AGRICULTURAL UNIVERSITY -PLOVDIV

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"*IN VITRO* CULTURES OF *FABIANA IMBRICATA* RUIZ. ET PAV., AS TECHNOLOGICAL MATRICES FOR THE PRODUCTION OF BIOLOGICALLY ACTIVE SUBSTANCES"

ABSTRACT

OF THE DISSERTATION FOR AWARDING THE EDUCATIONAL AND SCIENTIFIC DEGREE "DOCTOR"

Scientific specialty:

Selection and seed production of cultivated plants (Plant Biotechnologies)

Supervisors:

Assoc. Prof. PhD Svetla Yancheva

Corresponding Member Prof. DSc. Atanas Pavlov

Plovdiv, 2021

The thesis is written on 118 pages and contains 16 tables and 31 figures. Totally 194 literature sources were used, 3 of which in Cyrillic and 191 in Latin.

The research was performed in the period 2016-2019 in the Laboratory of Plant Biotechnology of the Agricultural University - Plovdiv; The Laboratory of Plant Biotechnology at the Institute of Fruit Growing - Plovdiv; The Laboratory for Analysis of the Department of Analytical Chemistry of the University of Food Technologies - Plovdiv; Laboratory of Applied Biotechnology, branch of BAS - Plovdiv.

The PhD Thesis was discussed and directed for the defense at an extended meeting of the Department of Viticulture and Fruit Growing at the Agricultural University - Plovdiv.

Acknowledgments

I would like to express special thanks to my research supervisors
Assoc. Prof. Dr. Svetla Yancheva and Corresponding Member. Prof. Ph.D. Atanas Pavlov,
to the colleagues from the Laboratory of Applied Biotechnologies, branch of BAS - Plovdiv, to Ch. Assistant Professor Dr. Radka Vrancheva, to the colleagues from the Laboratory of Plant Biotechnology at the Institute of Fruit Growing - Plovdiv.
Thanks to all the colleagues for the help and advice during the preparation of this dissertation.

I. INTRODUCTION

For centuries, plants have been used as medicines and a traditional source of many chemical products and secondary metabolites used in the pharmaceutical industry, cosmetics, food additives, biopesticides, aromatic components, dyes enhancers, and colorants and others. Frequently about 80% of all existing drugs are obtained directly or indirectly from plants. In recent decades, recent phytopreparations based on secondary metabolites of plants have been produced by cell cultures. One of the largest groups of biologically active secondary metabolites is polyphenols.

Plants are rich sources of phenols, molecules that can act as antioxidants to prevent heart disease, reduce inflammation, the incidence of cancer and diabetes, and reduce the rate of mutagenesis in human cells. Flavonoids, the other most common group in plant tissues, are often responsible, along with carotenoids and chlorophylls, for their blue, purple, yellow, orange, and red colors. The flavonoid family includes flavones, flavonols, iso-flavonols, anthocyanins, anthocyanidins, proanthocyanidins, and catechins.

Fabiana imbricata Ruiz. et Pav. is a valuable medicinal plant that is little known in Europe.

There are problems with its conventional propagation and breeding. Biotechnologogical methods open new opportunities for plant propagation and research. The development of suitable cell and tissue culture techniques allows for its mass propagation and distribution as an ornamental plant and its use to obtain biologically active substances.

The information presented so far determines the importance of the topic as a doctoral thesis and motivates us to research this area.

The results obtained by us could be of scientific and applied interest in solving some problems in the reproduction of the plant species *Fabiana imbricata* Ruiz. et Pav., through *in vitro* cultures and assessing their potential for obtaining biological substances.

II. PURPOSE AND TASKS OF THE DISSERTATION

This dissertation aims to evaluate the potential of *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. as technological matrices for the production of biological substances.

To realize this goal, the following tasks have been formed:

1. Obtaining plant material and introduction into *in vitro* culture.
2. Optimization of the micropropagation system of *Fabiana imbricata* Ruiz. et Pav.

2.1. Creating an optimized medium for multiplication.

2.2. Adaptation and acclimatization of regenerants.

2.3. Influence of the light source on the growth and development of the plants in the stage of multiplication.

2.4. Study on the efficiency of a bioreactor system with temporary immersion (TIS), type RITA® in the stage of multiplication of *Fabiana imbricata* Ruiz. et Pav.

3. Obtaining in vitro plant systems with different degrees of differentiation.from *Fabiana imbricata* Ruiz. et Pav.

4. Analysis of the antioxidant activity of different extracts from *Fabiana imbricata* Ruiz. et Pav.

5. Analysis to determine the polyphenolic profile of different extracts of Fabiana imbricata Ruiz. et Pav.

III. MATERIALS AND METHODS

The medicinal plant *Fabiana imbricata* Ruiz. et Pav. is distributed in the Andean region, prefers dry mountain slopes, stony and sandy soils (*Hoffmann et al., 1992 cited in Ratsch, 1998*), and has been used in ethnopharmacology for centuries.

The experimental work was performed with plants of the species *Fabiana imbricata* Ruiz. et Pav., purchased from the greenhouse *Paddock Plants* (England). The development of the plants during the following stages was studied: 1. Introduction to *in vitro* culture; 2. Multiplication; 3. Rooting; 4. Adaptation of *in vitro* plants to *ex vitro* conditions.

The experiments were carried out in the Laboratory of Plant Biotechnology AU-Plovdiv, Laboratory of Plant Biotechnology Institute of Arboriculture Plovdiv, Laboratory of Analytical Chemistry at the UFT-Plovdiv, and the Laboratory of Applied Biotechnology of BAS - Plovdiv.

1. Optimization of the culture medium for multiplication of *Fabiana imbricata* Ruiz. et Pav.

The experiment was performed in the Laboratory of Plant Biotechnology at AU-Plovdiv. An investigation was conducted to examine different concentrations of plant growth regulators (Table 1) towards optimizing the culture medium for multiplication. MS (Duchefa) basal medium supplemented with sucrose (30 g/l), Merk agar (6 g/l), and pH=5.8 (before autoclaving) was used.

Table 1. Concentration of the growth regulators in the propagation media (mg l-1).

Culture medium / Additions	A0	A1	A25	A3	A33
BAP	0	0.1	0.25	0.5	0.5
IBA	0	0.01	0.01	0.01	0.01
Activated charcoal (AC)	0	0	0	0	3000

All treatments consisted of five replications, each containing five explants, and the average data analysis was based on three independent experiments. The following indicators were counted: mean number of shoots per explant, mean plant height, mean number of roots, and mean root length. The effect of the propagation media was studied dynamically by data collection on days 14, 21 and 28, and analyzed by standard biometrical methods.

The plants adaptation and acclimatization *ex vitro* were performed in a growth chamber with gradually decreasing atmospheric humidity, temperature of 22±1°C, and 16/8 h photoperiod. A mixture of peat-perlite (3:1) with sand (50%) was used as a substrate for transplanting in pots. The percentage of surviving plants cultivated on media A0 and A33 was compared on days 7, 14, 21, and 28.

2. Effect of the light source on the growth and development of plants in the stage of multiplication.

The experiment was performed in the Laboratory of Plant Biotechnology at the Institute of Fruit Growing - Plovdiv. Explants cultivated on medium A33 were exposed to four variants of LED light (provided by Philips Green Power LED research module): white (LW), red (LR), blue (LB), LMix (Red:Blue:DeepBlue:White=1:1:1:1) and a control (FW) of white fluorescent tubes (OSRAM, 40 W). Explants

The cultivation of the plants was performed in glass vessels (volume 180 ml), containing 30 ml culture medium, in a growth chamber with a temperature of 24±1°C, a light intensity of 3000 lx, and 16/8h photoperiod. The average data analysis was based on three independent experiments with four weeks of subculture duration. The following indicators were counted: mean number of shoots per explant, mean plant height, multiplication coefficient, mean number of roots, and mean root length. The effect of the light on the propagation was studied dynamically by data collection on days 14, 21 and 28, and analyzed by standard biometrical methods.

3. Investigation of the efficiency of a bioreactor system with temporary immersion (TIS), type RITA® in the stage of multiplication of *Fabiana imbricata* Ruiz. et Pav.

The experiment was performed in the Laboratory of Plant Biotechnology at AU-Plovdiv. The experiment used explants (2 cm) from well-developed plants obtained *in vitro* on medium MS. The multiplication step was performed by comparing the two systems: solid medium A33 in glass vessels and liquid culture medium with the same composition (without agar) in a TIS bioreactor with 120 explants in a variant. The experiment consists of 24 replicates for glass vessels with five explants on solid medium and six bioreactors with 20 explants. The bioreactor containers were autoclaved, connected to sterile PTFE filters filled with 200 ml of liquid medium, and the explants were placed in sterile conditions. The immersion time of the explants in the culture medium was 15 minutes every 8 hours. Cultivation was performed in a growth chamber with a temperature of 24±1 ° C, light intensity 3000 lx (white fluorescent light), and 16 / 8h photoperiod. The data analysis was based on two independent experiments with a passage duration of four weeks. Reported were the following parameters: mean number of shoots per plant, mean plant height, coefficient multiplication, mean number of roots, and mean length of the roots.

4. Induction of callus culture on solid culture medium.

The experiment was performed in the Laboratory of Plant Biotechnology at AU-Plovdiv. The initial explants were prepared by segmenting a leaf and stem of 28-day *in vitro* plants *Fabiana imbricata* Ruiz. et Pav. For primary callus induction, the explants were placed on two culture medium composed on MS enriched with sucrose (30 g / l), MERK agar (6 g/l) and 2,4-D at concentration 0, 2 mg/l (MSD) and 4 mg/l (D) (*Denchev, et al., 1990*) at pH = 5.7. The experiment was performed in 5 replications (90 mm diameter petri dishes) containing 30 ml of culture medium, cultivated in a growth chamber with a photoperiod 16h light / 8h dark, and temperature 24±1°C.

4.1. Effect of the light source on the growth and development of callus culture of *Fabiana imbricata* Ruiz. et Pav.

Calus cultures were placed on culture medium MS, with sucrose supplement (20 g/l) and MERK agar (6 g/l), with pH = 5.7 and different concentrations of plant growth regulators (Table 2). Three callus lines set on a different culture medium (MSD, D, A) have been studied.

Table 2. Plant growth regulators (mg/l) used in the culture media for culturing the undifferentiated *in vitro* systems *Fabiana imbricata* Ruiz. et Pav.

PGR / Culture medium	2,4-D	Kinetin	NAA
MSD	0.2	-	-
D	4.0	-	-
A*	0.25	0.25	0.25
* Culture Media A has a doubled content of MS vitamins			

The effect of the light source was studied by exposure of the callus cultures to light provided by white fluorescent lamps (L) and red (R) light, and in the dark (D). Each variant consisted of 3 petri dishes with 10 calluses, placed on the three culture media. The duration of the experiment was 90 days, three subcultures in a chamber with 24°C, and 16/8 hours photoperiod.

Additionally, the influence of the light regime on the synthesis of secondary metabolites has been studied by collecting fresh biomass (1g) at day 0, 7, 14, 21, 28, and 35. Ten samples per variant of 3 independent subcultures were collected to analyze the metabolic composition.

4.2. Obtaining of plants cell suspension cultures.

The following experiment aimed to obtain a fast-growing homogeneous suspension culture. Fast-growing calluses obtained on MSD solid medium in light and darkness were transferred to a liquid culture medium MSD based on MS, with 2,4-D (0.2 mg/l). In sterile conditions, 3 g of callus was placed in a 30 ml liquid medium in 200 ml cultivation vessels. The experiment was in three replications. Cultivation was carried out on a rotary shaker at 110 rpm in the dark and temperature of 24°C, with a ten-day subculture period.

4.3. Effect the culture media efficiency and establishing the dynamics of plant biomass growth of suspension culture variants.

Preparatory stage - carried out for obtaining the necessary quantity of suspension culture by placing 30 ml inoculum in 30 ml of culture medium in 200 ml cultivation vessels. The experiment was set with three variants of MSD, D, and A (Table 2), with pH = 5.7, each repeated 3 times.

Experiment: 880 ml of liquid medium and 220 ml inoculum (8-day culture of the corresponding suspension) were mixed to homogenization in 2 liters flask. Each treatment

was prepared in 3 repeats with 10 samples (100 ml of the homogenized suspension divided in 10 sterile Erlenmaire flasks of 500 ml volume. Cultivation was performed on a rotary shaker (100 rpm) and 25°C in the dark. The collection and reporting were conducted at 0, 2, 4, 5, 6, 7, 8, 9, 10, and 11 days of the beginning of cultivation. Biomass has been collected for phytochemical analyzes.

4.4. Digital holographic microscopy for characterization of *Fabiana imbricata* Ruiz. et Pav. cell suspension cultures.

The experiment was performed at AU-Plovdiv. The light source is a diode laser (Lasiris) with a wavelength of 673.2 nm and an output of 7 mW. The laser radiation is focused onto a pinhole, after which a polarizer controls the intensity. The spherical wave passes through the object. The diffracted by the object and the nondiffracted wave interfere and were recorded as a hologram on a CCD sensor. The intensity and the phase were reconstructed numerically. USAF Test Target 1951 was used to calibrate the holographic microscope. The optimum distance between the laser source and the object was 30 cm to record holograms of *Fabiana imbricata* Ruiz. et Pav. cell suspension cultures. The distance from the object to the CCD camera in the experiments was 5 cm.

Plant cell suspensions were obtained by cultivating callus on solid MSD medium and subsequently transferred to different liquid culture media. . Suspension cultures were cultured on a rotary shaker at 110 rpm in darkness at 25°C with a subculturing period of 10 days. Different concentrations of growth regulators were used in the studied variants

Extraction of polyphenols

The extraction procedure was performed in the Department of Analytical Chemistry laboratory at the University of Food Technologies - Plovdiv.

Lyophilized samples of plants *in vitro*, plants *in vivo*, callus and cell suspension culture (100 mg) were extracted three times with 5 ml of 70% ethanol at reflux at 75°C. The resulting extracts were filtered through filter paper and combined (by exhaustive method). After evaporation on a rotary vacuum, the dry extracts were stored in the dark at 4°C and dissolved in a suitable solvent.

5. Methods for analysis of the metabolic profile of *Fabiana imbricata* Ruiz. et Pav.

To analyze the metabolic profile by *Fabiana imbricata* Ruiz. et Pav. cultures *in vivo* plants (overhead mass) obtained from *Paddock Plants* (England) and *in vitro* plants, callus of selected cultures, and cell mass of suspension culture were used.

5.1 Spectrophotometric determination of the content of common phenols, general flavonoids, and antioxidant activity.

Spectrophotometric analysis of the extracts was performed in the Department of Analytical Chemistry laboratory at the University of Food Technologies - Plovdiv.

5.1.1. Contents of total phenols and total flavonoids.

The total phenols were determined by the Folin-Ciocalteu (*Stintzing et al.,* 2005) spectrophotometric method modified as follows: To 1 ml of Folin-Ciocalteu (Sigma) reagent, 0.2 ml of the analysis extract and 0.8 ml of 7.5% Na2CO3 (Sigma) were added. The resulting mixture was left in the dark at room temperature for 20 min for the reaction course. After the reaction time expires, the light absorption of the sample was measured at 765 nm against a control sample prepared in the same manner containing the corresponding extract instead of extract. The results obtained are represented as mg of GAE (GAE) for G dry weight (DW) against standard rights constructed with different concentrations of Galic acid (Sigma), linear 0.02-0.10 mg /ml gallic acid.

The spectrophotometric determination of the total flavonoids was carried out according to a method of Kivrak et al. (2009): to the analyzed extract (1.0 mL) was added 0.1 ml of 10% aluminum nitrate (Sigma), 0.1 ml of 1M potassium acetate (Sigma) and 3.8 ml of water. After the reaction time (40 min, room temperature), the sample absorption was measured at 415 nm against a control sample containing 0.1 ml of water instead of aluminum nitrate. The results obtained are represented as mg equivalents of quercetin (QE) for G dry weight (DW), according to standard linear in the concentration 10-100 μg/ml quercetin.

5.2.2. Determination of antioxidant activity

5.2.2.1. DPPH method

To determine the antioxidant activity by the DPPH method by capturing the radical (*Kivrak et al.,* 2009). The analyzed extract (0.15 mL) was mixed with a freshly prepared solution of DPPH (0.1 mm in methanol) (Sigma) (2.85 mL). The reaction mixture is incubated in the dark at 37ºC for 15 min. The absorption of samples was measured at 517 nm against a control sample, ethanol.

5.2.2.2. ABTS method

The ABTS method (*Thaipong et al.,* 2006) was performed with minor changes. ABTS radical was generated by mixing equal amounts of 7.0 mM aqueous solution of 2, 2'azinobis (3)-ethylbenzthiazoline-6-sulfonic acid (ABTS) and 2.45 mM aqueous K2S2O8. The reaction mixture is staying in the dark for 16 h at room temperature. Before analysis, 2 ml of the ABTS radicals were dissolved in methanol (1:30) to give final absorption 1.0 ÷ 1.1 at 734 nm. The investigated extract (0.15 mL) was mixed in a tube with 2.85 ml of freshly prepared ABTS radical solution. A control sample of 0.15 ml ethanol was prepared instead of the extract. The reaction mixture was incubated in the dark for 15 min at 37°C, and the light absorption of the control and samples was reported spectrophotometric against ethanol at 734 nm wavelength.

5.2.2.3. FRAP method

The FRAP method (*Benzie and Strain,* 1996) was modified as follows: the FRAP reagent was prepared by mixing pre-prepared 0.3 M acetate buffer with pH 3.6, 10 mM 2,4,6-tripyridyl- S-triazine (TPTZ, Fluka) in 40 mM HCl and 20 mM Fecl3.6H2O in a ratio of 10: 1: 1. The test extract (0.1ml) was added to 3 mL of a FRAP reagent. The reaction mixture was incubated for 10 min at 37°C, and then the absorption of the samples was measured spectrophotometric against the control sample (0.1 ml of ethanol) at a wavelength of 593 nm.

5.2.2.4. CUPRAC method

The method described by *Apak et al.,* (2006) was used with modifications. The reaction was initiated by mixing 1.0 ml of 10 mM CuCl2.2H2O (Sigma), 1.0 ml of 7.5 mM Neocuropine in methanol (Sigma), 1.0 ml of 0.1 M ammonium acetate buffer pH 7.0, 0.1 ml of the analyzed extract, and 1.0 ml of distilled water. A control sample of 0.1 ml of ethanol is prepared instead of extract. The reaction mixture was incubated for 20 min at 50 °C. After cooling the mixture, the absorption of the samples was read at 450 nm against the control.
Light absorption was measured by spectrophotometer (Shimadzu UV / Vis mini 1240, Japan). Antioxidant activity all used methods was expressed as mM Trolox equivalents for g dry weight (DW) by pre-built standard with different concentrations (0.05, 0.1, 0.2, 0.3, 0, 4, and 0.5 mm) of Trolox (Fluka) dissolved in methanol (Sigma).

5.3. HPLC analysis to determine polyphenol compounds.

HPLC analysis was performed at the Department of Analytical Chemistry Laboratory of the University of Food Technologies - Plovdiv. Quantitative determination of polyphenol compounds was carried out using the HPLC system waters. For phenolic acid analysis, gradient elution with solution A (1% acetic acid, v / v) (Sigma) and solution B (methanol)

(Merck) was used. The phenolic acid detection was performed at two wavelengths λ = 280 and 360 nm, column temperature of 28 °C and the volume of the injected sample 20 μl. Standard show linearity in the range of 20-100 μg/ml with correlation coefficients above 0.998. For the determination of flavonoids, gradient elution with solution A (% acetic acid, v / v) (Sigma) and solution B (methanol) (Merck) was used. The flavonoid determination was carried out at a wavelength λ = 380 nm, column temperature 28°C, and volume of injected sample 20 μl. The flavonoids determination was by pre-prepared standard with different concentrations of standard solutions. The standard has shown linearity between 20-100 μg/ml with correlation coefficients above 0.998.

IV. Results and Discussion

***In vitro* culture establishment.**

Following the sterilization procedure described, over 98% of the explants demonstrated the beginning of development on a hormone-free medium (A0). The formation of shoots and roots was observed after four weeks of cultivation. Initially, the *in vitro* obtained plants were grown on A0 medium in two subcultures. In the subsequent experiments, the influence of the growth regulators concentration on proliferation was studied.

1. Optimization of the culture medium for multiplication of *Fabiana imbricata* Ruiz. et Pav.

Results counted on day 28 showed that the proliferation value in variant A0 was 5.04±0.68 compared to variant A3 – 5.64±0.70 (Fig. 1). Surprisingly, the explants cultivated on media A1 and A25 enriched with growth regulators (BAP, IBA) demonstrated lower proliferation than those grown on the hormone-free medium A0. Comparing the efficiency of the studied proliferation media, medium A3 demonstrated the highest proliferation capability, but the used concentration of the applied cytokinin (BAP) caused shoot development with symptoms of hyperhydricity (vitrification).

Data analysis of the mean plant height indicator showed that on day 14, the plants grew to 2.2 cm, reaching over 5 cm on day 28 (Fig. 1B). In all the treatments, similar values for the mean plant height were established. *Schmeda-Hirschmann et al.,* (2004) reported the development of a rapid *in vitro* propagation system for *Fabiana imbricata* Ruiz. et Pav. leading to the formation of shoots, calli, roots, cell suspensions, and plantlets. In their study,

the shoots derived from nodal sections were multiplied by branching new axillary buds using the temporary immersion system (TIS) with an average length of 5 cm.

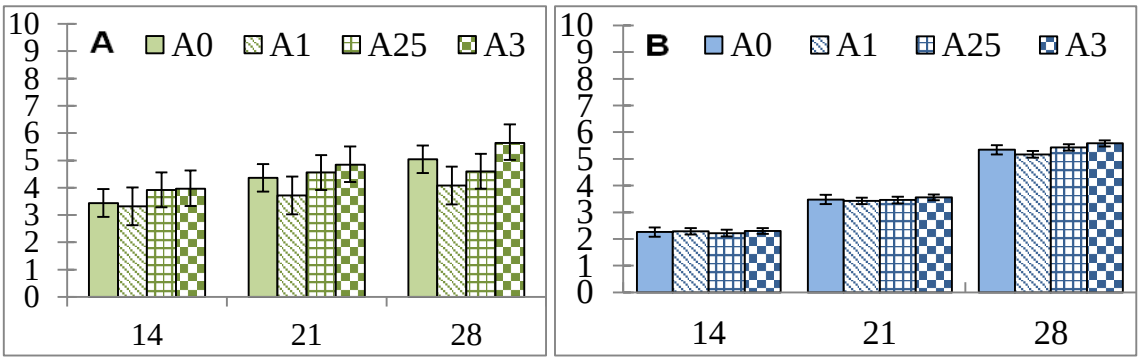


Fig. 1. Mean number of shoots per explant (*A*) and mean plant height (*B*) on days 14, 21 and 28, (SE)

An optimized proliferation medium (A33) was tested to overcome the physiological disorder vitrification. It had the same composition as A3 but with activated charcoal addition (0.3%). The application of activated charcoal positively influenced the growth and development of the plants, resulting in almost two-fold higher proliferation and absence of hyperhydricity. Cultivation of the explants on the A33 medium was characterized by the formation of 9.04±0.54 shoots per explant compared to A3 (5.64±0.70) on day 28 (Fig. 2A). The results for the mean plant height showed similar values for both variants again (Fig. 2B). 28 (Fig. 2A).

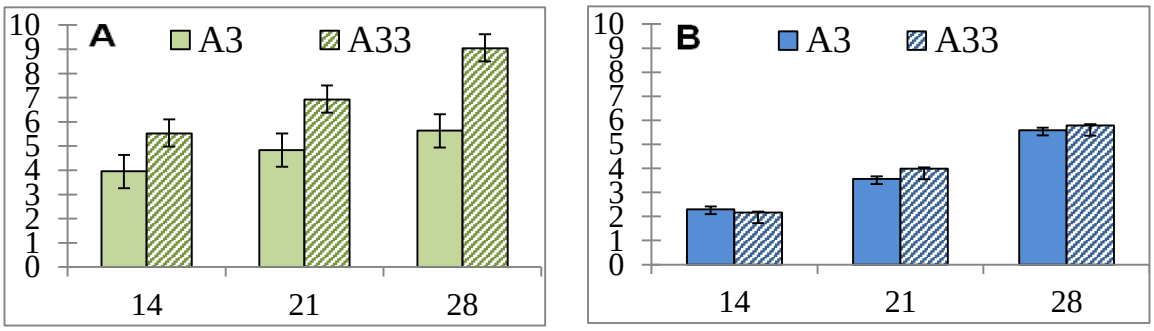


Fig. 2. Effect of activated charcoal addition on *Fabiana* micropropagation as (A) mean number of shoots per explant, and (B) mean plant height on days 14, 21, and 28, (±SE).

The presence of growth regulators was essential for plant morphogenesis and development (Fig. 3). Plants cultivated on A0 were characterized by a compact, well-formed shrub, dark green leaves, and short roots. Medium A1 induced shoots with different lengths, light green

stems, and the formation of small roots. Both media, A25 and A3, demonstrated high proliferation, but it was associated with shortened internodes and visible symptoms of vitrification. The growth behavior and plant development on A33 were strongly influenced by the addition of activated charcoal and characterized by the formation of symmetrically shaped shrubs and elongated roots compared to those grown on variant A0 (Fig. 3).

Fig. 3. Plant development on culture media A0 и A33.

All explants (100%) cultivated on different proliferation media produced roots during the multiplication stage. Data obtained on days 14, 21, and 28 confirmed that A0 and A33 were the most efficient media, inducing the formation of a high mean number of roots and mean root lengths (Fig. 3). At the end of the culture, the highest values of mean root numbers (8.96 and 7.04) were recorded for variants A0 and A33, respectively (Fig. 4A). However, data obtained for the mean root length indicator (Fig. 4B) demonstrated that placement on A0 resulted in the formation of short and thick roots compared to variant A33, where slim, long, and tender roots developed (Fig. 4).

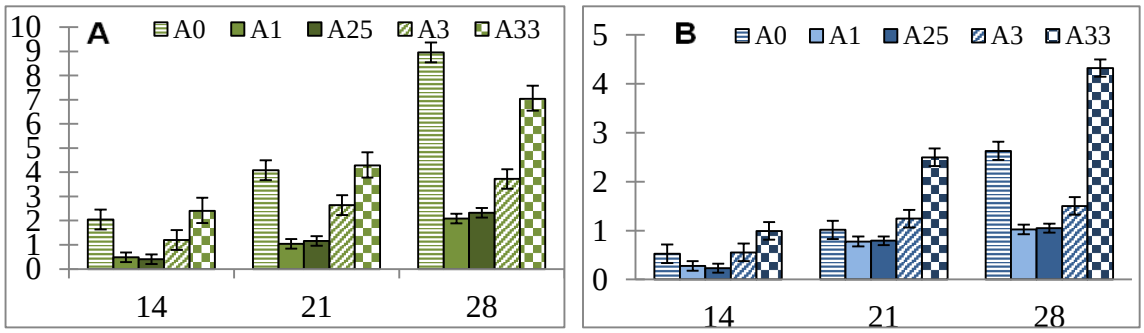


Fig. 4. Rooting capacity as mean root number (A), and mean root length (B) of plantlets cultivated on different propagation media on days 14, 21 and 28, (±SE).

Schmeda-Hirschmann et al., (2004) tested three initiation media in the experiment with *Fabiana imbricata* Ruiz. et Pav., also led to rhizogenesis. Root formation of single shoots mainly occurred after one month in the presence of IAA, IBA or NAA used alone or in combinations at concentrations 0.25 – 1.0 mg/l. Under these conditions, rooting occurred in 41.2-64.7% of explants, with the highest rate of 5.6 roots per explant (*Schmeda-Hirschmann et al.*, 2004).

1.1. Adaptation and plant survival

It was found that cultivation medium has a signifficant influence on the adaptation efficiency as a percentage of surviving plants. Following the acclimatization procedure, the plants grown on medium A0 demonstrated the highest rate (80%) of survival. In comparison, the percentage of vital plants originating from variant A33 decreased from 70% on day 7 to 30% on day 35 (Fig. 5 and Fig. 6). Based on these results, we suggest that the omission of the growth regulators in the last subculture before adaptation could be a valuable prerequisite for plant hardening, resulting in successful survival.

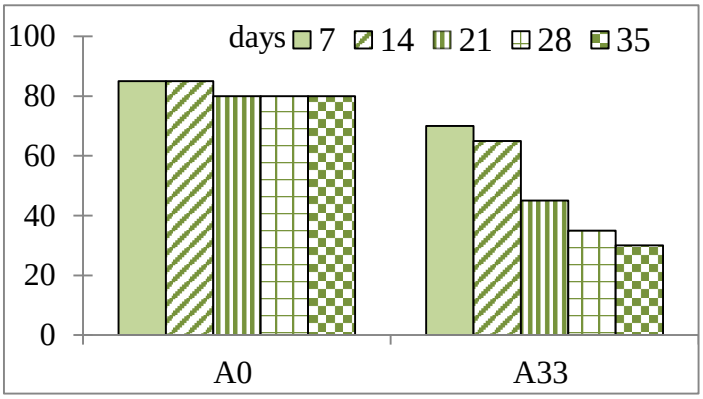


Fig. 5. Influence of the rooting medium on adaptation and plant survival.

Fig. 6. Survival of plants following 35 days of adaptation

2. Effect of the light source on the growth and development of plants in the stage of multiplication.

The results show that LED sources with different spectra have a specific influence on the growth and development of *in vitro* cultivated plants *Fabiana imbricata* Ruiz. et Pav. According to the indicator average height of the plants (Fig. 7), no significant differences were found in the values of the variants cultivated in the conditions of LED lights compared to the control, reported on the 28th day from the start of the experiment.

This experiment showed that the LED light sources with different spectra have a specific effect on *in vitro* grown *Fabiana* plants. The highest shoot formation 6.56±0.86 was established in the control treatment with white fluorescent light (FW), followed by LED white (LW) 5.56±0.82 (Fig. 7). Moreover, cultivated plants under LR had lower shoot formation (4.44±0.82) comparing to all studied treatments.

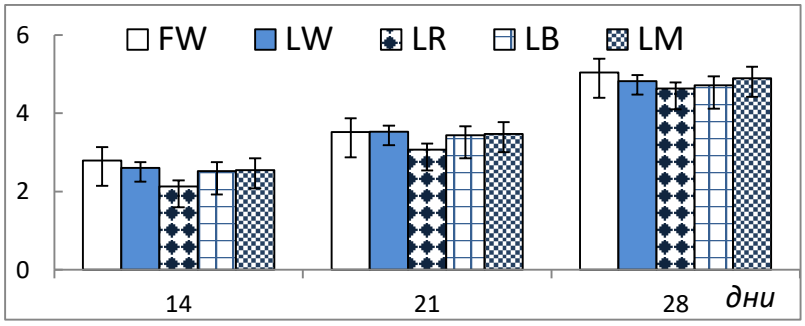


Fig. 7. Mean plant height [cm] on days 14, 21 and 28, (±SE)

The highest number of shoots was established under the FW - 6.56±0.86, followed by LW 5.56±0.82 (Fig. 8). The red LED - LR had the lowest proliferation efficiency (4.44±0.82) recorded on day 28.

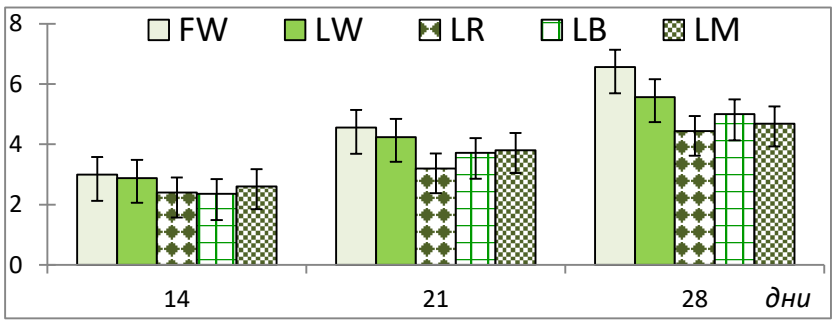


Fig. 8. Mean number of shoots per explant on days 14, 21, and 28, (±SE).

The high value was established again for the explants developing under FL (4.44±0.76) in comparison to the LED treatments, respectively LM (3.92±0.70), LR (3.36±0.86), LW (2.88±0.72), LB (2.64±.86) after 28 days of culture (Fig. 9).

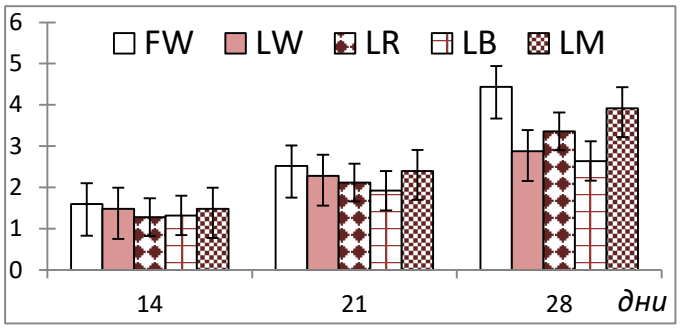


Fig. 9. Effect of the light source and spectra on *Fabiana* multiplication coefficient on days 14, 21, and 28, (±SE).

Concerning rooting process, a high average number of roots was found for the FW control (6.84±0.80) (Fig. 10A). Almost half fewer roots were reported in variant LW (3.36±0.64), while the other variants LR, LB, and LM, had significantly lower values. The same trend was observed for the average root length indicator (Fig. 10B) - the values for the LED lights were lower. The influence of light quality on rooting varies in plant species. The authors report that the lowest value for the average root length was obtained from red light treatment, with the growth regulator IAA added to the nutrient medium. *Nacheva et al.*, (2020) reported the potential of LEDs as an effective lighting system for rapid micropropagation of raspberries (*Rubus idaeus* L.). The combination of blue, red, pale red and white light (1:1:1:1) stimulates the growth and accumulation of biomass and the intensity of photosynthesis. Contrary, *Wang et a.,*. (2011) and *Poudel et al.*, (2008) reported that light spectra are not effective in influencing root length.

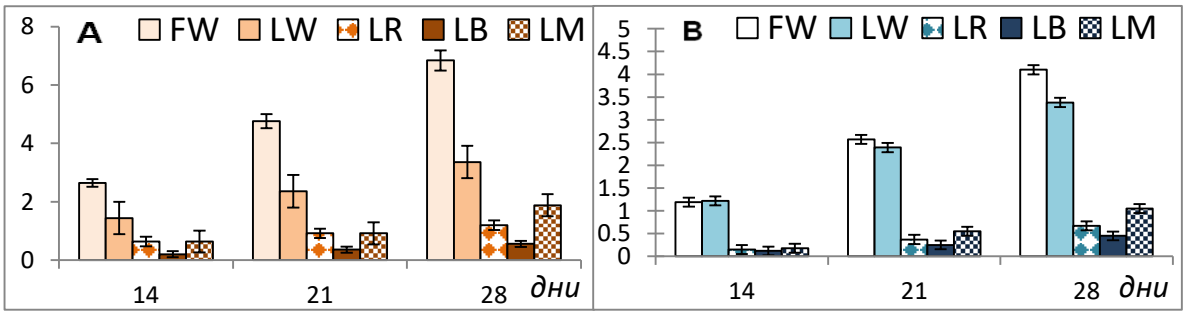


Fig. 10. Mean number of roots (A) and mean root length (B), [cm] on days 14, 21 and 28, (±SE).

Fluorescent lamps, the main light source commonly used for *in vitro* culture, have fixed emission spectra composed of multiple bands in the wavelength range of 320 to 800 nm, without the possibility of different illumination parameters, such as spectral and time characteristics (*Kurilcik et al.*, 2008). LEDs have several unique advantages, including the ability to control spectrum composition, durability, long service life, wavelength specificity, relatively cool radiating surfaces (*Li et al.*, 2010). *Dimitrova et al.* (2021) reported that pear seedlings grown *in vitro* under white LED light produced high fresh (FW) and dry (DW) biomass values, regardless of the cytokinin applied.

However, the performed experiment proved that in the micropropagation of *Fabiana imbricata* Ruiz. et Pav, white fluorescent light was more efficient than LED lighting systems, regardless of their advantages.

3. Investigation of the efficiency of a bioreactor system with temporary immersion (TIS), type RITA® in the stage of multiplication of *Fabiana imbricata* Ruiz. et Pav.

The experiment aimed to establish the efficiency and the possibility of optimizing the standard protocol for *in vitro* propagation of *Fabiana imbricata* Ruiz. et Pav. cultures using a temporary stirring system, type RITA® compared to conventional *in vitro* propagation. Both multiplication systems were assessed based on the reported data on the growth indicators (Fig.11) on day 28.

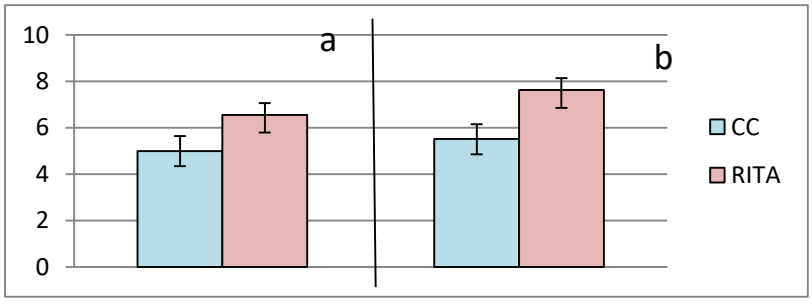


Fig. 11. Effect of cultivation in two *in vitro* systems in the stage of multiplication of *Fabiana imbricata* Ruiz. et Pav., as mean plant height (cm) (a) and mean number of shoots (b) on day 28 (±SE).

The average plant height indicator had a higher value in the explants cultured in TIS bioreactor (RITA) - 6.55±0.66 compared to conventional *in vitro* culture (CC) - 5.00±0.60, reported on day 28 (Fig. 11a). For the average number of shoots (Fig. 11b), a higher value

(7.62±0.76) was also reported for TIS bioreactor culture (RITA) while the result was in the conventional culture was lower (5.51±0.81).

Schmeda-Hirschmann et al., (2004) used a temporary immersion bioreactor (TIS) system to develop a rapid *in vitro* propagation system for *Fabiana imbricata* Ruiz. et Pav., leading to the formation of shoots, calluses, roots, cell suspensions and plants. This study reported that nodal sections produced branching, and the obtained shoots reached an average length of up to 5 cm.

The values of the growth indicators from the performed by us experiment with *Fabiana imbricata* Ruiz. et Pav. are in accordance with the findings of other authors, reporting that the shoot multiplication and shoot length are significantly better in TIS than in solid and liquid pineapple media (Ananas comosus L. Merr) (*Escalona et al.,* 1999), as well as in *Siraitia grosvenorii* (*Yan et al.,* 2010). A higher value of the multiplication for apple rootstock M26 was found in cultivation in TIS compared to solid medium (*Zhu et al.*, 2005). *Damiano et al.,* (2005) compared TIS culture and solid medium in apple (York 9), peach (cv. Yumyeong), cherry (cv. Biggareau Burlat), and plum (cv. Adara). They found no difference in multiplication between the two propagation systems.

The reported coefficient of multiplication had the same tendency, namely a higher value for the TIS bioreactor culture 5.98±0.63 than the conventional crop 4.01±0.51 (Fig. 12).

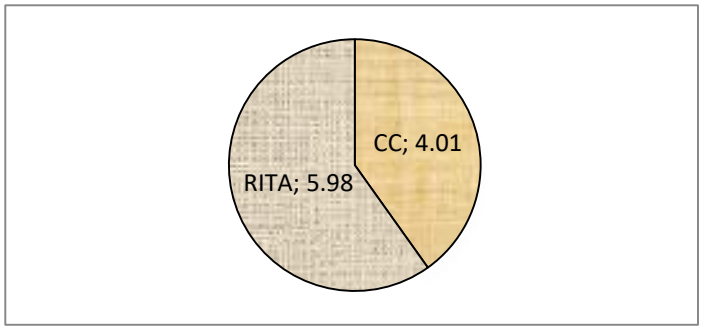


Fig. 12. Multiplication factor, recorded at day 28 ((±SE)

Silva et al., (2007) and *Scheidt et al*., (2009) established that temporary immersion resulted in a multiplication rate up to three times higher than the conventional pineapple micropropagation system. Also, *Piatczak et al.,* (2014) reported that the medicinal plant *Rehmannia* cultivated in TIS formed 21 shoots per explant in 60 days, three times more than the conventional protocol.

All explants (100%) in both test systems of cultivation form roots during the stage of multiplication. The values reported for the mean number of roots (Fig. 13a) are 5.70±0.69 for TIS bioreactor culture and 5.26±0.51 for conventional *in vitro* culture. A similar trend was found for the mean root length, respectively 3.74±0.37 for TIS bioreactor culture and 3.23±0.15 for traditional culture (Fig. 13b).

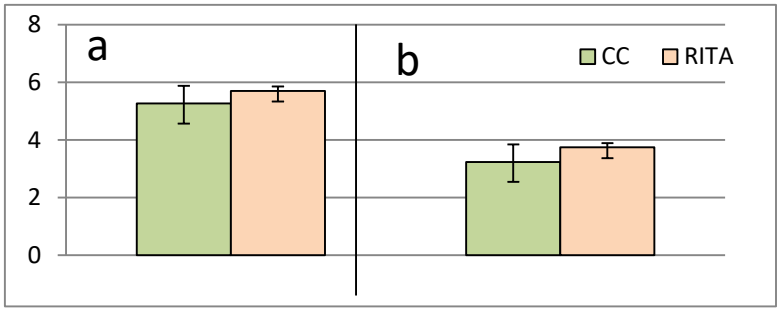


Fig. 13. Mean number of roots (a) and mean root length (в), [cm] on days 14, 21 and 28, (±SE).

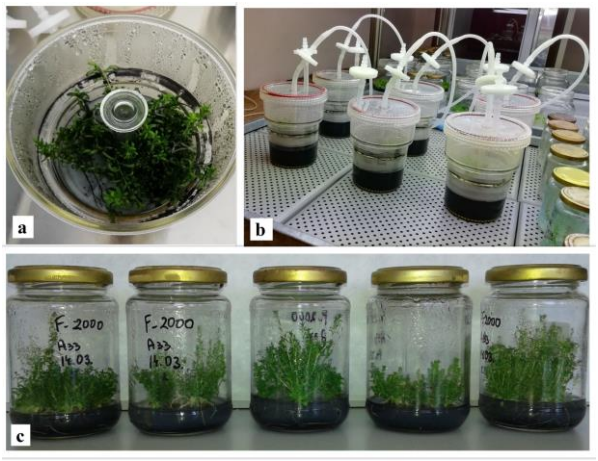


Fig. 14. Experimental view: temporary immersion system, type RITA® (a, b), compared to conventional system (c) in the multiplication stage of *Fabiana imbricata* Ruiz. et Pav. on the 28th day.

The obtained results can be explained by the fact that the solid medium in glass jars or bioreactors without forced ventilation is characterized by high relative humidity and accumulation of gases harmful to growth, due to poor gas exchange (*Georgiev et al.*, 2014). Improved gas exchange during forced ventilation reduces gas accumulation and relative

humidity in culture containers, and also minimizes the difference between the gaseous medium *in vitro* and *ex vitro* (*Roels et al.*, 2006).

Compared to the conventional micropropagation system, the temporary immersion system (type RITA®) is more efficient due to the reported higher values of the growth characteristics. Regardless of the high investment in bioreactor system equipment, the advantage is that it provides process automation, minimal space and production of large amounts of biomass (Fig. 14).

In conclusion, it was found that for the development of an effective micropropagation system of *Fabiana imbricata* Ruiz. et Pav. the following factors are essential:

- Optimal concentration of plant growth regulators (0.5 mg / l BAP + 0.01 mg / l IBA)
- Addition of activated charcoal as an antioxidant in the culture medium
- Cultivation under white fluorescent light
- Plant cultivation on a PGR-free medium in the last passage before adaptation *ex vitro*.
- Use of bioreactor system with temporary immersion.

4. Inducing a callus culture in a solid culture medium.

The process of callogenesis of MSD medium (0.2 mg/l 2,4-D) began on day 18 from the beginning of the experiment. The callus mass was characterized by rapid growth and by day 29 of cultivation covers almost the entire surface of the explants. The resulting callus mass (Fig. 15) had rapid growth, soft and crumbly consistency, and a relatively homogeneous structure. The primary callus was pale green, and then in the following passages, it turns to milky white. The calluses obtained on MSD medium were used in the subsequent experiments (1) to study the effect of the light source, (2) to obtain a fast-growing callus culture, (3) to induce a suspension culture, (4) to collect samples and to analyze the metabolic profile.

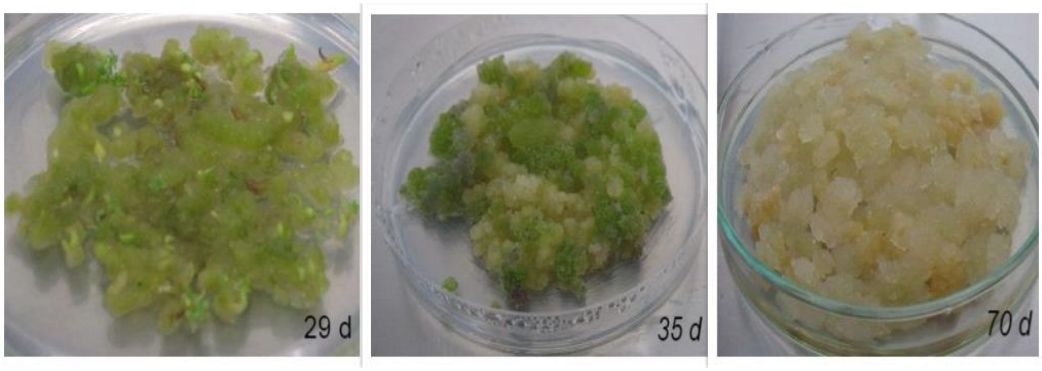


Fig. 15. Degrees of dedifferentiation and callus growth of MSD medium.

4.1. Effect of the light source on the growth and development of calus culture of *Fabiana imbricata* Ruiz. et Pav.

The experiment aimed to determine the influence of the light source on the growth and development of the obtained callus lines (MSD, D, and A) cultured on different media. Medium A, reported by *Schmeda-Hirschmann et al.* (2004) as efficient for callus induction, was included in the experiment, respectively. For analysis of the metabolic composition, a gram of fresh biomass was collected on days 0, 7, 14, 21, 28, and 35 of cultivation. Based on established culture differences, 10 samples per variant of 3 independent subcultures were taken to analyze all variants.

Characterization of callus cultures.

All callus cultures were characterized by growth rate and homogeneous structure (Fig. 16) and presented in fresh biomass (g) on day 28. It was found that under red fluorescent light with a 16/8 hours photoperiod, callus cultures reach their maximum growth. The highest biomass-producing cultures were RA (12.40±0.82), RMSD (11.35±0.85), RD (10.80±0.53). The treatments under white fluorescent light with photoperiod 16/8 had mean values of LMSD (6.65±0.17), LD (5.32±0.30), LA (7.60±0.20). The lowest weight had the callus obtained in the dark: DMSD (5.58±0.7), DD (6.43±0.22), and DA (6.30±0.42). It has been proven from the literature that the light source indicates the influence on the growth and development of plant crops, respectively regulates their secondary metabolism. Light is actively involved in the metabolic processes of plants, and *in vitro* culture is a means of producing biologically active compounds (*Sa´ez et al.,* 2013). However, plants have different reactions to the growth and production of the desired metabolites when exposed to different *in vitro* culture conditions. Fluorescent lamps are the main source of light used in *in vitro* cultivation. They have fixed emission spectra and are composed of many bands in the wavelength range from 320 to 800 nm (*Kurilc˘ik et al.,*2008). *Alvarenga et al.* (2015) report

that the quality and quantity of light regulates the secondary metabolism of yarrow (Achillea millefolium L.), the number, content, and profile of volatile components. Based on the mentioned effect and the results of this experiment, the collected material was used to study and determine the influence of light regime on synthesizing secondary metabolites.

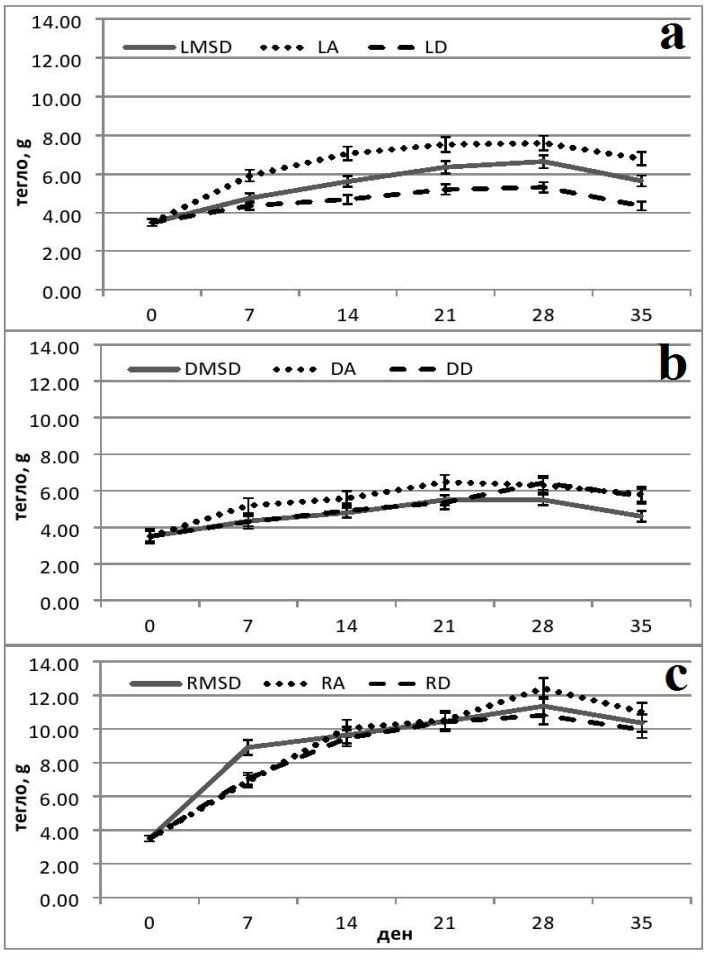


Fig. 16. Effect of the light source on the growth and development of calus culture of *Fabiana imbricata* Ruiz. et Pav., cultivation on variants culture medium: a- white light светлина; в- in- dark; c- red light, ±SE.

4.2. Obtaining of plants cell suspension cultures.

The next group experiments aimed to obtain a fast-growing homogeneous suspension culture, producing plant biomass for targeted phytochemical analyzes of the biologically active ingredients of *Fabiana imbricata* Ruiz. et Pav.

The obtained suspensions were subcultured for nine months, and 20 replicates showing homogeneity and a satisfactorily fast growth rate were available during the experiment.

4.3. Effect the culture media efficiency and establishing the dynamics of plant biomass growth of suspension culture variants.

After one month of initial subculturing, a sufficient suspension cultures amount was obtained to start the actual experiment. Variants MSD and A were characterized by a maximum increase in cell mass about eight days of culture compared to variant D, in which the biomass increased in 3-4 days and then followed a decrease. There was also a slight variation in the wet/dry biomass mass in the subsequent readings (Fig. 17). Variants MSD and A increased cell mass up to 3-fold, making them more effective suspension cultures for experimental purposes than variant D (4 mg/l 2,4-D). However, *Schmeda-Hirschmann et al.*, (2004) reported that the suspension culture of *Fabiana imbricata* Ruiz et Pav. doubles its biomass in one week only when the concentration of 2,4-D was increased to 5 mg/l. Regarding the color of the suspension cultures, the studied variants also differ. Variant MSD was milky white, while variants D and A had a darker tone.

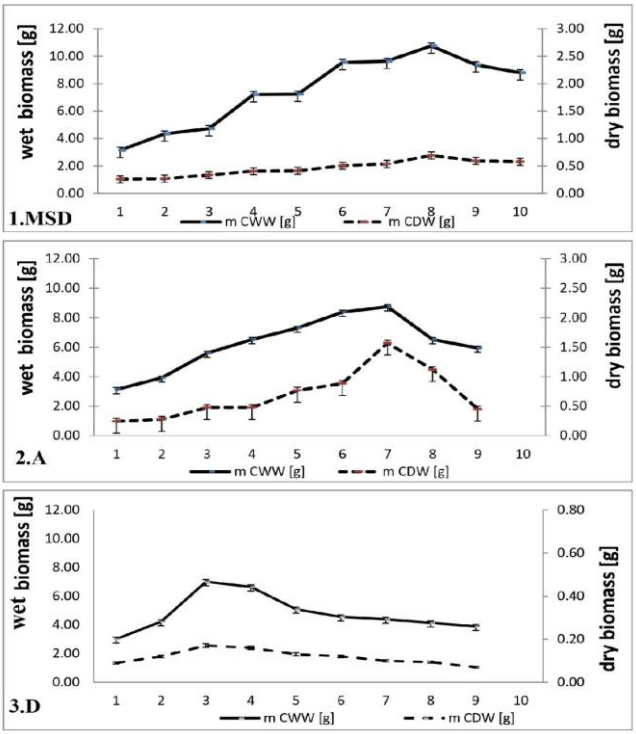
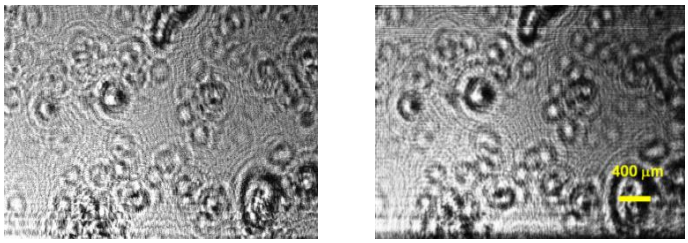


Fig. 17. Growth dynamics of suspension culture (MSD; A; D), ±SE.

4.4. Digital holographic microscopy for characterization of *Fabiana imbricata* Ruiz. et Pav. cell suspension cultures.

DIHM was applied to visualize the three different cell suspensions of *Fabiana imbricata* Ruiz. et Pav. named A, D, and MSD. Digital reconstruction of the recorded interference patterns was performed using the "HoloVision 2.2" software.

Small cell aggregates with dimensions between 120 and 180 μm have been observed in all suspensions. The large cell aggregates (140-180 μm) were characteristic for suspension A. The cell aggregates in suspension D had dimensions 120-150 μm (Fig.18). Because the plant cell walls have a natural tendency to adhere, obtaining homogeneous cell lines that consist only of single cells was impossible or problematic. Some progress has been made in selecting cell suspensions with increased cell segregation in the cultures with medium MSD. The proportion and size of the cell aggregates seem to be influenced by both - growth regulators and some nutrient medium constituents (such as calcium ions) in the culture.



a **b**

Fig.18. Images of cell suspension culture D: a) digital hologram; b) the numerically reconstructed wavefront intensity of a).

This experiment with digital holographic microscopy (DHM) was applied for the first time to measure the size of cell clusters in the suspension cultures. Histological examination of suspension cultures is a slow and complex process, and the application of DHM allows the assessment of their development in dynamics. The present investigation indicates that DHM can provide efficient morphological analysis of cells without prior study for cell division and migration.

5. Phytochemical characteristics of cultures of *Fabiana imbricata* Ruiz. et Pav.

5.1. Spectrophotometric determination the content of common phenols, general flavonoids and antioxidant activity.

5.1.1. Contents of total phenols and total flavonoids.

The literature review did not reveal any information regarding the content of total phenols, total flavonoids, and antioxidant activity of *Fabiana imbricata* Ruiz. et Pav. in vitro

cultures. In this sense, the present study presents data for analyses of total phenolic compounds and antioxidant activity for the first time and has an original contribution.

The reported results for callus culture extracts ranged between 4.17±0.11 mgGAE/g DW (DD) and 7.00±0.11 mgGAE/g DW (LMSD) (Table 3).

Higher content of total phenols was established in the analyzed biomass extracts of plant cell suspensions. It could be suggested that in deep cultivation, the cultures probably increase the biosynthesis of phenolic compounds and explaining the higher results. The values obtained for total phenols in the analyzed cell suspensions ranged from 5.48±0.07 mgGAE/g DW (MSD REP 2.9) to 20.72±0.13 mgGAE/g DW (A9) (Table 4). The tested plant extracts showed values respectively 11.34±0.11 mgGAE/g DW (*in vitro* plant) and 24.46±0.11 mgGAE/g DW (*in vivo* plant). It is noteworthy that the concentrations of the phenolic compounds for the plant extracts analyzed are close but in cell suspensions decreased in half. It is known that the antioxidant capacity of plant extracts is mainly due to the presence of phenolic acids and flavonoids in them (*Jaberian et al.*, 2013).

5.2.2. Determination of antioxidant activity

The antioxidant activity of the extracts obtained from the *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. was analyzed spectrophotometrically by four widely used methods. Usually, several methods are used to determine the antioxidant activity of plant extracts, which provide complete information on how the components of the extract react with the model *in vitro* systems. In the present study, the antioxidant activity of aqueous-ethanolic extracts from *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. was determined by analyzing their ability to capture free DPPH and ABTS radicals or by their ability to reduce metal ions in FRAP and CUPRAC methods. Results are presented as millimoles of Trolox equivalent/gram dry weight (mMTE/g DW) in Tables 3 and 4.

5.2.2.1. DPPH method

The activity of the tested callus extracts varied between 10.49±0.20 mMTE/g DW for RMSD and 41.08±0.53 mMTE/g DW for DA. The extracts obtained from plant cell suspensions showed higher antioxidant activity than callus cultures, with values obtained between 4.49±0.13 mMTE/g DW for MSD REP2.7 and 70.91±0.27 mMTE/g DW for A9 (Table 3 and 4).

The indicator established was 43.96±0.17 mgGAE/g DW for the plant *in vitro* and 97.95±0.56 mgGAE/g DW for the plant *in vivo*, respectively two times higher. It could be explained by the fact that in natural conditions, different stress factors influence plant

metabolism, which directs them to synthesize and accumulate more metabolites for environmental protection.

5.2.2.2. ABTS method

The analysis performed by this method showed the lowest results for RMSD (34.46±0.53 mMTE/g DW) and the highest for LMSD (77.63±1.12 mMTE/g DW) of callus culture extracts. Values obtained from suspension cultures ranged from 35.28±0.85 mMTE/g DW (MSD REP 2.7) to 167.03±0.54 mMTE/g DW (A9). It should be noted that undifferentiated cultures demonstrated an antioxidant capacity, approximately comparable to the ABTS radical in the analyzed plants *in vitro* - 86.68±0.74 mgGAE/g DW and the result obtained from a plant in vivo - 145.38±0.74 mgGAE/g DW.

5.2.2.3. FRAP method

The lowest antioxidant activity detected by the FRAP method was 114.15±0.84 mgGAE/g DW reported for the suspension culture extract MSD REP 2.7 and the highest for A9, respectively 664.03±1.42 mgGAE/g DW (Table 4). For callus cultures, the results obtained were between 136.71±2.01 mgGAE/g DW for RMSD and 361.59±1.75 mgGAE/g DW for RA. Half-lower values of 349.27±2.46 mgGAE/g DW for the plant *in vitro* were reported compared to 694.83±5.51 mgGAE/g DW for the plant *in vivo* (Table 3)

5.2.2.4. CUPRAC method

Using the CUPRAC method, the results for callus extracts ranged from 40.53±0.68 mMTE/g DW in RMSD to 179.97±1.15 mMTE/g DW in RA culture. Higher antioxidant activity was reported for the extracts of cell suspensions, with a maximum value in variant A9 (274.59±1.42 mMTE/g DW) and minimum in MSD 2.9 (59.05±0.82 mMTE/g DW).

The in vitro plant extract sample demonstrated 252.90±1.88 mMTE/g DW, while *in vivo plant* 335.98±0.79 mMTE/g DW (Table 3, 4).

There were no reports concerning spectrophotometric analysis of the antioxidant activity of *in vitro* or *in vivo* cultures of the plant *Fabiana imbricata* Ruiz. et Pav., in the available literature.

Undoubtedly, the results obtained by us for the content of total phenols in the studied *in vitro* systems, and their antioxidant capacity show a great variety. In conclusion, we can say that the obtained *in vitro* plants, callus culture RA, and suspension culture A have a higher antioxidant capacity than other *in vitro* cultures, close to that of the sample extract of the *in vivo* plant.

Table 3. Spectrophotometric determination of total phenols, total flavonoids, and antioxidant activity of callus cultures, *in vitro* and *in vivo* plants from *Fabiana imbricata* Ruiz. et Pav., (mg GAE/g DW).

Variants*	total phenols	total flavonoids	DPPH	ABTS	FRAP	CUPRAC
LMSD	7.00±0.11	**2.34±0.05**	**33.61±1.75**	77.63±1.12	217.74±1.05	77.75±1.74
DMSD	4.34±0.03	0.62±0.04	15.93±0.96	53.45±0.40	173.14±0.38	84.93±2.06
RMSD	6.59±0.05	0.67±0.02	10.49±0.20	34.46±0.53	136.71±2.01	40.53±0.68
LA	4.45±0.03	1.70±0.01	22.06±0.28	53.34±0.56	190.20±0.63	74.34±0.47
DA	5.16±0.05	1.43±0.05	41.08±0.53	53.85±0.52	232.98±2.02	66.32±0.45
RA	**15.08±0.18**	1.81±0.03	31.43±0.18	**77.85±0.45**	**361.59±1.75**	**179.97±1.15**
LD	6.95±0.03	1.75±0.02	33.24±0.25	66.04±0.21	278.15±0.54	88.09±0.82
DD	4.17±0.11	1.22±0.02	18.02±0.15	49.30±0.56	184.85±0.66	53.90±0.66
RD	4.91±0.02	1.15±0.02	17.40±0.09	55.38±0.11	165.55±0.92	81.51±0.88
***in vitro* plant**	11.34±0.11	2.42±0.04	43.96±0.17	83.68±0.61	349.27±2.46	252.90±1.88
***in vivo* plant**	24.46±0.52	4.76±0.09	97.95±0.56	145.38±0.74	694.83±5.51	335.98±0.79

** Legend : L-white light, D- in the dark, R-red light, MSD, D, A - culture media*

Table4. Spectrophotometric determination of total phenols, total flavonoids and antioxidant activity of suspension cultures from *Fabiana imbricata* Ruiz. et Pav., mg GAE/g DW.

	total phenols	**total total flavonoids**	**DPPH**	**ABTS**	**FRAP**	**CUPRAC**
D2	6.38±0.09	1.01±0.03	9.70±0.33	68.61±0.39	165.42±0.81	77.12±0.81
D3	7.51±0.05	1.06±0.06	14.36±0.19	79.27±0.61	220.45±0.77	89.62±0.77
D4	7.76±0.25	0.88±0.10	11.68±0.49	90.15±0.81	209.03±0.32	102.19±1.39
D5	7.58±0.15	1.03±0.06	12.65±0.24	85.87±0.69	231.28±0.83	106.87±0.83
D6	8.04±0.08	0.87±0.06	11.70±0.26	75.58±0.56	247.02±0.90	92.77±0.90
A5	14.67±0.15	**2.29±0.02**	**67.72±0.13**	121.15±0.51	451.72±1.36	148.22±1.57
A6	**16.97±0.14**	1.44±0.02	49.57±0.71	**146.23±0.84**	575.14±1.46	178.57±0.84
A7	15.54±0.37	1.27±0.04	49.38±0.48	121.97±0.53	471.05±1.40	175.80±1.21
A8	13.86±0.09	1.62±0.01	39.38±0.66	104.56±0.78	244.99±0.78	146.40±1.34
A9	20.72±0.13	1.47±0.04	70.91±0.27	164.03±0.54	**664.03±1.42**	**274.59±1.42**
MSD REP 2.7	5.49±0.05	1.38±0.02	4.49±0.13	35.28±0.85	114.15±0.84	67.28±0.80
MSD REP 2.8	6.31±0.05	1.38±0.04	8.79±0.18	42.11±0.27	137.65±0.40	88.36±0.80
MSD REP 2.9	5.48±0.07	1.98±0.03	16.41±0.46	47.67±0.11	137.86±0.62	59.05±0.82
MSD REP 2.10	5.67±0.08	1.47±0.02	13.27±0.33	45.76±0.30	140.03±0.85	64.66±0.87
Legend : MSD, D, A- culture medium; REP- repetition ; 2,3,4,5,6,7,8,9,10- day sample taken with a high biomass						

5.3. HPLC analysis to determine polyphenol compounds.

HPLC analysis of the biosynthetic potential of plants *in vitro*, *in vivo*, calluses, and cell suspensions obtained from *Fabiana imbricata* Ruiz. et Pav was performed to search for a highly effective *in vitro* system producing biologically active ingredients. Information on phenolic constituents of *Fabiana imbricata* Ruiz. et Pav. found in the available literature showed that the plant had been analyzed for the first time by HPLC-DAD-ESI-MS, identifying 10 compounds, 9 of which were characterized based on UV spectrum and MS. fragmentation models. The compounds include n-hydroxyacetophenone derivatives, scopoletin, rutin, chlorogenic acid, and three quercetin glucosides (*Cristina et al.,* 2012). It was foud that *Fabiana imbricata* Ruiz. et Pav. contains many secondary metabolites: rutin, scopoletin, coumarin, oleanolic acid, and some sesquiterpenes (*Brown*, 1994a; *Razmilic et al.*, 1994) as well as D -manno- heptulose, perseitol, and D-glycero-D-manno-octulose (*Richtmeyer*, 1970).

Oleanolic acid (OA) is the basic triterpene acid in the aerial parts of *Fabiana imbricata* Ruiz. et Pav., showing a gastroprotective effect in rats and mice, with very low toxicity (*Astudillo et al*., 2002). Chlorogenic acid is one of the main components of the polar extracts of *Fabiana imbricata* Ruiz. et Pav. Biological activities reported for scopoletin include an anti-inflammatory (*Muschietti et al*., 2001) and hepatoprotective effect (*Kang et al*., 1998), monoamine oxidase inhibition (*Yun et al*., 2001), and inducible nitric oxide synthetase (*Kang et al*., 1999; *Kim et al*., 1999). Polyphenolic compounds have high biological activity and are widely used in the pharmaceutical and cosmetic industries.

It was essential to establish how the cell differentiation degree of some *in vitro* cultures and the growth conditions influence the synthesis of the polyphenol compounds. Callus cultures, characterized by soft and crumbly texture, homogeneous structure, and relatively fast-growing, were selected. On this basis, the callus cultures (MSD, D, A) grown under white and red light (photoperiod 16/8) and in the dark were analyzed.

Impressive was the found great variety in the amounts of synthesized phenolic acids in the aqueous-ethanolic extracts of the studied *in vitro* cultures. Among the light-cultured variants, callus culture LD with a high content of protocatechinic acid (342.47±24.17 mg/g DW), vanilla acid (941.89±51.2 mg/g DW), and caffeic acid (903.54±42.3 mg/g DW) compared to the other two variants (LMSD and LA) which had lower concentrations.

Callus culture DA produced in the highest amounts protocatechinic acid (1114.43±52.4 mg/g DW) and chlorogenic acid (772.59±23.7 mg/g DW) and culture DMSD - caffeic acid (1043.72±77.6 mg/g DW).

It is noteworthy that only callus RA synthesizes gallic acid (27.08±0.29 mg/g DW) and ferulic acid (63.25±11.09 mg/g DW), a high amount of salicylic acid (3320.94±102.8 mg/g DW) and rosemary acid (26.64±0.34 mg/g DW). Table 5 shows all other callus cultures and the detected phenolic acids in small amounts or traces compared to the above.

Table 6 presents the results from HPLC analysis of *in vitro* plants that synthesized almost double chlorogenic acid (2821.57±94.3 mg/g DW) compared to the sample extracted from a plant *in vivo* (1552.33±66.7 mg/g DW). This finding confirms that chlorogenic acid (3-O-caffeoylquinic acid) is an integral part of the raw drug infusion (*Quispe et al.,* 2012).

Among suspension cultures, cell suspension A is distinguished by the highest concentrations of synthesized phenolic acids compared to the other two. Traces of p-coumaric acid were found in all suspensions. It was also established that all suspensions (D, A, and MSD) were characterized with high values of phenolic compounds around the day they reach maximum biomass produced (Table 7).

The ability of callus cultures to synthesize flavonoids (Table 8) is relatively less than in diversity but quantitatively within close limits, such as the synthesis of phenolic acids. (-) - Epicatechin was synthesized in the callus cultured under red light, as the highest amount was reported for RA (1358.67±96.3 mg/g DW). Epicatechin has several benefits in humans; it stimulates muscle fibers growth, regulates blood sugar levels, lowers cholesterol levels to natural for the body, and improves brain and heart health. RD synthesized a significant amount of quercetin (3057.8±159.8 mg/g DW) compared to other callus cultures. The flavonols kaempferol, quercetin, and rutin were identified from the plant's aboveground parts by *Hörhammer et al.* (1973). In the infusion of aerial parts, *Quispe et al.,* (2012) identified rutin, quercetin-O-rhamnose-dihexoside and quercetin-diramhanoside hexoside.

Callus cultures grown in the dark and red light synthesized (-) - Epicatechin and those in the photoperiod 16/8h - rutin at high levels, compared to cell suspensions cultured in the dark. According to *Ionkova et al*. (2009), high concentrations of 2,4-D lead to the synthesis of flavonoids in *in vitro* cultures of *Astragalus missouriensis* Nutt., while an increase in cytokines (kinetin) leads to increased yields of rutin and hyperoside. The results obtained by us do not show such dependence. Most likely, the light spectrum and the degree

of differentiation of the *in vitro* cultures obtained by us had a more substantial effect on the biosynthesis of some biologically active ingredients.

Suspension cultures (Table 9) demonstrated: (+) - Catechin in suspension D5 (1214.37±105.0), (-) - Epicatechin in A8 (1888.68±124.1), Rutin in MSD rep 2.8 (573.89±42.3) and Quercetin at D2 (1872.14±137.9) in higher amounts than callus cultures.

It was reported that the main compounds in the infusion are metabolites with several proven biological effects. Chlorogenic acid (*Naveed et al.,* 2018), rutin (*Hosseinzadeh and Nassiri-Asl,* 2014), quercetin (*Carvalho et al.,* 2017), scopoletin (*Benoit et al.,* 2012; *Ferreira et al.,* 2017), and oleanolic acid (*Pollier and Goossens,* 2012; *Yin,* 2012) are compounds of growing interest for health promotion and their potential therapeutic applications (*Schmeda-Hirschmann and Theoduloz*, 2019).

Table 10 shows the concentration of flavonoids synthesized in the two analyzed plant extracts (*in vitro* plant and *in vivo* plant). Higher amounts of rutin and quercetin were found in the *in vivo* plant extract. The establishment confirms the early reported (Schmeda-Hirschmann and Papastergiou (1994) that untreated herbal drug contains in its secondary metabolites rutin and coumarin, scopoletin well as oleic acid, and some sesquiterpenes.

Rutin has been reported to prevent reflux esophagitis and gastric secretion in rats by inhibiting gastric acid secretion, oxidative stress, inflammatory cytokine production, and intracellular calcium mobilization in PMNs (*Shin et al.,* 2002).

The results of the HPLC analysis showed a difference in the reported amounts of phenolic compounds in the callus, suspensions, and plant cultures of *Fabiana imbricata* Ruiz. et Pav. Moreover, some compounds of primary and secondary metabolism demonstrated dependence on the degree of cell differentiation, leading to the use of tissue cultures as a source of secondary metabolites (*Rodríguez-Sahagún et al.,* 2012).

Based on the results obtained, it could be concluded that the degree of differentiation, the composition of the nutrient medium, and the light conditions were identified as factors determining the metabolic profile of analyzers *in vitro* systems. It is important to note that in *Fabiana imbricata* Ruiz et Pav. the dominant phenolic compounds were:

- callus cultures - protocatechin, chlorogenic, caffeic and salicylic acid, (-) epicatechin, quercetin
- • suspension cultures - the highest amounts of gallic and caffeic acid, epicatechin
- • plants *in vitro* - chlorogenic acid,

- plants *in vivo* - syringic acid and rutin

Based on the synthesized amounts of phenolic compounds in the analyzed plant *in vitro* culture systems, we suggest that they could be used as a technological matrix for producing valuable phenolic acids and flavonoids, with potential application in the pharmaceutical and cosmetic industries.

Table 5. HPLC analysis of phenolic acids synthesized in callus cultures from *Fabiana imbricata* Ruiz. et Pav., expressed in mg/g DW.

	Gallic acid	Protocatechinic acid	Chlorogenic acid	Vanillic acid	Caffeic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Salicylic acid	Rosemary acid
LMSD	nf	72.35±3.02	259.4±22.15	nf	636.14±25.17	nf	*trace*	*trace*	344.89±36.15	13.9±0.10
LA	nf	*trace*	219.97±31.2	nf	561.16±28.9	nf	*trace*	*trace*	318.79±27.3	11.2±0.12
LD	nf	342.47± 24.17	281±17.2	941.89±51.2	903.54±42.3	nf	*trace*	*trace*	305.67±41.2	23.53±0.21
DMSD	nf	375.52±22.3	724.75±34.4	nf	1043.72±77.6	251.67±16.7	*trace*	*trace*	264.05±29.4	*trace*
DA	nf	1114.43± 52.4	772.59±23.7	348.36±27.4	619.57±26.5	nf	*trace*	*trace*	347.24±42.5	*trace*
DD	nf	361.08±23.7	643.72±22.6	275.66±17.6	908.82±18.4	nf	40.51±1.6	*trace*	283.31±27.8	nf
RMSD	nf	nf	198.33±13.2	nf	506,1±34.7	219.06±18.4	*trace*	*trace*	235.93±22.7	19.1±0.29
RA	27.08± 0.29	nf	186.65±16.5	294.09±16.7	585.08±22.6	239.57±21.5	512.4±18.7	63.25±11.09	3320.94±102.8	26.64±0.34
RD	nf	nf	nf	nf	702.29±31.4	217.29±18.6	*trace*	*trace*	308.64±32.2	nf
Legend: nf - not found; trace, L-white light, D- in the dark, R-red light, MSD, D, A-culture medium										

Table 6. HPLC analysis of phenolic acids synthesized in *in vitro* and *in vivo* plant from *Fabiana imbricata* Ruiz et Pav., expressed в mg/g DW.

	Gallic acid	Protocatechinic acid	Chlorogenic acid	Vanillic acid	Caffeic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Salicylic acid	Rosemary acid
in vitro plant	nf	324.15±21.6	**2821.57±94.3**	nf	nf	575.39±42.16	*trace*	*trace*	228.48±32.4	*trace*
in vivo plant	nf	*trace*	1552.33±66.7	388.5±32.3	661.78±37.14	**6080.16±106.7**	351.74±37.8	*trace*	257.2±24.7	153.67±13.4
Legend: nf– not found; trace										

Table 7. Quantity of phenolic acids synthesized in plant cell suspensions from *Fabiana imbricata* Ruiz. et Pav., expressed в mg/g DW.

	Gallic acid	Protocatechinic acid	Chlorogenic acid	Vanillic acid	Caffeic acid	Syringic acid	p-Coumaric acid	Ferulic acid	Salicylic acid	Rosemary acid
D2	78.43±19.5	nf	212.16±19.2	331.32±29.7	588.79±22.4	275.58±22.6	*trace*	*trace*	222.04±19.8	14.47±0.07
D3	861.06±87.3	116.08±86.1	318.13±33.5	411.92±34.7	641.28±37.6	296.19±37.0	*trace*	*trace*	380.95±29.8	35.34±0.10
D4	999.36±91.5	131.49±62.6	388,55±29.6	475.04±45.2	647.82±61.4	329.26±26.8	*trace*	*trace*	496.75±36.5	40.23±0.09
D5	1056.17±98.2	151.6±33.7	381.22±24.3	507.09±43.1	905.36±74.2	328.1±31.4	*trace*	*trace*	510.79±37.1	38.77±0.16
D6	913.66±68.9	145.67±77.5	333.67±23.8	472.66±27.3	690.79±44.2	353.72±26.7	*trace*	*trace*	484.56±25.6	34.98±0.05
A5	688.28±82.3	371.25±55.1	393.91±31.5	479.69±39.1	603.46±39.5	328.8±15.6	*trace*	*trace*	437.74±46.9	18.78±0.07
A6	806.11±96.8	499±22.3	**509.92±41.6**	**568.54±34.5**	648.52±36.7	347.68±24.6	*trace*	8.27±0.13	480.73±36.7	22.43±0.11
A7	1405.84±101.4	536.1±21.5	462.29±37.1	555.83±30.9	653.32±57.2	438.41±36.8	*trace*	15.82±0.32	604.98±29.3	22.73±0.12
A8	**2202.75±126.4**	**731.58±56.4**	414.06±22.2	498.69±29.3	**698.39±44.3**	491.65±24.5	*trace*	145.55±21.5	663.73±56.7	26.78±0.15
A9	891.17±79.8	544.62±55.2	364.53±25.5	466.88±37.2	695.68±36.7	**870.56±34.9**	*trace*	140.05±21.9	**814.47±34.5**	**43.56±0.19**
MSD	nf	nf	239.66±19.7	nf	nf	nf	*trace*	*trace*	414.93±44.	nf
MSD	27.62±0,17	nf	392.5±37.6	nf	nf	nf	*trace*	*trace*	510.95±49.	nf
MSD	nf	nf	243.55±28.7	nf	nf	nf	*trace*	*trace*	485.67±46.	nf
MSD	nf	nf	264.99±34.1	nf	nf	nf	*trace*	*trace*	474.92±37.	nf
Legend : nf– not found, trace MSD, D, A- culture medium; REP- repetition ; 2,3,4,5,6,7,8,9,10- day sample taken with a high biomass										

Table 8. Quantity of flavonoids synthesized in callus cultures from *Fabiana imbricata* Ruiz et Pav., expressed in mg / g DW.

	(+)-Catechin	(-)- Epicatechin	Hesperidin	Rutin	Quercetin
L MSD	*nf*	*nf*	*trace*	109.12±15.6	141.52±19.7
L A	*nf*	*nf*	*trace*	514.35±19.7	549.51±26.7
L D	*nf*	*nf*	*trace*	523.53±21.1	198.7±16.5
D MSD	*nf*	1009.59±69.0	*trace*	93.78±0.94	397.28±28.9
D A	*nf*	876.9±29.5	*nf*	168.19±12.8	*trace*
D D	465.06±57.5	1161.52±96.3	*trace*	345.37±31.0	*trace*
R MSD	*nf*	874.1±29.4	*trace*	71.33±0.11	*trace*
R A	*nf*	1358.67±89.5	*trace*	157.63±0.82	71.98±19.1
R D	376.1±29.4	928.73±59.5	*nf*	191.78±21.5	3057.8±159.8

Legend: nf - not found; trace, L-white light, D- in the dark, R-red light, MSD, D, A-culture medium

Table 9. Quantity of flavonoids synthesized in suspention cultures from *Fabiana imbricata* Ruiz. et Pav., expressed in mg / g DW.

	(+) Catechin	(-) Epicatechin	Hesperidin	Rutin	Quercetin	Kaempferol
D2	nf	nf	*trace*	167.51±27.1	**1872.14±137.9**	*trace*
D3	878.46± 19.5	1126.34±6.7	*trace*	239.56±23.7	*trace*	*trace*
D4	1117.57± 91.5	1181.41±84.1	*trace*	273.89±64.2	*trace*	*trace*
D5	1214.37± 105.0	1242.18±57.7	*trace*	269.16±23.7	*trace*	*trace*
D6	**1189.73± 99.2**	1150.6±46.7	*trace*	268.63±36.7	*trace*	*trace*
A5	968.26± 62.5	1254.26±79.1	*trace*	208.07±29.7	*trace*	*trace*

A6	1071.48± 49.7	1464.47±95.4	*trace*	224.46±26.7	*trace*	*trace*
A7	1103.58± 82.4	1556.42±92.7	*trace*	245.19±34.1	*trace*	*trace*
A8	**1165.27± 52.4**	**1888.68± 124.1**	*trace*	317.99±37.4	*trace*	*trace*
A9	1093.8± 63.4	1339.54±87.2	*trace*	241.09±31.6	*trace*	*trace*
MSD rep 2.7	nf	1026.75±72.3	*trace*	**482.24±85.2**	364,82±37.1	*trace*
MSD rep 2.8	nf	1032.63±73.4	*trace*	573.89±42.3	1356,27±111. 2	*trace*
MSD rep 2.9	nf	1041.98±67.5	*trace*	373.96±33.1	*trace*	673.16±42.2
MSD rep 2.10	nf	nf	*trace*	337.55±23.0	*trace*	635.81±36.5
Legend : nf– not found, trace MSD, D, A- culture medium; REP- repetition ; 2,3,4,5,6,7,8,9,10-day sample taken with a high biomass						

Table 10. Quantity of flavonoids synthesized in *in vitro* and *in vivo* plant from *Fabiana imbricata* Ruiz. et Pav., expressed in mg / g DW.

	(+) Catechin	(-) Epicatechin	Hesperidin	Rutin	Quercetin	Kaempferol
in vitro plant	*nf*	996.31±57.3	*trace*	534.84±75.6	557.88±61.3	*trace*
in vivo plant	627.16±47.3	*nf*	2029.76±173.4	31243,35±303.5	984.08±57.6	*trace*
Legend: nf– not found; trace						

V. CONCLUSIONS

1. A plant material *in vitro* was obtained from initial plants *Fabiana imbricata* Ruiz. et Pav., that is suitable for further use as a producer of biologically active substances.

2. An optimized nutrient medium for micropropagation of *Fabiana imbricata* Ruiz. et Pav., has been developed in which the balance of growth regulators provides a high multiplication rate.

3. The addition of activated carbon (AC) to the nutrient medium has a stimulating effect on shoot proliferation. It leads to overcoming unfavorable physiological disorders such as hyperhydration and malformations of shoots and roots.

4. The use of nutrient medium without growth regulators in the last subculture is a prerequisite for hardening regenerants and ensures a high survival of plants during *ex vitro* adaptation.

5. Light is an essential factor for the growth and development of *Fabiana imbricata* Ruiz. et Pav., plants as white fluorescent light is most suitable for the multiplication stage than LED sources.

6. Induced callus cultures of *Fabiana imbricata* Ruiz. et Pav. differ in morphology and growth characteristics. Based on fast-growing, soft, crumbly consistency and homogeneous structure, three callus lines suitable for further metabolic analyses were selected.

7. The three selected callus lines (MSD, D, and A) were also appropriated for the induction of plant cell suspensions.

8. For the first time, an experiment with digital holographic microscopy (DHM) was performed, which is easily applicable for determining the number and size of cells and cell clusters in suspension cultures of Fabiana imbricata Ruiz. et Pav.

9. Suspension cultures are a preferable system for producing target metabolites. They reach a maximum biomass increase for a short cultivation period (8-10 days) than callus cultures and *in vitro* plants (28 days).

10. The polyphenolic profile of plants *in vivo* and *in vitro*, calluses, and the resulting plant cell suspensions from *Fabiana imbricata* Ruiz. et Pav., was determined by HPLC analyzes.

11. For the first time, spectrophotometric analysis of *in vivo* and *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. was performed, demonstrating the high antioxidant activity of the

analyzed extracts. The most increased antioxidant activity was reported for callus culture RA cultivated in red light, *in vitro* plants, and suspension culture A, comparable with the plant *in vivo*.

12. The light (regime, spectrum, and photoperiod) and suitable nutrient medium significantly affect the secondary metabolites production from undifferentiated *in vitro* systems. The combination and change of these parameters could be a useful approach towards modeling the synthesis of target biologically active substances:

* callus culture D / light - Vanilla acid (941.89±51.2);

* callus culture D / red light - Quercetin (3057.8±159.8).

* MSD / dark - Caffeic acid (1043.72±77.6);

* DA / dark - Protocatechinic acid (1114.43±52.4), chlorogenic acid (772.59±23.7);

* A / red light - Salicylic acid (3320.94±102.8) and Rosemary acid (26.64±0.34), (-) - Epicatechin (1358.67±89.5);

* A / light - Routine (514.35±19.7),

13. The diversity of the synthesized polyphenolic compounds in the studied *in vitro* systems with different degrees of differentiation has been proved by HPLC analyzes:

* *in vitro* plant - Chlorogenic acid (2821.57±94.3)

* *in vivo* plant - Syringic acid (6080.16±106.7)

* suspension cultures A8 - Syringic acid (870.56±34.9), Rosemary acid (43.56±0.19), (+) - Catechin (1165.27±52.4), (-) - Epicatechin (1888.68±124.1);

* suspension cultures D2 - Quercetin (1872.14±137.9);

* MSD rep 2.10 - Kaemferol (673.16±42.2).

14. The HPLC analysis revealed the synthesis of Gallic acid (27.08±0.29) and Ferulic acid (63.25±11.09) only in callus RA (red light) and suspension A8 - Gallic acid (2202.75±126.4) on day 8 of cultivation.

15. Depending on the degree of differentiation of *Fabiana imbricata* Ruiz. et Pav., *in vitro* systems, the potential as technological matrices for obtaining target metabolites has been determined.

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VII. PUBLICATIONS CONNECTED TO THE DISSERTATION

1. **Halkoglu P. S**., Yancheva S. D., Pavlov A. I. and Mihaylova E. M., 2019. Digital Holographic Microscopy for Characterization Of *Fabiana Imbricata* Ruiz & Pav. Cell Suspension Cultures. Special Issue of the 8th International Advances in Applied Physics and Materials Science Congress (APMAS 2018) ACTA PHYSICA POLONICA A; Vol.135, 1132-1135. http://przyrbwn.icm.edu.pl/APP/PDF/135/app135z5p76.pdf

2. **Halkoglu P.,** Yancheva S. and Pavlov A., 2019. *FABIANA IMBRICATA* RUIZ ET PAV. MICROPROPAGATION. Bulgarian Journal of Agricultural Science, 25 (5), 1001–1006

3. **Halkoglu, P**.; Yancheva, S., 2019. Effect of the light source on *Fabiana imbricata* Ruiz. et Pav. micropropagation. Journal of Mountain Agriculture on the Balkans, Vol. 22, No.1, pp.337-347.

VIII. PARTICIPATION IN SCIENTIFIC FORUMS

- ✓ 5¯th INTERM 2018 - International Congress on Microscopy & Spectroscopy, Oludeniz, Turkey
- ✓ 22nd scientific conference with international participation "EcoMountain 2019", Troyan, Bulgaria

SUMMARY

The thesis aimed to evaluate the potential of *in vitro* cultures of *Fabiana imbricata* Ruiz. et Pav. as technological matrices for the production of bioactive substances.

As a result of the study, *in vitro* plant material from *Fabiana imbricata* Ruiz. et Pav. was established and used for obtaining cultures with different degrees of differentiation. Subsequently, they were evaluated as potential biological matrices for phenolic compounds production.

A system for multiplication and successful adaptation has been developed by optimizing the nutrient medium and cultivation conditions. The white fluorescent light source was found to be most suitable for the multiplication of *Fabiana imbricata* Ruiz. et Pav., compared to LED light sources. The experiment conducted with an automated temporary immersion system (type RITA®) showed greater efficiency due to the reported higher values of the indicators characterizing the growth than the conventional in vitro system. The main advantage of the bioreactor system is that it provides an automation process, minimal space, and the production of large amounts of biomass, despite the high cost of equipment investment.

Based on the fast-growing, soft, crumbly consistency and homogeneous structure of the induced callus cultures, three callus lines suitable for further study were selected. The established suspension cultures (MSD, D, and A) were analyzed, and the most productive of them was suspension A.

For the first time, an experiment with digital holographic microscopy (DHM) was performed to measure the size of cell clusters in suspension cultures of *Fabiana imbricata* Ruiz. et Pav., which proves that it can be successfully used for: cell counting, measuring the size of cells and cell clusters, analysis of cell culture viability, and others.

The polyphenolic profile of *Fabiana imbricata* Ruiz. et Pav., was determined by HPLC analysis in plants *in vitro, in vivo,* calluses, and derived from them the plant cell suspensions, sowing that they are producers of the valuable substances:

• callus cultures - protocatechin, chlorogenic, caffeic and salicylic acid, (-) epicatechin, quercetin.

• suspension cultures - the highest amounts are reported for gallic and caffeic acid, epicatechin.

• *in vitro* plant is dominated by chlorogenic acid, while *in vivo* plant is dominated by syringic acid and rutin.

For the first time, spectrophotometric analysis of the antioxidant activity of *in vitro* and *in vivo* cultures of *Fabiana imbricata* Ruiz. et Pav was performed. The results obtained for the studied *in vitro* systems' total phenolic content and their antioxidant capacity showed a great variety. According to the modified Folin-Ciocalteu method used - most common phenols contain callus culture RA (15.08±0.18), suspension culture A6 (16.97±0.14) compared to samples *as in vitro* plant (11.34±0.11) and *in vivo* plant (24.46±0.52).

For total flavonoids, callus culture Lmsd (2.34±0.05) is comparable with the sample of *in vitro* plant (2.42±0.04) and suspension culture A5 (2.29±0.02), which had half of the *in vivo* plant extract content (4.76±0.09).

Target metabolites are gallic, protocatechuic, chlorogenic, vanilla, syringic, caffeic, salicylic, and rosemary acids. The *in vitro* cultures with different degrees of differentiation showing a higher antioxidant capacity close to that of the *in vivo* plant are:

• callus culture LD for Vanilla acid (941.89±51.2), DMSD for caffeic acid (1043.72±77.6), DA for Protocatechin acid (1114.43±52.4), RA for Gallic acid (27.08±0.29), Ferulic acid (63.25±11.09), and the highest synthesis of Salicylic acid (3320.94±102.8), Rosemary acid (26.64±0.34), (-) - Epicatechin.

• suspension culture A - in the most significant amount synthesizes gallic acid from all analyzed cultures and half of the other phenolic acids and flavonoids.

• The *in vitro* plants are of interest as Chlorogenic Acid, Syringic Acid, Rutin, Quercetin producers.