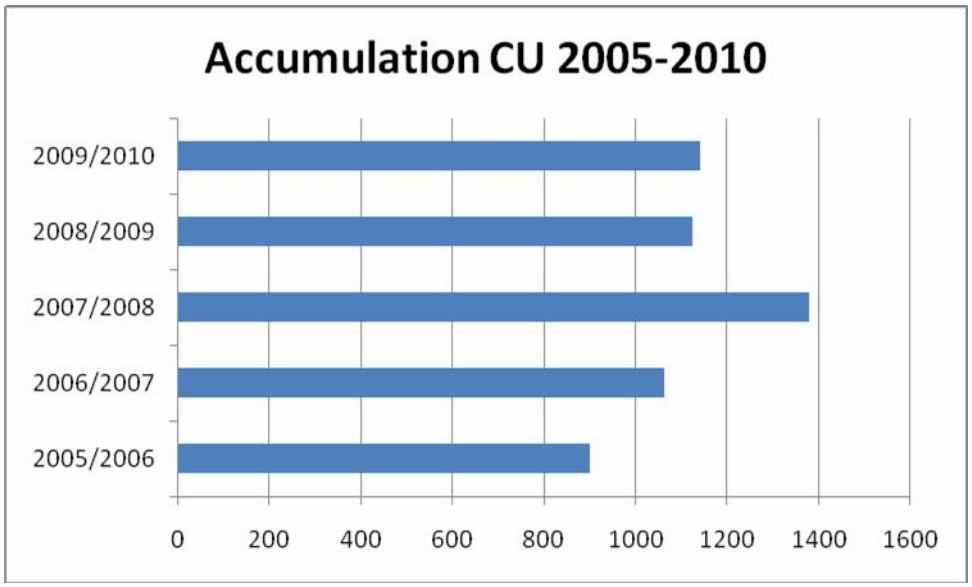



**Fig.1** Accumulation of CU over a period of 6 years (2005 - 2010)

The study of the dynamic of the accumulation of chill units over months (october - april) between the years 2005 – 2010, highlights 2 accumulation peaks, one in the month of november and the other one in the month of february or march (**Fig.2**). Larger quantities of chill units are accumulated in november, the average ove 5 years being 275,2 CU. In the month of february the average of the accumulations is 173,7 CU and in the month of march it is 241 CU. The large number of chill units which are accumulated in march, constitute and important restrictive factor in the extension of surfaces on which species with low cold requirements are cultivated. The fulfilment of these requirements determine the early emergence of the trees from the stage of endodormancy and the entrance into ecodormancy, a stage in which the trees are more exposed to cold. Endodormancy is caused by internal factors and it has a genetic determinism (Samish, 1954). It is a progressive mechanism which evolves until reaching the deep rest state (Lang et al., 1987), and it implies the expressions of genes with a stabilizing role for the cellular membranes under the effect of low temperatures (Faurobert et al., 2006). Ecodormancy is determined by the action of unfavourable factors after the beginning of vegetation such as cold and the stress of draught, which induce critical signals which stop the growth of buds (Lang, 1987, 1996; Horvath et al., 2003).

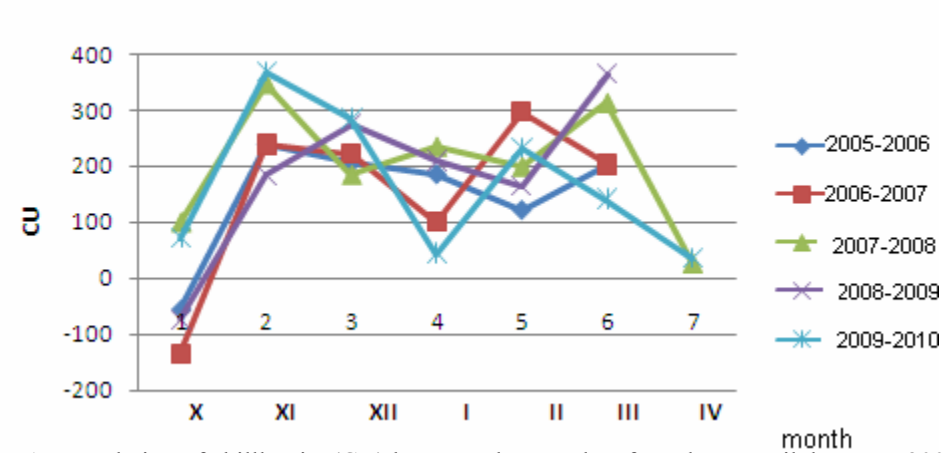



**Fig.2.** The Accumulation of chilll units (CU) between the months of october – april, between 2005 – 2010

For a series of varities being cultivated in the USA, the cold requirement is between 650 CU (Snow Queen) and 900 CU (Snow Zee) ( Lorimer et al., 2006). In **Fig. 3** one can see that the conditions in the studied area (N – W Romania), the cold requirements are satisified after aprox. 100 – 115 days from the debut of the CU accumulation.

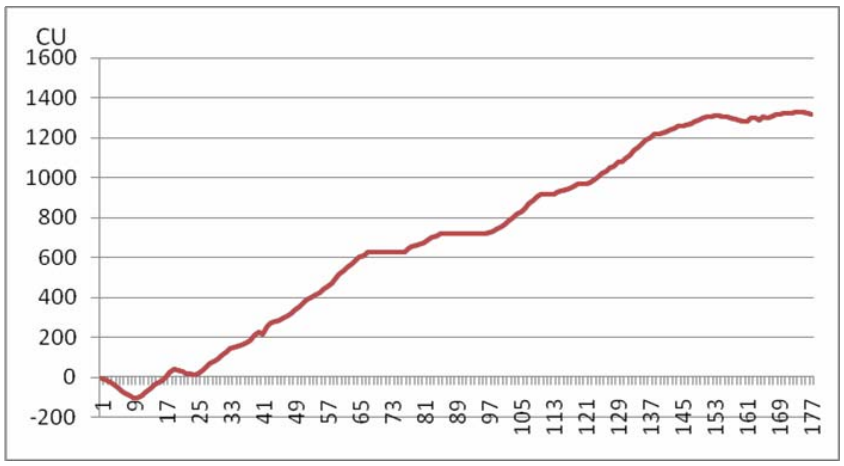
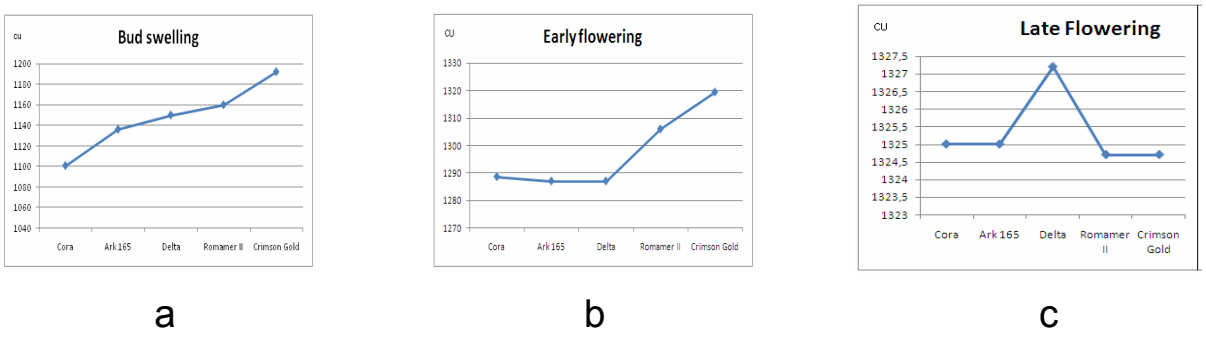



Fig.3 The dynamic of chill unit accumulation over a period of 177 days, between october 1st 2009 and - april 14 2010

The phenophase of the swelling of the buds in the cultivations taken into they study started in 26.02.2010 with the accumulation of 1192 CU with the varitty Crimson Gold **(Fig. 4a).** In the fall of 2009, the accumulation of CU started on 17 october (2,4 CU). The fall of leaves took place on the last part of november. The first negative temperatures were recorded at the beginning of november (01.11 - 3,2 $^0$C; 01.11. - 3,7 $^0$C ; 03.11 – 3,5$^0$C ) and the constant accumulation of CU on 03.11.2009. The debut of the forced rest period is associated with the fall of leaves (Fuchigami et al. 1987, Naor et al, 2003, Guerriero, et al, 2006) and also with the constant accumulation of chill units (Richardson et al., 1974; Erez et al., 1979; Ruiz et al., 2007).




a b c

**Fig. 4** a. Phenophase of bud swelling. b. Phenphase of early flowering. c. Phenophase of late flowering

The difference between the accumulation of chill units between the first (Cora) and last of the cultivations (Crimson Gold) studied in this phenophase is 91.2 CU and the time difference is 7 days.

The phenophase of early flowering (starting with the moment in which 10% of the flowers are open, according to Adato (1990), debuted on 26.03.2010 (CU accumulation 1288.6) and ended on the 05.04.2010 (CU accumulation 1319.5) with the cultivation Crimson Gold (**Fig. 4b**)

The phenophase of late flowering starts on the 06.04.2010 with the cultivation Cora and ends on the 13.04.2010 with the cultivation Crimson Gold. (**Fig. 4c**).

***B). Modificatoins of the biochemical processes in relation to the accumulation of chill units***

For all cultivations taken into account we have recorded a seasonal dynamic regarding the content of phenolic compounds. (**Fig. 5**). The greatest quantity of phenolic compounds has been recorded on the 28.01.2010, for the cultivations Delta (0.0021± 0.083 mgGAE/g fresh weight) and Romamer (0.0018± 0.038 mgGAE/g fresh weight). On the 09.02.2010, the greatest concentration of phenolic compounds was reached by the cultivation Romamer (0.0017 ± 0.84 mgGAE/g fresh weight) followed by the cultivation Delta (0.0015± 0.10 mgGAE/g fresh weight). In the following period (24.02.2010) there was a sudden drop in the content of phenolic compounds. For example in the case of the cultivation Romamer, the quantity of phenolic compounds was 0.00065± 0.01 mgGAE/g fresh weight. In the beginning of March (08) as well as at the end of the month (29), there was a growth in the content of pehnolic compounds in relation to the end of february (24). In the case of cultivation Delta, it was the period with the highest concentration of phenolic compounds compared

to the other cultivations taken into account (0.0014 ± 0.01 mgGAE/g fresh weight respectively 0.0013± 0.02 mgGAE/g fresh weight). The smallest quantity of phenolic compounds was recorded during the month af April, for the cultivations Crimson Gold, Romamer II and Ark 165, the values obtained were almost idential to the ones from 24.02.2010.

The drop in the content of phenolic compounds in relation to rising temperatures can be an important indication regarding the role they have in the protection of buds during endodormancy.

A close relationship between the activity in the apple's buds, of poliphenoloxidase and the endogenous content of phenoles has been described by Wang et al., 1991. They have observed that while the content of endogenous phenols has decreased, the activity of poliphenoloxidase has increased. There has not been any evidence of an opposite relationship between poliphenoloxidase and peroxidase. The conclusion has been that endogenous phenoles can modify the activity of poliphenoloxidase and peroxidase as stimulants or inhibitors (Szecskó et al., 2002).

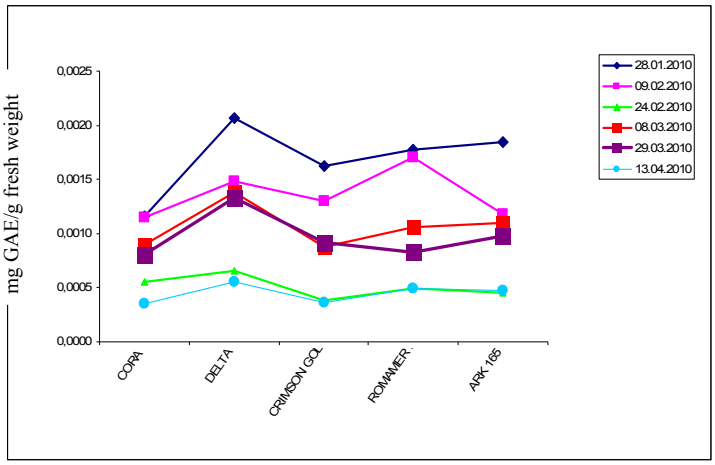



Fig.5. The quantity of phenolic compounds (mgGAE/g fresh weight) at 5 cultivations of nectarines during an interval between 26 February 2010 and 13 April 2010

The peroxidase activity (**Fig. 6**) has, like in the case of phenolic compounds, a seasonal dynamic which is similar for all five studied cultivations. Unlike the quantities of phenolic compounds, in the case of peroxidase the greatest enzyme activity has been recorded during the month of April (13.04.2010) with values between 0.1889 ± 0.001 U/mg protein, for the cultivation Cora, and 0.1015± 0.002 U/mg protein for the cultivation Romamer II. The most reduced enzyme activity has been recorded during the month of January.

A comparison between the dynamic of peroxidase activity and phenolic compounds shows that while peroxidase activity registers declines for the determinations carried out during the month of January, February and the beginning of March (28.01; 09.02; 24.02; 08.03) and growths at the end of March (29.03) and April (13.04) in the same intervals there is an opposite result for phenolic compounds. The results we have obtained confirm the observations of Wang et al., 1991, according to which endogenous phenoles can inhibit the peroxidase activity.

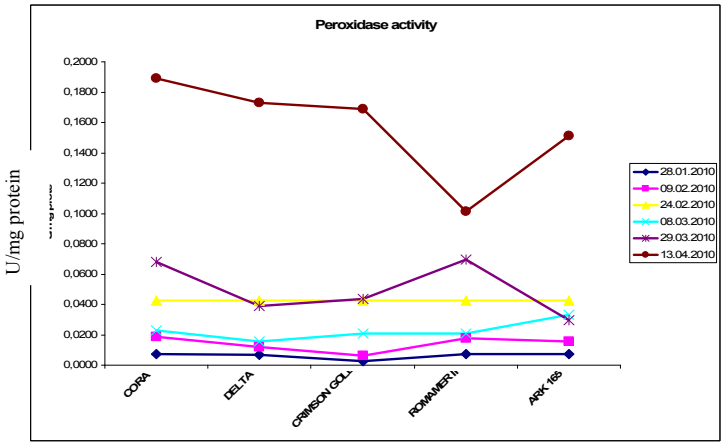



Fig.6. The enzyme activity of peroxidase (U/mg protein) for 5 cultivations of nectarines during an interval between 26 February 2010 and 13 April 2010

We could not establish a correlation between the earlier or later cultivation in the case of peroxidase nor in the case of phenolic compounds.

Reported to the periods in which the determinations had been carried out fluctuations with small amplitudes of the activity of peroxidase during the cold period and larger fluctuations together with the growth in median temperatures could be observed.

The protein content has a characterisitc dynamic for every cultivation taken into account. The greatest protein quantity has been obtained at the end of January and the beginning of February. In January, the values were between 0.190 ± 0.01 mg BSA/ mg fresh weight for the cultivation Crimson Gold and 0.070 ± 0.002 mg BSA/ mg fresh weight for the cultivation Cora. Small differences of the quantitiy (**Fig. 7**) of protein can be observed between successive determinations during the months of March (08.03; 29.03), the lowest values were registered during April (13.04).

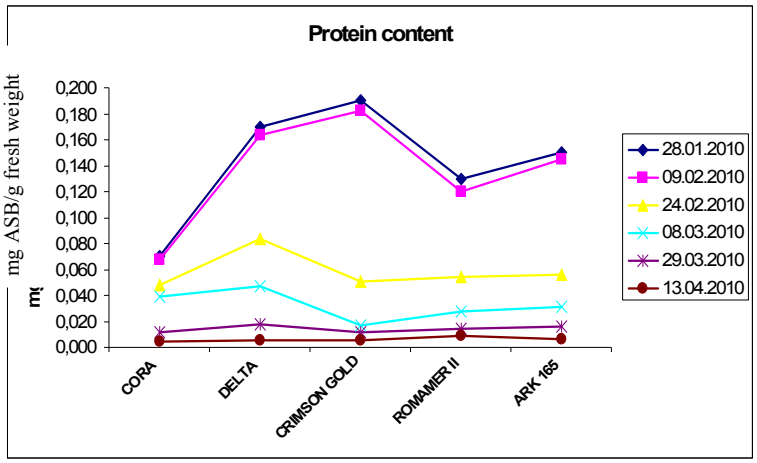



**Fig.7** The quantity of proteins (mg ASB/mg fresh weight) for five cultivations of nectarines during an interval between 26 February 2010 and 13 April 2010

Numerous studies (Burak et al., 1992; Tamássy et al., 1992; Thomashow, 1999; Iba, 2002) have been concerned with the specifying the role played by proteins in the plant in the growth of resitance to cold. Results have shown that a high content of soluble proteins and a low level of free aminoacids can be associated with the growth of frost resistance.

## CONCLUSIONS

The difference between the accumulation of chill units between the first (Cora) and the last (Crimson Gold) of the cultivations studied, which have entered the phenophase in which the buds begin to bulge is 91.2 CU and the difference is 7 days. The phenophase from the beginning of the bloom debuted on the 26.03.2010 (accumulated CU 1288.6) and ended on the 05.04.2010 (accumulated CU 1319.5) with the cultivation Crimson Gold. The phenophase of the end of blooming starts on the 06.04.2010 with the cultivation Cora and ends on the 13.04.2010 with the cultivation Crimson Gold.

The content of phenolic compounds has had a seasonal dynamic for all varieties which have been studied. In relation to the period analysed, the maximum quantity of phenolic compounds was recorded for all cultivations at the end of January, two cultivations stood out, Delta and Romamer II (0.0021± 0.083 mgGAE/g frees weight, respectively 0.0018± 0.038 mg GAE/g frees weight). The smallest quantity of phenolic compounds was registered during the month of April, for the cultivations Crimson Gold, Romamer II and Ark 165. Unlike the levels of phenolic compounds, in the case of peroxides the greatest enzyme activity was registered during the month of April with values between 0.1889 ± 0.001 U/mg protein, for the cultivation Cora and 0.1015± 0.002 U/mg protein for the cultivation Romamer II. The smallest enzyme activity was registered during the month of January. The protein content has a dynamic which is characteristic for every cultivation in the study. The greatest quantity of protein was obtained at the end of January and the beginning of February, when the trees are confined to their most restful period.

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