Chapter 11
EFFECTS OF SOME COMBINED TREATMENTS OF PVY INFECTED POTATO PLANTLETS CV. ROCLAS

C.L. BĂDĂRĂU[1,2] F. DAMȘA[2] A. NISTOR[2]

[1] *University Transilvania of Brașov, Faculty of Food and Tourism / Department of Engineering and Management in Food and Tourism, Brașov, Romania*

[2] *National Institute of Research and Development for Potato and Sugar Beet, Brașov, Romania*

badarau_carmen14@yahoo.com, carmen.badarau@unitbv.ro

1. Introduction

For solving food shortages at the beginning of this millennium, the potato proves to be a product with promising perspectives. Considered by some a common product, cheap food, poor peopleôs

food and the plant of poor areas, the potato is actually a product that helps improving the daily diet being rich in carbohydrates, vitamins and minerals. For Romania, the potato is a strategic food, contributing to the national food safety system. Our country is ranked on the third position in Europe in terms of area cultivated with potatoes (after Poland and Germany).

Genetic and physiological characteristics of the potato and its cultivation technology features require compliance with strict requirements on the health of the planting material. In the growing season, plants are infected with viruses rapidly leading to deterioration by gradual decrease in production capacity. Losses due to virus diseases are much higher than in case of other plants because potato multiplication is made through a vegetative manner.

Virus diseases lead to the reduction of farmersô income from agricultural or national communities. Damages and economic losses are due primarily to the reduction of plant growth leading to reduced production or even its destruction. This is why protective measures of culture against viral infections, diagnosis and **control of virus diseases** play an important role in potato production technology and multiplication.

Distributed worldwide, *potato virus Y* (PVY, *potyvirus* genus, family *Potyviridae*) is a major economic disease agent for the crops. This pathogen causes losses in solanaceous crops such potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and tomato (*Lycopersicum esculentum)* [1, 2]. PVY in potato received (in the

last period) a big attention because this pathogen is one of the most economically important problems in seed potatoes in the world. This virus is responsible for serious decreases yield and quality tubers, but the main problem in seed potato production is the requirement for a strict PVY tolerance limits for certified lot of seed. High levels of PVY are responsible for the rejection of many seed potato lots. Also, a significant reduction of the crop value was noticed and in a certified seedôs shortage, too, especially for certain varieties highly susceptible to PVY infection [3].

The methods employed to eliminate viruses from plants like meristem culture, chemotherapy and thermotherapy are technically demanding and time consuming. Electrotherapy, however, is a simple method of virus eradication without the need to use any special or expensive equipment. In this technique, the electric current is applied to plant tissues in order to disrupt or degrade viral nucleoprotein and eliminate its virulence activity [4, 5]. Sometime the electrotherapy technique is not more efficient than other conventional techniques in eliminating viruses from plant tissues. However, it seemed to be more effective, faster, easier and less demanding than other methods in regenerating virus-free plants [6, 7]. It can also be effectively combined with chemotherapy as demonstrated earlier [6, 8, 9].

It has been postulated as a hypothesis that viral nucleoproteins could be denatured by when it is exposed to electric current [5]. Until now, the basis of this observation is still poorly understood.

1.1. Objectives

The study aimed to evaluate the effects (on chlorophyll and anthocyanin content and on the multiplication rate) of several electrotherapy treatments used for PVY elimination in potato plants and find out the best one both for virus eradication and for an optimal next plants development. This study could be a challenge for food science and emerging technologies, by their contribution in the growing process of a health, virus free vegetable material for food industry.

2. Why this effort for eliminate Potato virus Y? In brief about PVY and their world spread

Potato virus Y (PVY, *Potyvirus)* threatens seed potato growing in areas worldwide as one of the most damaging viruses (infections may reduce production by 50-90% depending on the strain type, infection type, resistance of varieties). In order to prevent this inconvenience absolute priority requires initial identification of the material free of viruses and other diseases transmitted through tubers, using rapid and accurate diagnostic methods, and using of varieties resistant to the attack of viruses.

In the last three decades new PVY strains have emerged, some of them (e.g. PVY $^{(N)\,W}$) induce barely visible symptoms during the growing season (often being unnoticed during visual inspection) and

others (e.g. PVY $^{(N)\ NTN}$) produce symptoms on tubers, causing the so-called the necrotic ring staining of tubers**.** Due to the fact that these viral strains may affect the resistance of some potato varieties compared to other strains of the virus Y (PVY ^{o} and PVY ^{c}) numerous varieties that were considered resistant passed into the category of sensitive ones, which affected the production of the potato in our country. The damage caused by this pathogen agent is both quantitative (significant reduction of production) and qualitative (commercial depreciation of tubers). In case of cultivation of sensitive varieties under favorable conditions, financial losses can be important both for potato consumption (it can become unmarketable) as for seed potatoes (it will be downgraded or rejected from certification).

Massive imports of potato in last decades, the continuous "migration" of seed potatoes from one area to another, climate change, inadequate treatments for disease vector control (especially aphids), viral pressure, resistance of varieties are just some several factors that may favor the spread of aggressive strains of the virus Y that recently appeared in the culture.

Until now, the world has been doing research on the etiology and epidemiology of the pathogen implicated in necrotic ring staining of tubers, viral genome was studied, ways to differentiate between Y virus strains and PVY^{NTN} strain were looked for [10].

Potato virus Y (PVY), type member of the *Potyvirus* belonging to the *Potyviridae* family, is a common virus

that can infect many cultivated species of *Solanaceae* family: potato, tobacco, tomatoes, peppers [11].

Potato virus Y (PVY) was described for the first time by Smith in 1931 (UK). For a long time, PVY isolates were classified according to foliar systemic and local symptoms in three main groups PVY^{O}, PVY^{N}, PVY^{C}, depending on the symptoms induced in *Nicotiana tabacum* and *Solanum tuberosum* varieties (by Bokx and Huttinga, 1981, Singh and al., 2008). While the virus PVY^{O} is present all over the world, the virus PVY^{N} was mentioned in Europe, South America, Africa, India [3, 8, 9, 12-18].

It should be noted that it was not always possible the classification of different isolates based on multiple properties [19, 20]. In recent years, PVY isolates were found apparently intermediate between PVY^{O} and PVY^{N} groups, because they share symptoms, serological and genomic properties with the two groups [21- 23]. Thus relatively recent there were identified new subgroups of viruses $PVY^{N:}$

- PVY^{N-Wi} [14] and PVY^{N-O} [24-28] that have PVY^{N} pathotype and PVY^{O} serotype.
- PVY (NTN) often associated with destructive virus that causes PINTC on sensitive species. More recently, molecular genotype of PVY^{NTN} was described as follows: NA-PVY (NTN) [25-26] and NE-11 PVY (NTN) [7, 29-30]. PVY in 2000 was considered quarantine plant pathogen in USA and Canada [31].

PVY (NTN) strains produce symptoms on tubers, causing the so-called necrotic ring staining of potato tubers (PINTC). Being very aggressive, these strains can overcome existing resistance to infection with other strains of potato virus Y (PVY^o and PVY^c) [29, 32].

Necrotic ring staining of potato tubers (PINTC) occurred in the early 1980s in Hungary and was described by Beczner and al. in 1984, as the Potato tuber necrotic ring spot disease (PTNRD). It was later found in many European countries, the Middle East and the United States. In our country was discovered in 1988, both in experimental varieties and some varieties in culture [33, 34].

In Hungary, the number of varieties and the percentage of tubers affected with symptoms varied according to the area of cultivation and years, ranging between 4 and 24 varieties, namely between 1 and 24%. On susceptible varieties Rosalie and Mona Lisa infection rates with necrotic ring staining reached 70% and 50% respectively [35].

In Slovenia, necrotic ring staining erupted in 1988 and since then has spread continuously, but not only on potato but also on other *Solanaceae* (tomatoes, peppers, *Solanum nigrum,* etc.), so today is one of the most damaging diseases of potato. According to estimates by [5, 36], the percentage of infection of plants in the field in the main potato growing areas was over 50%. The consequences of this epidemic in Slovenia were: blocking the national production system of seed potato due to the negative effect on the quality of

seed potatoes produced in Slovenia, substantial increase in imports of potato for seed and hence of financial expenses, abandoning the cultivation of the most popular potato in Slovenia, namely Igor that was grown on an area of 18.000 hectares and represented about 60% of the area cultivated with potato, because of high sensitivity.

An intensification of research on varieties susceptibility and occurrence of necrosis on tubers in Germany according to the reports of Weidemann and Mais (1996), necrotic ring symptoms on potato tubers were observed starting with the autumn of 1983, when the damage was frequent, and since then necrotic ring staining of potato tuber has spread so much that it became a serious problem. Research made by Hanne and Hamm (1999) showed that this disease has a negative effect on germination capacity of the tubers, causing their weight reduction, commercial depreciation of tubers.

In very favorable years for the production and spread of the disease (hot and dry summers, early emergence of aphid vectors) real epidemic occurs with damage important for both seed potatoes and consumption potatoes, particularly in susceptible and very susceptible varieties [4, 17].

Most countries which discovered the presence of necrotic PVY strains have acted to prevent the spread of this pathogen. Initially funds were allocated for research on resistance to infection with this particularly aggressive viral strain, after which there was a drastic revision of the structure of potato varieties, decision making forums

intervening in time to limit expansion of tuber necrotic ring staining induced by PVY necrotic strains that are in a permanent "genetic reviewing-recombination".

In our country, the disease has been reported sporadically in some susceptible potato varieties, beginning in the fall of 1988, in some experimental plots, further more plant protection inspectorates in the country have reported the presence of this disease in the state and private producers [37].

3. Material and Methods

3.1. Biological material

Solanum tuberosum L. vitro plants cv. Roclas, tested virus free, were obtained from the Biotechnology Department of National Institute of Research & Development for Potato & Sugar Beet Brasov [33, 37-39]. The vitro plants were transferred to greenhouse conditions 30 days. For obtaining positive material, a part of these plants were mechanically inoculated [33, 37] using a PVY secondary source variety Record (necrotic strains). The plants had previously tested positive by ELISA for PVY^N, to confirm the occurrence of single infection in the selected material. Plants with similar levels of virus concentration were used to obtain stem segments containing axillary buds for electrotherapy. Tissue samples infected mother plants growing in the greenhouse were used as positive control.

3.2. Electrotherapy treatments and regeneration of virus-free plants

Each infected plant provided for approximately 3 nodal cuttings that were subsequently used for electrotherapy treatment. From each stem one node was cut for the control (untreated by electrotherapy) and the stem segments remaining were immersed in natrium chloride solution (1M) in an electrophoresis tank and exposed to electric currents of 40, 50 and 100 mA for 5, 10 and 20 minutes (Table 1) using a power supply (Tehsys E250V) [38, 39].

Table 1. *Variants of the treatments with electric current (intensity/ times)*

Variant	V0	V1	V2	V3	V4	V5	V6	V7	V8	V9
Treatment (mA/min)	0/0	40/5	40/10	40/20	50/5	50/10	50/20	100/5	100/10	100/20

After treatment, the stems were surface sterilized and rinsed three times in distilled water. Explants were prepared by dividing stem segments into nodal cuttings with a single axillary bud. The cuttings were cultured in test tubes containing MS medium. The experiment was repeated three times for each electrotherapy treatment. Stem segments excised from infected potato plants were transferred three times in MS medium (sub-culture S1-26 days, sub-culture S2 -30 days, sub-culture S3-28 days). Only before the subcultures S1 and S2 the material was treated by electrotherapy. Plantlets obtained in all the subculture were divided into single node

cuttings (about 1cm length) and sub-cultured on a fresh MS medium [40].

Multiplication rate was estimate by count the single nodes for each plant, in all the variants.

Therapy Efficiency Index (TEI) For notice an electrotherapy treatment leading to high rates of virus elimination and plant regeneration, the Therapy Efficiency Index (TEI) was used [5]. This indicator was estimated with the following relation:

$$TEI = percentage\ of\ regenerated\ plantlets \times percentage\ of\ virus\text{-}free\ samples/100$$

3.3. DAS ELISA test

The analysis was performed following the protocol Clark and Adams (1977) [41]. For preparation leaf samples, a press with roles was used and for the tuber samples extraction and distribution, an extractor Microlab 500B/C **(**Hamilton**)** was used. The antiserum and conjugated used for viruses detection were obtained in our laboratory [34]. All experiments were repeated three times. Rinsed microplates filled with substrate solution (p-nitro-phenyl-phosphate) were incubated one hour and the absorbance values were estimated at 405 nm (A_{405}) using a Tecan SunRise reader (software Magellan). The samples that have A_{405} values exceeding the cut-off (two times the healthy control samples average) were considered PVY infected [33, 37-39].

3.4. Evaluation of the chlorophyll and anthocyanin content

Monitoring the vegetative state of healthy regenerated plants was done by estimation:

- chlorophyll content of leaf (portable device SPAD 502 Chlorophyll Meter). The values determined by the equipment used, indicated the relative amount of the chlorophyll quantity present in the plants leaves, measured by the transmittance of leaf at the two wavelengths, 650 nm (red) and 940 nm (near infrared ï NIR) (Figure 1a).

- anthocyanin content on the leaf (portable device ACM 200 plus, Antocianin Chlorophyll Meter). As in the case of evaluation the chlorophyll content, values indicated by device represents the relative sum of anthocyanin content present in leaves, estimated by transmittance of plant material, measured at two wavelengths, characteristic for anthocyanin pigment analysis (510 nm and 700 nm) (Figure 1b). These tests were made after 36 vegetation days.

3.5. Statistical analysis.

Data were analysed by ANOVA and Duncanôs Multiple Range Test and scored as significant if $P<0.05$ (IBM SPSS Statistics software). For example, in the figure 2 there are presented the means of 3 repetitions, values not followed by the same letter are significantly different ($P=0.05$) according to Duncanôs test).

Fig. 1. *Evaluation the chlorophyll (a) and the anthocyanin content (b).*

4. Results and Discussions

4.1. Multiplication rate

The toxic effect of the electrotherapies (several variants of electric current intensities, time of the treatment) was estimate by a specific indicator: multiplication rate (number of single nodes steam obtained from the first node propagated). To the second and third subculture were used only two explants of apex. The experimental

results (Figure 2) highlight the significant influence, which had the current intensity and duration of treatment on the rate of explants *in vitro* multiplication, in case of all the variants. Thereby, it was observed that the multiplication rate increased compared to untreated control, for all variants, benefic effect of electrotherapy being significant in all the subcultures.

The multiplication rate evaluation, for variants that has been used the maximum current intensity (100mA) revealed different results, depending on the subculture. To material infected with PVY was observed a decrease in multiplication rate, especially to the long duration (20 minutes), particularly at subculture 2, at exposure of material to the electric current 50 and 100 mA (Figure 2).

Maximum values of multiplication rate were observed in variants that used the biggest intensity of the electric current (100mA) (figure 2). Significantly high multiplication rates were recorded at S1 subculture, significantly higher values was observed at variant V9 (100mA/ 20 minutes) The biggest differences compared with to untreated control, were recorded for the variants of treatment more severe, both for the average number of leaves/plantlet, and for the mean values of the stem / plantlet.

Application of electrotherapy on the potato cultivar Roclas resulted in partial elimination of PVY from potato tissues when the most severe treatments were applied (100 mA for 10-20 minutes) [33, 37]. In spite of developing of many virus-free plants [1] increasing levels of biological indicator values were observed in all

variants even if the regenerated plantlets remain infected [33, 37]. For evaluate the electrotherapyôs success in producing virus-free material, there are very important to calculated the plant multiplication rate and to observe the next development of the plants [37]. Usually, this indicator depends upon several factors, including genotype, physiological state of the explant, culture medium, the cultivation conditions and the interactions between these factors [36, 42].

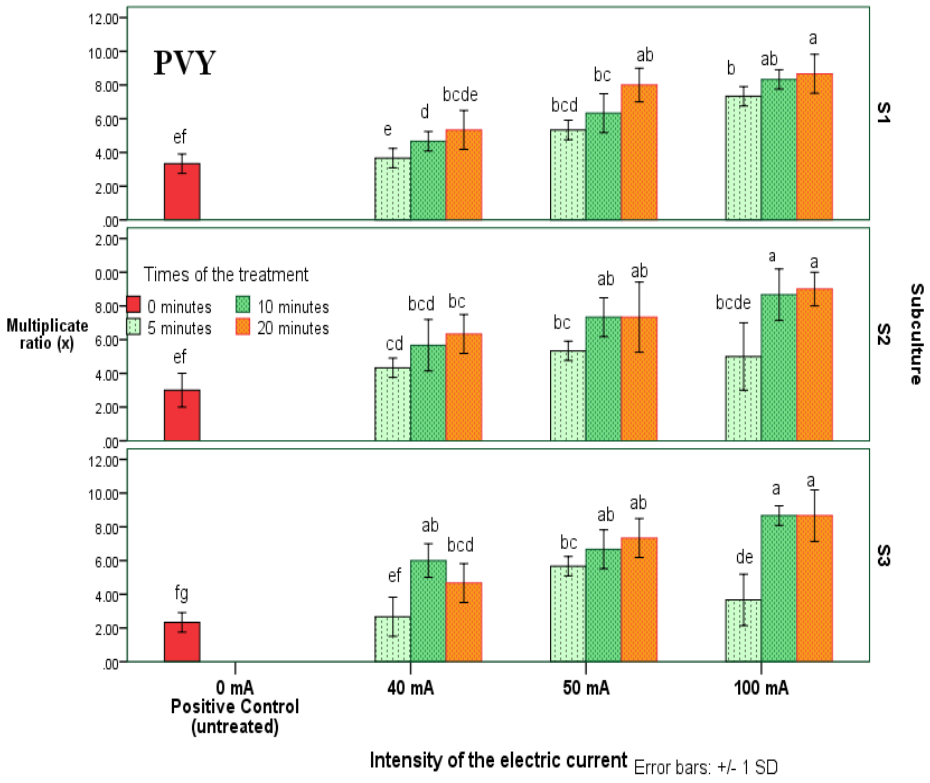

Fig. 2. *Effects of the electrotherapy on the multiplication rate of PVY infected material.*

The electric pulses are also reported as stimulants of plant differentiation *in vitro*, being demonstrated that in several experiments, the mild current could be beneficial for regeneration of potato plant tissues [5, 36, 38, 39, 42].

4.2. Therapy efficiency index

Application of electrotherapy on the potato variety Roclas resulted in successful elimination of PVY from potato tissues when the most severe treatments were applied (100 mA for 10-20 minutes). In spite of developing of many virus-free plants, diminishing levels of virus concentration were observed in all variants even if the regenerated plantlets remain infected, for both viruses (figure 3). But the beneficial effects of the electrotherapy in the production of virus-free material depend upon 2 essential experimental indicators: plant regeneration and virus elimination rates [5, 36, 38, 39, 42].

Usually, plant regeneration depends upon several factors, including genotype, physiological state of the explant, culture medium, the cultivation conditions and the interactions between these factors [8-9]. The electric pulses are also reported as stimulants of plant differentiation *in vitro* [2].

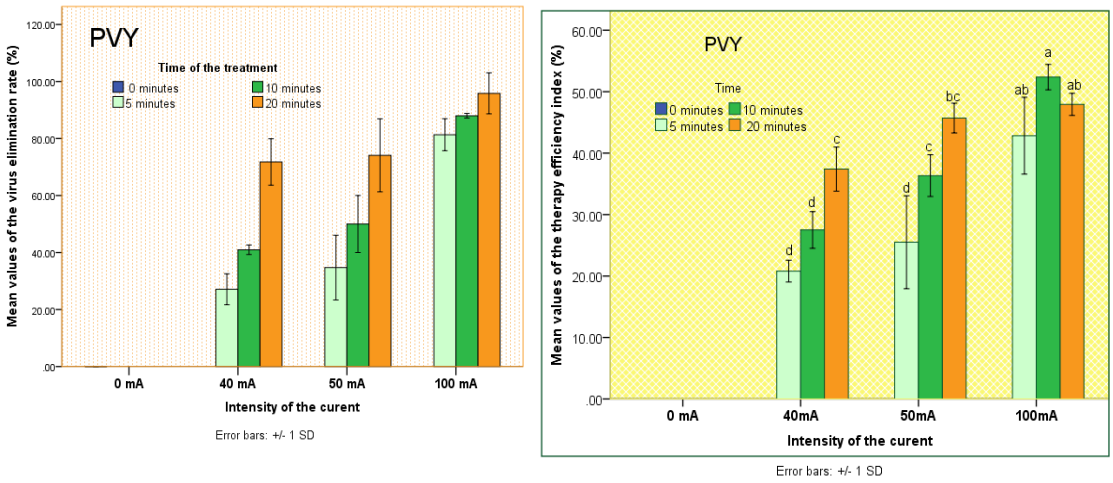

Fig. 3. *Effects of electrotherapy on the virus elimination rate and the therapy efficiency index.*

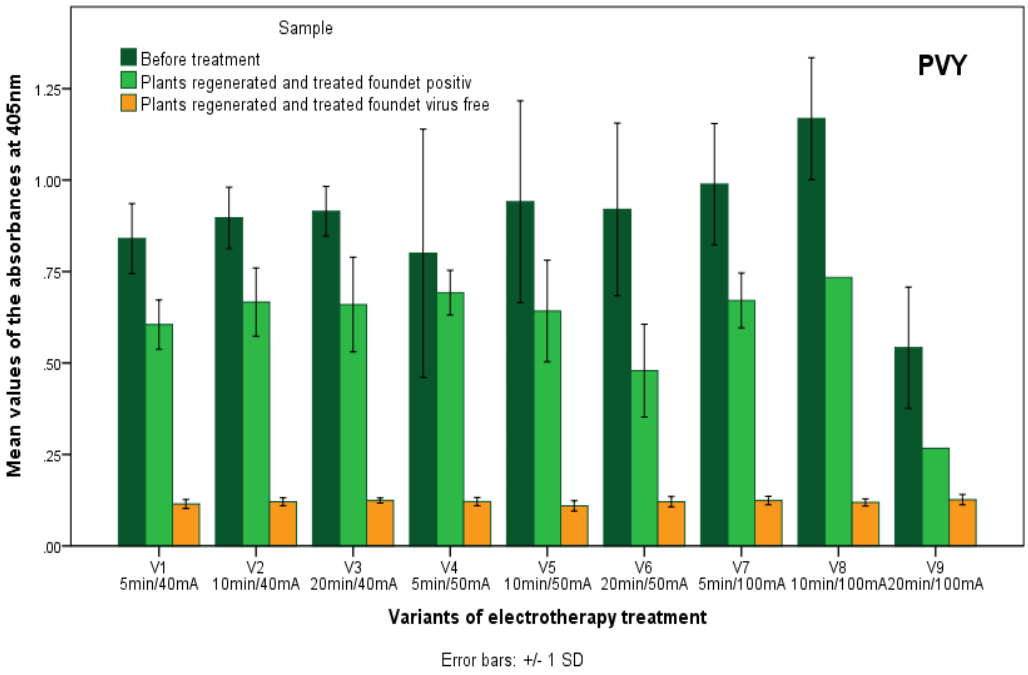

Fig. 4. *Results of ELISA tests applied on the plants before and after the treatments.*

Plant regeneration rate estimated as the ratio of the number of regenerated plantlets to the total number of cultured explants was 50.0-77.2% for PVY infected and treated plantlets in the cultivar used in this study. The three electric currents of 40, 50, 100mA resulted in 65.6%, 70.0% and 54.0% mean plant regeneration for PVY infected material (Table 2). Usually, higher intensities of electric current adversely affected the survival of explants and thus plant regeneration.

Table 2. *Effects of electrotherapy treatments on regeneration rate of PVY infected plantlets**

Variant	**Regenerated [a]/ treated[b]**	**%**	**± STDEV**
V0	5/24	20.8	±4.194
V1	37/48	77.1	±13.38
V2	27/40	67.5	±15.12
V3	25/48	52.1	±13.19
V4	26/35	74.3	±9.311
V5	30/41	73.2	±12.43
V6	30/48	62.5	±2.887
V7	21/40	52.5	±1.925
V8	25/42	59.5	±13.57
V9	24/48	50.0	±12.72

[a] number of regenerated plantlets;
[b] number of explants treated
STDEV standard deviation
*Roclas cultivar infected explants, results are the mean of three experiments.

According to earlier studies, most species of the *Leguminosae*, with a few exceptions, are difficult to regenerate *in vitro* [43]. The recalcitrance of large-seeded legumes to *in vitro* regeneration could be the result of a long history of inbreeding and selection for high-performing genotypes, which could have lead to a reduction in the genetic variability in modern varieties [43]. But regeneration rate in potato is variable and depends on the genetic material.

4.3. Estimation of the anthocyanin and chlorophyll content

Between experimental variants were observed significant differences concerning plant development acclimatized. Probably that, the severity of electroshocks treatment initially applied to biological material contributed to the differential development of acclimatized plant. The first observations revealed pronounced effect that had particularly current intensity, respectively exposure duration of plantlets to electrotherapy treatments.

The simple correlation coefficient Pearson revealed significantly higher values regarding chlorophyll content (as compared to the negative control) in the case of plants regenerated from the infected material with PVY, variant 100mA/10minutes. Plants regenerated from material infected with PVY virus behaved differently, chlorophyll content being for variant 100mA/10minutes similar with the negative controls (Table 2).

Table 2. *The correlation between pigment content and type electrotherapy*

		Material used for obtaining regenerated plants (Healthy, PVY infected)	Variant of treatment (Healthy,100mA/ 5min, 100mA/10min, 100mA/20min)
Chlorophyll content (units ACI)	Correlation coefficient Pearson	-0.374**	-0.245
	Significance threshold	0.005	0.074
	N*	45	45
Anthocyanin content (units AAI)	Correlation coefficient Pearson	0.226	0.138
	Significance threshold	0.100	0.319
	N*	45	45

*For each variant were tested 5 regenerated plants, for each plant was determined mean value for three determinations (in different parts of foliage), in three repetitions (N=5x3x3).
** Correlation is significant for $p<0.01$.

Within the elimination of viruses PVY by electrotherapy was noticed a significant decrease of chlorophyll content compared to the control in case of variant 100mA/20minutes. The evolution of values for the mean number of leaves and the mean length of the plant was similar to chlorophyll content estimated for regenerated plants. Regarding the content of anthocyanin, there were significant differences between values recorded in the experimental variants as was checked using simple correlation coefficient Pearson (Table 2).

Compared to the negative control, however, it was found small

increase of anthocyanin content in case of material initially infected with PVY (but the values were not statistically supported) (Table 2).

As opposed the content of anthocyanin, we remark that monitoring of chlorophyll content indicate some changes in plant physiology, being observed effects of electrotherapy treatments over plantlets regenerated from material inoculated with PVY.

The results of the present research work show that the multiplication rate of explants *in vitro* is influenced by electrotherapy treatment and depends upon the electric current intensity. Many papers suggest that the multiplication rate of virus-free plants obtained after electrotherapy is higher than that of plants exposed to more conventional virus elimination techniques including in vitro tissues culture and chemotherapy [18, 31, 32, 35, 42]. In our study, for PVY infected material, we obtained good results regarding the multiplication rate when higher intensities of the electric current was used (100mA/10minutes). In this situation, compared to the negative control, it was found small increase of anthocyanin content in case of material initially infected with PVY (but the values were not statistically supported). Monitoring of chlorophyll content indicate some changes in plant physiology, being observed effects of electrotherapy treatments over plantlets regenerated from material inoculated with PVY.

5. Conclusions

This study revealed that the electrotherapy (100mA, 10 minutes) of PVY infected material had beneficial effects on the multiplication rate of the plants. Further investigations are needed for improvement and optimization of these techniques, because it is possible to obtain virus free material using electrotherapy treatment [33, 37].

As opposed the content of anthocyanin, we remark that monitoring of chlorophyll content indicate some changes in plant physiology, being observed effects of electrotherapy treatments over the healthy biological material regenerated from plantlets infected with PVY and treated.

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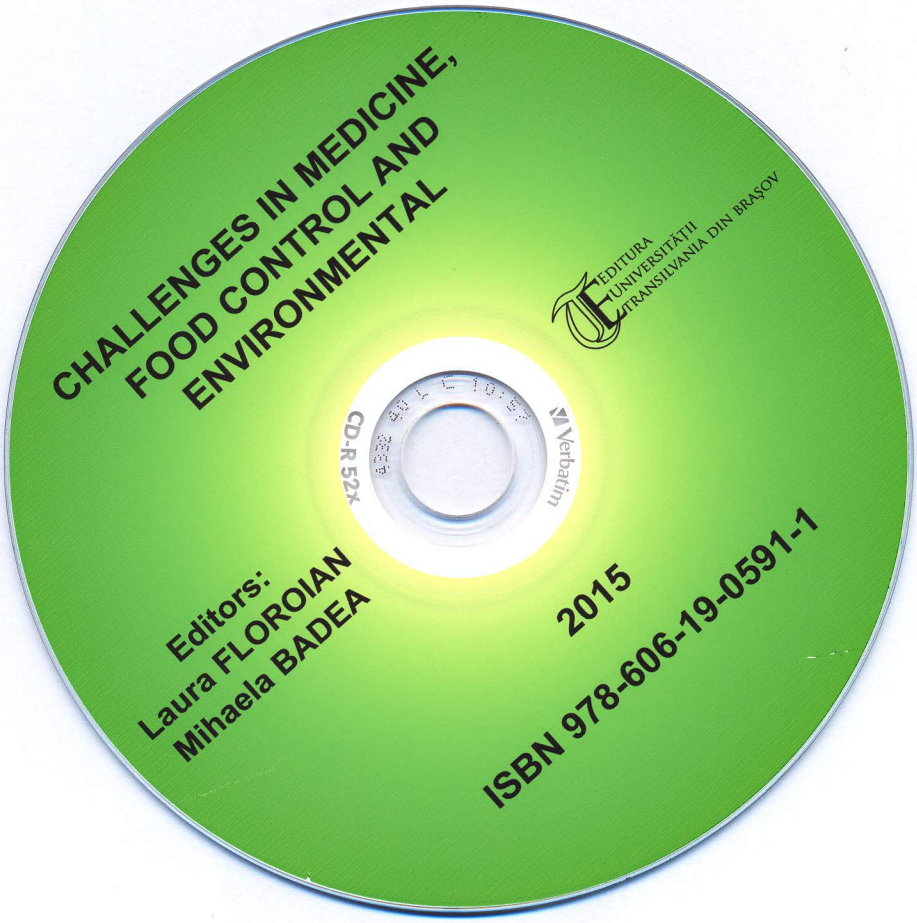
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