Elemental Analyse for Investigating the Influence of Chemotherapeutics on Virus Infected Grapevine *in vitro* Cultured

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In vitro cultures of V. vinifera L., cv. Feteasca neagră 7 Od. infected with grapevine fanleaf virus (GFLV) have been investigated on the influence of chemotherapeutics ribavirin and oseltamivir, added in medium, in order to block viral replication and virus-free plants regeneration. The concentrations of nitrogen, carbon, sulphur and hydrogen in grapevine plantlets were dosed, as nutrients having clear role in plant growth and development. The viricides determined a significant increasing of nitrogen in plantlets as compared to the control, after 60 and 90 days of treatment, while the carbon concentration decreased. The sulphur and hydrogen concentrations were not significantly influenced by the mixture of chemotherapeutics. Despite these modifications, the micropropagation processes continued until new grapevine plants regeneration. Therefore, ribavirin and oseltamivir could be used with the aim of grapevine healthy plant obtaining.

Keywords: *nutrient, ribavirin, oseltamivir, grapevine, in vitro*

Any element or chemical compound which contributes to the organism metabolism is considered as nutrient. Sixteen chemical elements are known to be important to a plant growth and survival. The non-mineral nutrients (hydrogen, oxygen, and carbon) are found in the air and water and in photosynthesis process the plants use energy from the sun to change carbon dioxide and water into starches and sugars. Nitrogen is a part of all living cells and is a necessary part of all proteins, DNA, RNA, hormones, enzymes and metabolic processes involved in the synthesis and transfer of energy. Also, it is a part of chlorophyll, the green pigment of the plant that is responsible for photosynthesis and helps plants with rapid growth, increasing seed and fruit production and improving the quality of leaf and forage crops. Sulphur is necessary for production of protein, promotes activity and development of enzymes and vitamins, helps in chlorophyll formation, improves root growth and seed production, helps with vigorous plant growth and resistance to cold [1].

Dosage of these elements in soil and plants can provide information on possible changes in the mineral metabolism of plants.

The objective of this study was to analyse the effects of chemotherapeutic agent (ribavirin and oseltamivir) on nutrients content of *in vitro* grown *V. vinifera* L., cv. Feteasca neagră 7 Od., GFLV- infected. Thus, the amounts of nitrogen, carbon, sulphur and hydrogen in regenerated plantlets were determined. *In vitro* culture medium ensures the nutritional needs of plantlets and the addition of other substances, in our case ribavirin and oseltamivir, with viricide role, can lead to malfunctions which can cause the death of explants.

Experimental part

The biologic material was represented by GFLV- infected grapevine plantlets belonging to *V. vinifera* L., cv. Feteasca neagră 7 Od., cultured on basal medium Murashige-Skoog [2], growth regulators specific to species [3], supplemented with a mixture of chemotherapeutics involved in viral multiplication blocking, ribavirin and oseltamivir. Viricides concentration varied by an experimental model with three variants, as follows: V1= 40 mg/L ribavirin + 40 mg/L oseltamivir; V2 = 20mg/L ribavirin + 40 mg/L oseltamivir; V3 = 20mg/L ribavirin + 80 mg/L oseltamivir. Experimental variants and culture conditions were described elsewhere [4].

Ribavirin *(1-[(2R,3R,4S,5R)-3,4-dihydroxy-5-(hydroxymethyl)oxolan-2-yl]-1H-1,2,4-triazole-3-carboxamide)* was purchased from Sigma-Aldrich, USA and oseltamivir was used as oseltamivir phosphate *(3R,4R,5S)-4acetylamino-5-amino-3(1-ethylpropoxy-1-cyclohexene-1-carboxylic acid, ethyl ester, phosphate*), with commercial name Tamiflu, produced by Hofmann-La Roche Germany.

After 60 and 90 days of treatment, the grapevine plantlets have been analysed to assess the effect of chemotherapeutics in medium, on plants growth and development, comparatively with control (C) represented by the infected plantlets cultivated on medium without viricides.

Simultaneous determination of nitrogen, carbon, sulphur and hydrogen in dehydrated plant by dry combustion is a method for evaluation of quality of obtained biomass.

The first stage, catalytic oxidation, was carried out at a temperature of 1150°C with the removal of halogens, water vapour and oxides of carbon. Next step, of reduction, occurred at 850°C, in helium flow, allowing the nitrogen oxides to molecular nitrogen. The desired components for measuring (N_2, CO_2, SO_2 and H_2O) have been separated with the aid of specific absorption columns. The percentage contents in C, N, H and S have been determined by means of thermoelectric cells.

The apparatus used was Elemental analyser Vario Macro (Gerhardt, Germany) with Vario EL-Vario macro CNHS soft.

Biological samples typically require 50-150 mg of dry matter per analysis.

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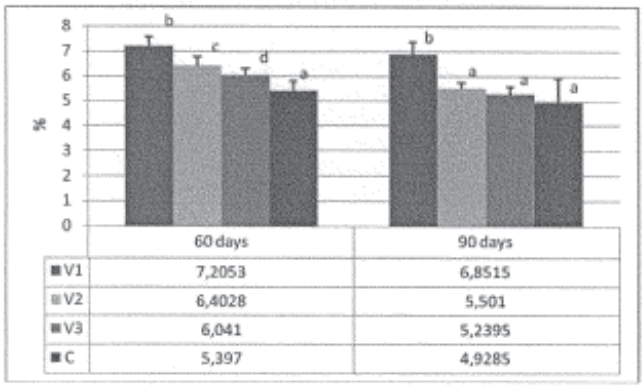

Fig. 1. Nitrogen content in GFLV- infected grapevine *in vitro* plantlets cv. Fetească neagră 7Od. in the presence of chemotherapeutics ribavirin and oseltamivir, after 60 and 90 days of treatment.V1,V2, V3 = variants of chemotherapeutics concentration, C = control. The values are means, the bars represent the standard deviations, the letters indicate the significance of differences at P<0.05

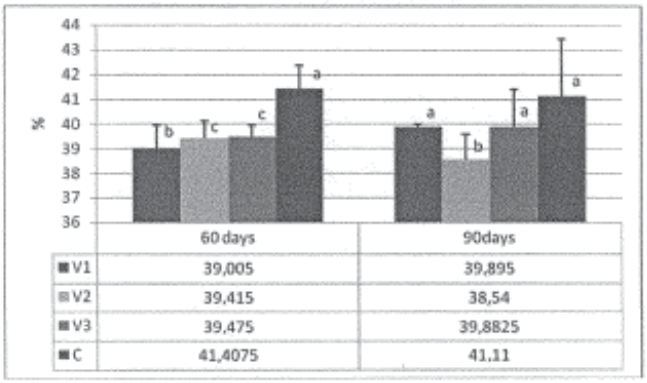

Fig. 2. Carbon content in GFLV- infected grapevine *in vitro* plantlets cv. Fetească neagră 7Od. in the presence of chemotherapeutics ribavirin and oseltamivir, after 60 and 90 days of treatment. V1,V2, V3 = variants of chemotherapeutics concentration; C = control. The values are means, the bars represent the standard deviations, the letters indicate the significance of differences at P<0.05

In comparison with Kjeldahl method for determination of total nitrogen (humid mineralization), the used method is, essentially, a dry oxidation and happens in a short period, with practical zero consumption, of chemical reagents, giving accurate and reproducible results.

All the analyses were performed in triplicate. The values in the figures indicate the mean values ± s.d. Differences between the variants and control were analysed by one-way ANOVA, taking $P < 0.05$ as significant according to LSD test.

Results and discussions

The presence of viricides ribavirin and oseltamivir in the medium determined a significant increasing of nitrogen content in GFLV- infected grapevine plantlets on all experimental variants, comparatively with the control, after 60 days of treatment. The highest value has been recorded on V1, as the medium with the highest concentration of ribavirin and the lowest of oseltamivir. After another subculture on medium with viricides (90 days of exposure), the nitrogen content increased comparatively with the control, the values registered on V1 being significantly higher, but not more than in the previous subculture. Therefore, the nitrogen concentration in grapevine plantlets cultured on medium with chemotherapeutics was influenced by the chemical drugs concentration, not by the number of treatment days (fig. 1).

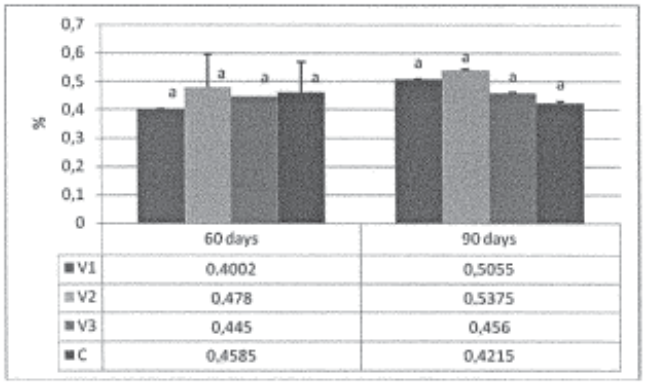

Fig. 3. Sulphur content in GFLV- infected grapevine *in vitro* plantlets cv. Fetească neagră 7Od. in the presence of chemotherapeutics ribavirin and oseltamivir, after 60 and 90 days of treatment.V1,V2, V3 = variants of chemotherapeutics concentration; C = control. The values are means, the bars represent the standard deviations, the letters indicate the significance of differences at P<0.05

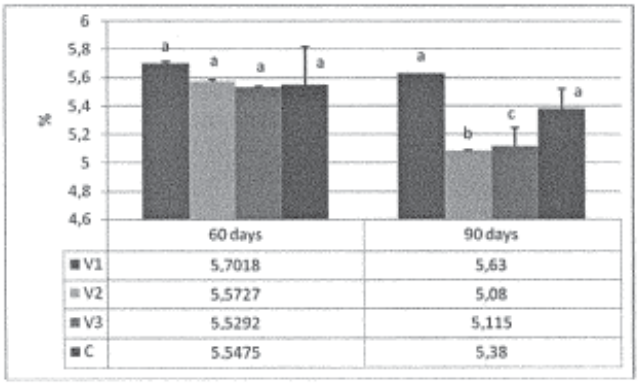

Fig. 4. Concentration of hydrogen in grapevine plantlets cultivated on media containing viricides (ribavirin and oseltamivir), belonging to GFLV–infected Fetească neagră 7Od. cv., after 60 and 90 days. V1,V2, V3 = variants of chemotherapeutics concentration; C = control. The values are means, the bars represent the standard deviations, the letters indicate the significance of differences at P<0.05

After 60 days of treatment, the chemotherapeutics determined a significantly decrease of carbon content on experimental variants comparatively with the control, registering the lowest value on V1, the variant with the highest concentration of ribavirin. The values of carbon concentration after 90 days of therapy were comparable on experimental variant with those registered after 60 days of culture on medium containing viricides (fig. 2).

Sulphur concentration was not influenced by the presence of chemotherapeutics in the medium after two treatment periods; the recorded values were not significantly different as compared to the control (fig. 3).

The dosage of hydrogen content led to significantly lower values on V2 and V3 after 90 days of treatment as compared to the control (fig. 4).

The application of chemotherapeutics in medium affected clearly the concentration of different nutrients dosed in grapevine plantlets. Ribavirin and oseltamivir determined a significant increase of nitrogen concentration in explants, that manifested by poor differentiation of small shoots, an agglomeration of adventive buds which could not be used in the micropropagation process.

This phenomenon was also observed by other researchers which reported increases of the nitrogen concentration in grapevine rootstock plantlets when ammonium nitrate or sodium chloride have been added in

media, in the frame of *in vitro* studies of the influence of additional quantities of nitrogen, or tolerance to salinity.

As expected, up to a certain concentration, the supplementation with nitrogen in medim determined the same variation in grapevine plantlets, reflected by well trained shoots with normal leaves, intense green colour, flexible tissues. Larger N-addition produced non-desired shoots with very dark colour, abnormaly large shaped leaves and fragile tissue [5]. The rootstocks explants behaved in a similar manner in the culture by adding sodium chloride at various concentrations [6, 7]. This is the reason that the influence on plants growth and development of each substance added in medium should be tested.

In this paper it was necessary to establish the concentrations of chemotherapeutics agents that the grapevine can tolerate in medium, in the context which these substances are necessary to regenerate virus-free plants, their action being to block the viral multiplication.

Conclusions

Various concentrations of ribavirin and oseltamivir added in medium, involved in viral multiplication blocking and virus- free grapevine plants regeneration were determined modifications in plants metabolism, reflected in changes of concentrations of some nutrients as nitrogen, carbon, sulfur and hydrogen.

The nitrogen concentration registered significant increasing under the influence of chemotherapeutics, while the content of carbon decreased. The sulphur and hydrogen concentrations have not indicated significant modifications as compared to the control.

Between the two chemotherapeutics, ribavirin caused the strongest response of plants, the highest differences in nutrient composition occurring on variants with the highest concentrations of ribavirin and the lowest of oseltamivir.

Despite the effects of chemotherapeutics on growth and development of virus-infected grapevine cultures, the micropropagation processes continued until the virus-free plants regeneration.

The elemental analysis applied to plants has become very important in the environmental sciences, in particular to analyse the effects of chemotherapeutic agents on nutrients content.

The subject was also studied in [8].

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