AgroLife Scientific Journal - Volume 11, Number 2, 2022
ISSN 2285-5718; ISSN CD-ROM 2285-5726; ISSN ONLINE 2286-0126; ISSN-L 2285-5718

BIOMASS AND VALUABLE METABOLITES DYNAMIC ACCUMULATION IN STRAWBERRY CALLUS CULTURES

Monica-Elena MITOI, Alexandra-Gabriela CIOCAN, Carmen-Rodica MAXIMILIAN, Florența-Elena HELEPCIUC, Gina-Carmen COGĂLNICEANU

Romanian Academy, Institute of Biology Bucharest, Department of Developmental Biology, 296 Splaiul Independenței Street, 060031, Bucharest, Romania

Corresponding author email: florenta.helepciuc@ibiol.ro

Abstract

Strawberry is one of the most commonly cultivated and consumed plant, due to its organoleptic properties and nutritional values, exerting multiple beneficial health effects. In this study, two distinct strawberry callus lines obtained in different lighting conditions were characterized. The biotechnological potential of these cultures can be demonstrated by biomass and secondary metabolites accumulation. For this reason, growth rate, cellular morphology and viability were assessed. For metabolic potential evaluation, biochemical analyses of total phenolic and flavonoid content, anthocyanin pigments concentrations and antioxidant capacity were performed. The red callus line displayed higher growth rate, total phenolic content and antioxidant activity than the white line. Flavonoids and anthocyanins were present only in the red line. The biomass and these metabolites accumulation varied between subcultures, but were maintained at a high rate, being superior to values reported in planta. Our results show that both callus cultures lines were highly proliferative and the red one being a source of valuable bioactive secondary metabolites, with high antioxidant activity.

Key words*: strawberry callus; total phenolic content; flavonoid content; anthocyanin pigments; antioxidant activity.*

INTRODUCTION

Fragaria x ananassa Duch. (*Rosaceae*), also known as cultivated strawberry, is a spontaneous octoploid hybrid between two American octoploid species*, Fragaria chiloensis* Duch. and *Fragaria virginiana* L. (Almeida et al., 2007; Yuan et al., 2019). It is one of the most intensely cultivated and studied species, due to its attractive appearance, its nutritional and health-related properties, being rich in more than 300 compounds such as folates, vitamin C, polyphenols, flavonoids, anthocyanins pigments, ellagic acid and sulfur compounds (Zhang et al., 2010; Yuan et al., 2019).

The flavonoids represents the most important class of secondary metabolites in strawberry. The patterns of synthesis and accumulation of polyphenols, along with the effects that their accumulation exerts on developmental phases, have been partially figured out. Thus, it was evidenced that in the initial stages of ripening, an abundance of proanthocyanidins are present. During the middle stages, when the receptacle acquires the well-known red colour, high concentrations of anthocyanin pigments, flavonols and other phenolic compounds are synthesized (Schaart et al., 2013). In the last stages of receptacle development, anthocyanins are found in much higher quantities than other flavonoids, such as flavonols or catechins. Responsible for these fluctuating levels are internal (genetic) and external (environmental) factors (Almeida et al., 2007).

In nature, plants synthetize these compounds in low quantities and only in certain developmental stages. One possible solution for mass-production of these essential compounds is represented by *in vitro* technology, which has increased in popularity over the last decades, due to its economic, environmental-friendly and efficient nature. Nowadays, it is applied in fields such as agriculture (for creating highly productive and adaptable crops), medicine (for developing vaccines and drugs), in food industry (for isolation of natural colorants) and in cosmetics (for production of anti-ageing treatments). For these purposes, cells suspension or callus culture are mostly recommended, as they stimulate both cellular proliferation and metabolite production in higher quantities in comparison to strawberry plants.

The aim of this study was to assess the biotechnological performances of two distinct *Fragaria x ananassa* Duch. callus culture lines. The biomass accumulation of *Fragaria x ananassa* Duch. callus culture was estimated through growth rate, percentage of dry weight and cell viability assay. The metabolic potential was evaluated by biochemical analysis of the main class of secondary metabolites from strawberry which presented economic interest (polyphenols, flavonoids and anthocyanin pigments), in relation with antioxidant activity of the callus crude extracts.

MATERIALS AND METHODS

Plant material and cultures growth

For the initiation of the callus culture, *Fragaria x ananassa* Duch. achenes of the Temptation variety were achieved from S.C. Agrosel SRL (Romania), batch 149506. The biotechnological protocols for obtaining the 2 types of calli is the subject of the patent pending application A/00808 from 4.12.2020. Both callus lines were grown on the same nutritive medium, MS (Murashige and Skoog, 1962) supplemented with 2.4-dichlorophenoxyacetic acid, 1-naphtylacetic acid and kinetin, but in different lighting conditions: in the presence of light a pigmented callus line (red) was obtained and in the dark an unpigmented callus line (white) was isolated.

For the growth dynamic experiments, three successive subcultures (passages) with 33 repetitions were used. Each Petri dishes of 6 cm in diameter was inoculated with 0.3 g of callus (± 0.002), under sterile conditions, on the same MS nutritive medium formula. The callus cultures were grown in a Fitotron Weiss-Galllenkamp SCG 120 (Weiss Technik, Loughborough, UK) at 25°C with a photoperiod of 16 h light with 2000 lux intensity for the red line and in the dark for the white line. The calli were subcultured every 21 day on the same nutritive medium.

Gravimetric parameters determination

For the estimation of biomass accumulation for each subculture, the weight of the final callus and the initial callus (inoculum) were measured using Highland®HCB302 portable precision balance (Adam Equipment, UK). The growth rate was defined as average ratio between the final mass of callus and initial mass of inoculum of all repetitions. The dry weight was estimated by dehydration of final callus to 80 °C in oven for approximately 3 h up to constant weight. The results were expressed as percentage of g dry weight/g fresh weight.

Light microscopy analysis

For both types of callus, squash specimen samples were prepared by placing isolated parts of callus on a glass slide, adding a drop of sterile distilled water and placing the cover glass on top. Using a matchstick, the plant material was evenly distributed and analyzed under optical microscope.

Evaluation of callus culture cell viability

Cell viability was determined by the 2.3.5-triphenyltetrazolium chloride (TTC) assay method adapted from Towill and Mazur (1975), which illustrates mitochondrial enzymes activity in viable cells. These enzymes reduce colorless TTC to a red compound, called formazan. The small cell groups were isolated from biomass of white callus while for the red callus line, small pieces from 3 layers (red, white, black) were isolated by filtration. The callus fragments were incubated with the TTC solution, then placed in the dark, at room temperature, for 24 hours, after that the callus pieces were collected and washed with distilled water. For the extraction of formazan, the callus fragments were incubated in 3 ml of 96° ethanol, at 65°C, for 30 minutes, until complete discoloration of the tissue. The absorbance of the extracts was measured at 485 nm, using the Spectronic Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific) and compared to blank with ethanol solution. The formazan content was expressed as OD_{485nm}/g fresh weight (FW).

Extraction of phenolic compounds

Initially, the determinations were performed on calli subjected to extraction with 100% methanol, in a ratio of 1:5 (m/v), for 2 days at 300 rpm continuously shaking at room temperature. Subsequently, different extractants such as absolute methanol, ethanol, ethanol acidified with 2N HCl (85:15 v/v) and distilled water were tested. In the growth dynamics experiments, the absolute ethanol and the acidified ethanol were used as extractants.

The homogenates were centrifuged twice at 10000 RCF for 15 minutes at room temperature and the final supernatant was stored at 4°C.

Evaluation of total polyphenolic content

The total polyphenolic content was determined according to the method described by Mihailovic et al. (2013). The reaction mixture consisted of 0.5 ml of appropriately diluted extract, 2.5 ml of 11-fold diluted Folin-Ciocâlteu reagent and 2 ml of 7.5% Na_2CO_3 solution. The mixture was kept at room temperature for 30 minutes and then the absorbance was measured at 765 nm using Helios Gamma UV-Vis spectrophotometer (Thermo Fisher Scientific). Three repetitions of the same variant were performed and the average of the values representing the total phenol content was expressed in gallic acid equivalents/g fresh or dry weight (mg EAG/g FW or DW).

Evaluation of flavonoids content

The estimation of the flavonoid content of the extracts was performed according to the method described by Zhishen et al. (1999), with small adjustments. Thus 0.5 ml of dilute extract corresponding to each sample was supplemented with 2 ml of distilled water and 150 µl of 5% $NaNO_2$ solution. The mixture was incubated at room temperature for 5 min and then 150 µl of 10% $AlCl_3$ solution was added. After 6 minutes, the reaction was sttoped with 1 ml of 4% NaOH. The reaction mixture was made up to a total volume of 5 ml with distilled water. The absorbance of each sample was determined at 510 nm against blank. The flavonoid concentration was estimated on a standard curve with rutin between100-1000 µg/ml. Three repetitions of the same variant were performed. The average of the values representing the flavonoid content was expressed in rutin equivalents/g fresh or dry weight (mg ERU/g FW or DW).

Determination of monomeric anthocyanins concentration

The differential pH method for anthocianins assay (Giusti and Wrolstad, 2001) was used, based on the reversible color change in a pH range of monomeric anthocyanin pigments from the colored oxonium form that exists at pH 1 to the colorless hemicetonic form that predominates at pH 4.5. Two dilutions per sample were prepared in 25 mM KCl-HCl buffer pH 1, respectively, in 0.4 M CH_3COONa- HCl buffer pH 4.5. The absorbance of each sample was measured at 520 nm and 700 nm, against blank prepared with distilled water. The absorbance of each diluted sample was calculated according to the formula:

$A = (A_{520} - A_{700})_{pH\ 1.0} - (A_{520} - A_{700})_{pH\ 4.5}$

The difference in pigment absorption at 520 nm and 700 nm is proportional to the pigment concentration:

Monomeric anthocyanin pigment (mg/l) = (A x MW x DF x 1000)/(ε x 1)

where: MW = molecular weight (449.2 g/mol C-3-G); DF = dilution factor; 1= optical path (1 cm); ε = molar absorptivity (26900 l x mol^{-1} x cm^{-1} for C-3-G);

The results were expressed as cyanidin-3-glucoside equivalents/g fresh or dry weight (µg C-3-G/ g FW or DW).

Index of pigment degradation, polymerization or oxidation

The index for degradation of anthocyanins can be obtained by determination of the absorbance values of the sample treated with 0.68M $K_2S_2O_5$ (potassium metabisulphite) solution and distilled water. Monomeric anthocyanin pigments will combine with bisulphite to form a colorless sulfonic acid adduct while the mixtures of colored anthocyanins-tannins (i.e. polymerized compounds) will be resistant to bleaching. A volume of 2.8 ml from each diluted sample were mixed with 0.2 ml of $K_2S_2O_5$ solution or 0.2 ml of distilled water. The difference in absorbance at 420 nm, 520 nm and 700 nm in the bisulphite sample represented the polymeric color. The percentage of polymerized anthocyanins from color density in water serves as the degradation index.

Determination of antioxidant capacity by DPPH method

According to the method proposed by Marxen et al. (2007), 100 µl of diluted extract were mixtured with 2.25 ml methanol and 150 µl of 1.27 mM DPPH methanolic solution. In parallel, a control was made in which the sample was replaced with the extraction solvent. After 30 minutes of incubation at room temperature and in the dark, the absorbance at 515 nm was measured. The estimation of the antioxidant capacity was performed by calculating the differences between the control and the sample. A standard curve with Trolox (synthetic antioxidant analog of α-tocopherol) as antioxidant was used for antioxidant activity estimation.

Three repetitions of the same variant were performed, and the average of the values representing the antioxidant capacity was expressed in Trolox equivalents/ g fresh or dry weight (mM Trolox/ g FW or DW).

Statistical analysis

SPSS (Statistical Package for Social Sciences) was used for statistical analysis of results. T-test was performed and were considered statistically significant different the results with $p<0.05$.

RESULTS AND DISCUSSIONS

Callus growth, morphology and cell viability

The growth rates of callus cultures varied between subcultures (Figure 1), but the differences were not statistically significant for both callus lines. Higher growth rates for callus red line were registered, compared to the white one. Cultivation in this *in vitro* system ensured an average growth rate more than 10-fold mass increase, for both lines, with 12-fold increase for the white callus and 17-fold increase for red callus, referring to a growth period of 21 days. The percentage of dry weight was above 5% for the red line, more than the white line. For the red line the increase of the dry weight was associated with the increase of the growth rate whereas in the white callus an increase of the dry weight and the decrease of the growth rate was observed.

The macroscopical examination (Figure 2) and dissection, squash analyses and cell viability test highlighted the structure of both type of calli. The absence of light ensured the initiation of a white callus, homogeneous in terms of cell pigmentation and morphology. The presence of light led to the formation of a red callus, with a visible cellular stratification in relation to the gradient of light: a discontinuous basal layer with grey nuclei consisting mainly of senescent or nonviable cells; a continuous, white and dense median layer, composed of meristematic cells, engaged in cell divisions, which ensures the growth of the callus; a red upper layer, with homogenous pigmentation, consisting of specialized cells, stimulated by the presence of light which intensely synthesize the anthocyanin pigments that give its characteristic color.

The light microscopy analysis of squash specimens certified the proliferative potential of the two lines of callus, by the presence of the small, relatively round cells which were intensively involved in cellular division processes (Figure 3). Moreover, it also revealed a series of cellular morphological differences between the 2 lines. Thus, the white callus cells displayed a predominantly elongated, reniform shape, were linearly arranged and were unpigmented, but rich in cytoplasmic inclusions of starch granules (Figure 3a). On the other hand, the red callus cells displayed an oval or round shape, were smaller in size (indicating a more intense proliferative process), clustered and rich in red anthocyanin pigments (Figure 3b, 3c).

To assess the cell viability of callus cultures, we applied the TTC assay method, which highlights the level of respiratory processes in viable cells (Towill & Mazur, 1975). In both callus lines, a decrease in cell viability was evident for third subculture (Figure 4), but given the aforementioned observations, it is possible to reveal a phase of secondary metabolites overproduction, in which the percentage of meristematic cells was lower. Certainly, it is difficult to ensure an equal distribution of these two cell types, in order to obtain a callus culture that is both highly proliferative and productive in compounds of interest. In order to illustrate cell viability as accurately as possible, it is recommended to apply several tests, which could also detect metabolic dormant cells, such as the method coupling TTC with Evans Blue (Busso et al., 2015).

These dynamic phases of callus growth, in which the cells that divide are predominant, followed by the specialization phase with the accumulation of metabolites and the declining phase characterized by the decrease in the number of viable cells are characteristic cyclic phenomena in callus cultures.

Evaluation of the polyphenols, flavonoids production and antioxidant capacity in the red and white callus line

The first analyses of the methanolic extracts revealed that the red callus line had a total polyphenol content much higher than the white line (Figure 5), measuring 2.874 mg GAE/g FW.

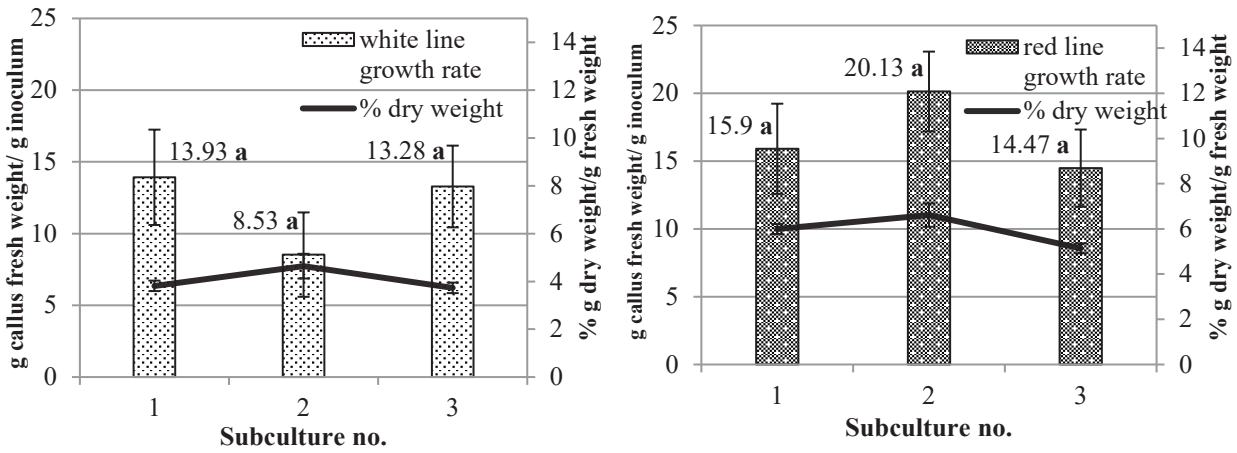

Figure 1. Growth dynamics in white and red lines of strawberry callus cultures (the average followed by the same letter represent unsignificant statistical differences between samples)

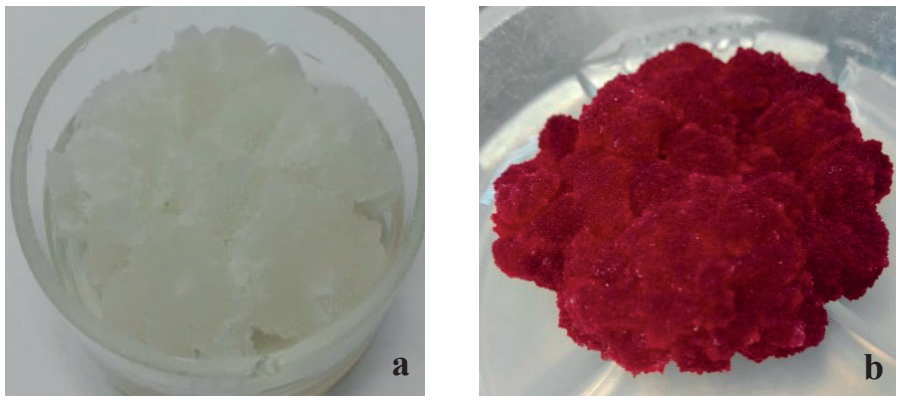

Figure 2. Macroscopical aspect of white (a) and red (b) strawberry callus

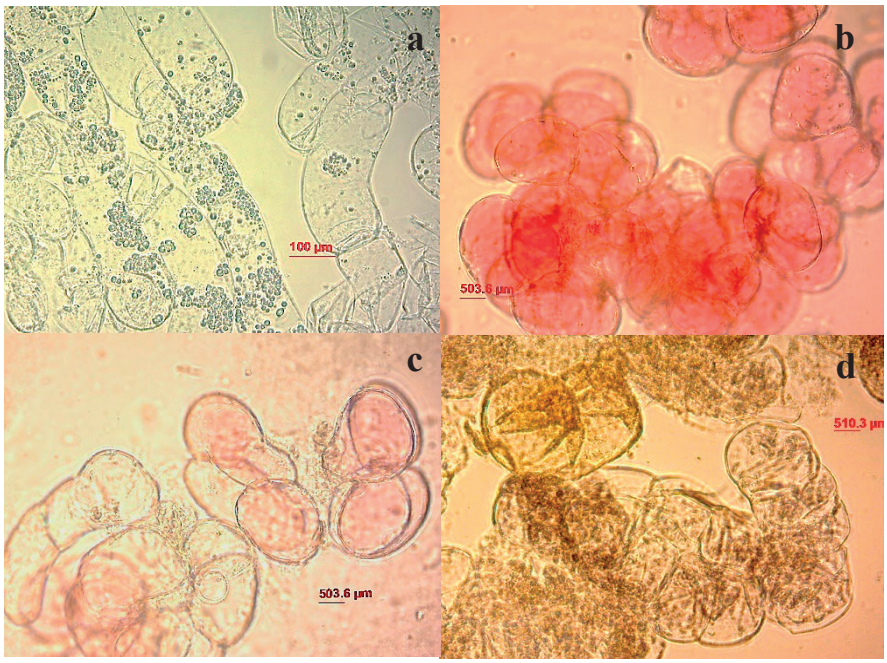

Figure 3. Squash analyses of white strawberry callus with elongated cells rich in starch granules (a) and red strawberry callus from upper layer overlapping red cells with round-shaped (b), the middle layer with small, isodiametric cells in division together with cells containing red pigment (c) and basal layer with clustered cells in grey nuclei (d)

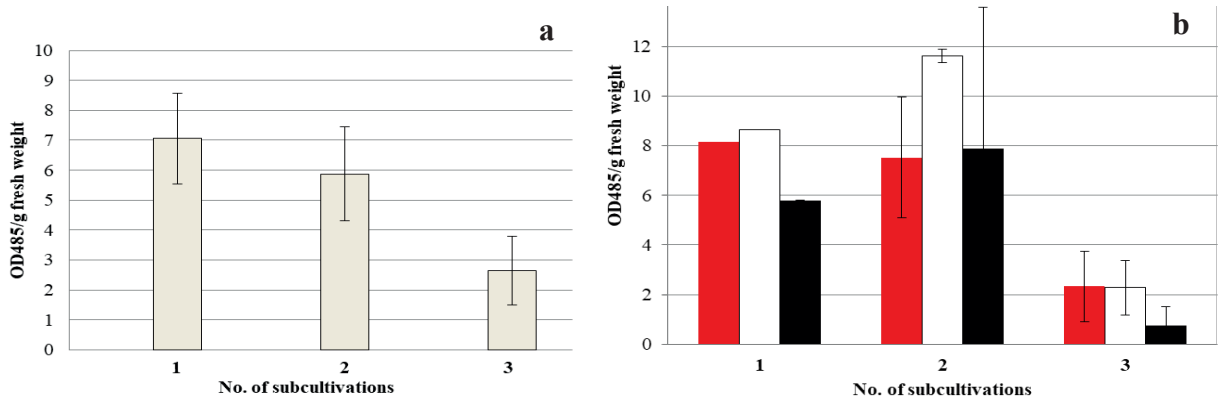

Figure 4. Evaluation of cell viability in white callus line (a) and red callus line, based on stratification pattern: upper layer-red columns, middle layer- white columns and basal layer-black columns (b)

This value was significantly higher than polyphenol content in strawberry fruits, as reported by Nowicka et al. (2019), who analysed the total polyphenol content in the fruits of 90 varieties of *Fragaria x ananassa* Duch., during two seasons and determined a total polyphenol concentration mean of 73.2 mg/100 g.

Polyphenols are compounds found in many plant species, they exert numerous beneficial effects for human health, that is why there is a growing interest in developing methods to ensure their large-scale production.

The presence of flavonoids was reported only in the red callus culture, at a concentration of 4.57 mg RUE/g FW (Figure 5). The red callus extract had a higher flavonoids concentration compared to strawberry fruits. The flavonoids concentrations in fruits of 90 varieties of *Fragaria x ananassa* Duch were reported between 5.31 - 5.47 mg/100 g (Nowicka et al., 2019).

The highest antioxidant activity was recorded in the methanol extract of red callus (Figure 5), with a value of 18.366 mM Trolox/g FW, which is mainly related to polyphenol and flavonoids content.

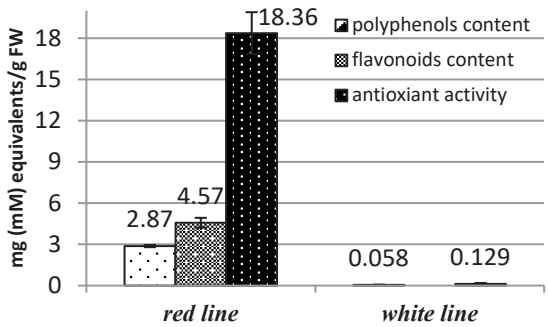

Figure 5. Comparative evaluation of analysed biochemical parameters in methanol extracts

Several studies correlate the antioxidant activity of *Fragaria x ananassa* Duch plants with the concentrations of polyphenolic compounds, especially with the amount of tannins (Nowicka et al., 2019) and anthocyanin pigments (Chaves et al., 2017). Comparing the antioxidant activity of the fruits of 90 varieties of *Fragaria x ananassa* Duch. by applying the DPPH method expressed as Trolox equivalents, Nowicka et al. (2019) detected an antioxidant activity between 751.57- 765.06 µmol/100 g. It is evidently that red callus line was highly successful and our *in vitro* cultures synthetized high concentrations of antioxidant compounds.

Metabolic content of red callus line using different type of solvent extraction

In order to highlight the potential of the red callus to synthetize polyphenols, flavonoids and anthocyanins, several extractants were tested. The results of solvent extraction efficiency ranked acidified ethanol as the best extractant for total polyphenols and anthocyanin pigments (Figure 6A, 6C), while methanol extraction ensured the highest concentration of flavonoids (Figure 6B). In fact, due to acid hydrolysis of the HCl the number of the exposed -OH groups which are assay with Folin-Ciocalteu reagent increased. In response, more hydroxyl groups released involve a higher antioxidant capacity. We used the acidic extraction to highlight the anthocyanin charge, however this extract does not really reflect the composition of red calli.

Acid hydrolysis releases monomers from polymeric structures and therefore increases the monomeric phenols and anthocyanins concentration which enhances antioxidant capacity of the extract (Figure 6D). Besides,

acidification of the extraction medium can have positive implications in preserving the extracts. Thus, there is a decrease in the oxidation index of anthocyanins from 70% in water to 16.134% in acidified ethanolic extract for red strawberry calli (Figure 6C).

The differences between acidified ethanol and methanol extraction are not significant and taking into account the effects of acid hydrolysis on the molecular composition of the extracts, the methanol would be the most suitable for the extraction of polyphenolic and flavonoid compounds.

Dynamic of metabolite accumulation in red callus successive subcultures

The dynamic of metabolites accumulation was investigated only for red callus line, because in the white callus line the values of polyphenols content and antioxidant capacity were very low. The extraction of metabolites was accomplished in the ethanol and acidified ethanol, avoiding the methanol toxicity in extracts subsequently exploited for the pharmaceutical purposes.

The variation pattern observed after three successive subculturing was the same for all analysed biochemical parameters: polyphenolic and flavonoids content, monomeric anthocyanins concentration and antioxidant capacity. A decrease in all the monitored parameters after the 2nd passage and a tendency to return to the initial values after the 3rd passage were observed (Figure 7). Metabolite accumulation varied inversely proportional with biomass accumulation (Figure 1). Thus, growth rate was the highest and the metabolite accumulation was lower in the second subculture while after the first and third passages growth rates were lower but biosynthesis of secondary metabolites was actively increasing. The squash analyses showed that the callus cellular composition of (Figure 3) comprised of two cell types: meristematic cells, responsible for callus growth and specialized cells, ensured the synthesis of secondary metabolites. In the second subculture, characterized by active callus growth, the meristematic cells predominated, while in the first and third subculture the specialized cells became majority and the accumulation of secondary metabolites increased. Cellular growth and metabolism can be represented as phase-shifted sinusoidal curves, so the maximum growth phase can be correlated with a minimum of the metabolic synthesis phase and vice versa, the ratios between them changing all the time (Lindsey and Yeoman, 1983).

The biochemical parameters analysed for each subculture were correlated with three exceptions. In the first subculture, the concentration of flavonoids in the acidified ethanolic extract was lower than in the ethanolic extraction (Figure 7B). Also, a significant increase in the concentration of all metabolites in correlation with the antioxidant capacity was observed in the acidified ethanolic extract obtained from the third subculture (Figure 7). In contrast, all parameters analysed in the ethanolic extract presented values below those registered for the first subculture. These findings suggest that metabolite composition changed during successive subcultures and acidification releases more monomeric compounds from polymeric structures. These qualitative and quantitative changes could be associated with decrease of cell viability (Figure 4b) and also with predominance of senescence and cell death phenomena from the third subculture.

The concentration of anthocyanin pigments in acidified extract from the second subculture tends to exceeded the values corresponding to the first subculture (Figure 7C). However, the anthocyanin concentrations in ethanolic extract maintained the variation pattern with the lowest value for the second subculture. This proves that metabolite composition, in this case of anthocyanin pigments, changes from one subculture to another. Also, a large difference in monomeric anthocyanin concentrations between the two types of extraction indicates anthocyanins polymerization in the second subculture.

The determination of the degradation index (polymerization) of anthocyanins sustains our supposition (Figure 8). The highest percentage of polymerized anthocyanins was recorded in the 2nd subculture.

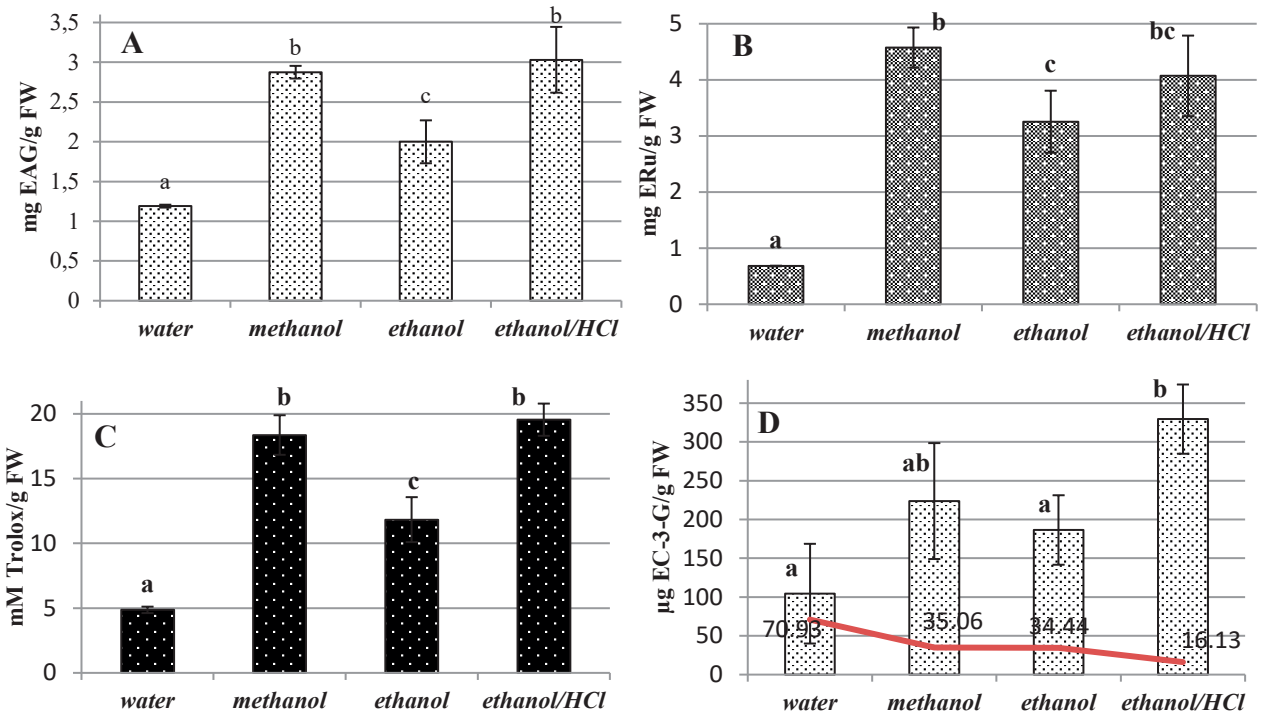

Figure 6. Comparative evaluation of red callus total phenolic content (A), flavonoids content (B), anthocyanins concentration (C) and antioxidant capacity (D) in different extractants. The different letters above the columns represent statistically significant differences between the samples, with $p<0.05$)

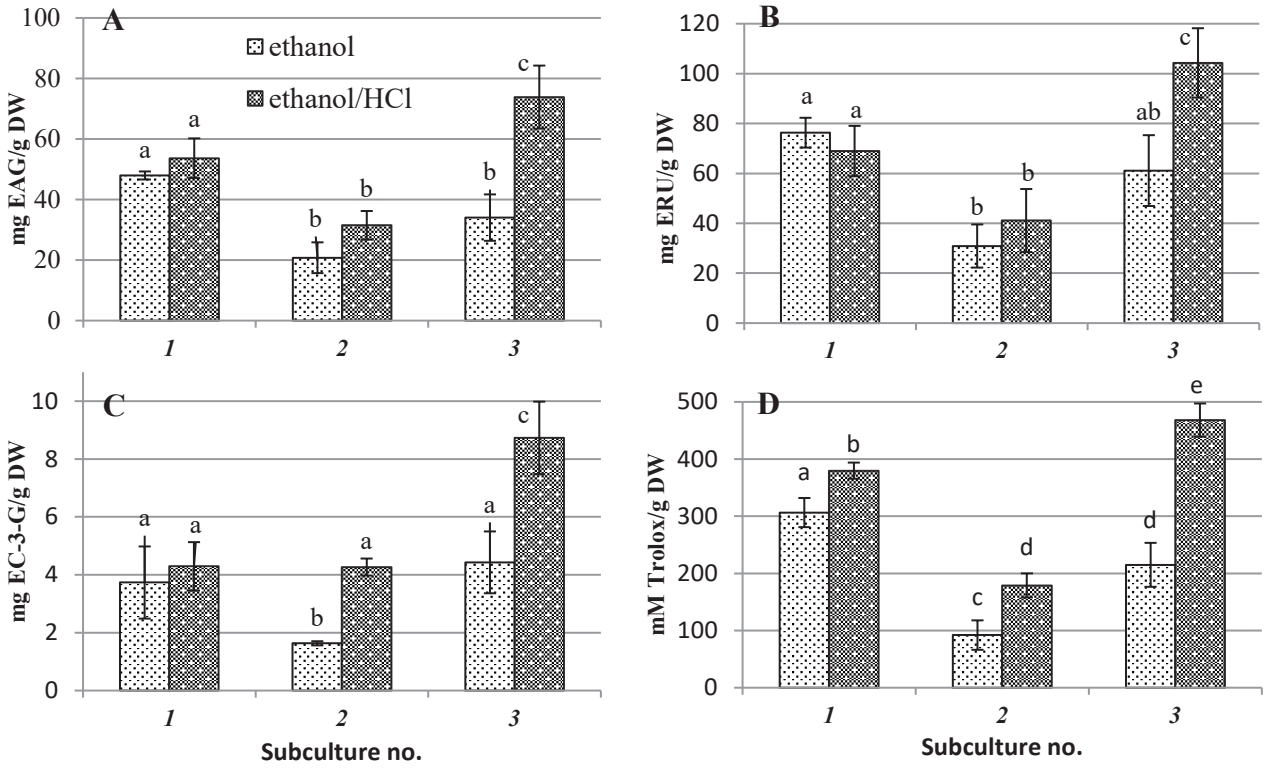

Figure 7. Dynamic changes of metabolite accumulation in red callus line in three successive subcultures: total phenolic content (A), flavonoids content (B), anthocyanins concentration (C) and antioxidant capacity (D). The different letters above the columns represent statistically significant differences between the samples, with $p<0.05$

This high polymerisation index correlated with the lower values of the monomeric anthocyanin concentration suggest that there is no decrease in the anthocyanin's synthesis, rather their polymerization and complexation with tannins.

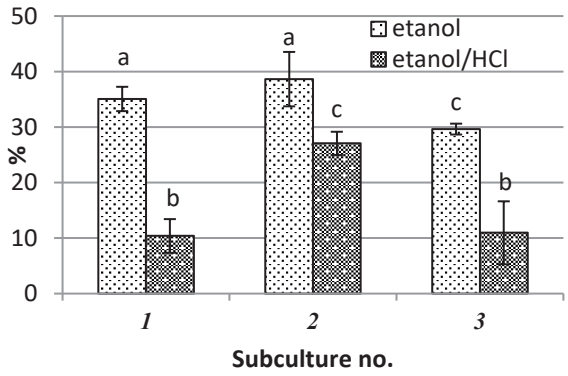

Figure 8. Evaluation of degradation index in red callus extracts in three successive subcultures

The biotechnological performance monitoring at the end of the three successive subculturing periods showed an increased accumulation of metabolites associated with a high growth rate and cell viability for the first subculture. This was followed by an increase in the growth rate of callus associated with increased cell viability, especially in the second layer (Figure 4b), but also a decrease in the accumulation of metabolites, at the end of the second subculturing period. In the third subculture, a restoration of the metabolite accumulation accompanied by decrease in callus growth and cell viability was noticed. These findings suggest that our callus is a heterogeneous system with cells in various stages of development as shown by the analysed squash preparations. Depending on the proportion of cells in a certain stage of development, respectively meristematic, specialized or senescent, the callus presented increased and decreased biomass curves and metabolites accumulation. Considering these cyclical changes, the red strawberry callus culture remains a highly-performance biotechnological system for biomass production in a short period of time with secondary metabolites in higher concentrations towards classical crops production.

CONCLUSIONS

The present study described a biotechnological potent *in vitro* system in *Fragaria x ananassa* Duch., which comprised of biomass production with valuable metabolites synthesis. The absence/presence of the light determined the isolation of two morphologically and functionally distinct callus cell lines. The pigmented (red) strawberry callus culture line displayed a superior proliferative capacity and a much more complex metabolite content. Finally, our results will facilitate further development of methods for mass production of interest compounds, which are found in significantly lower concentrations in plants or imply high costs if produced by chemical synthesis.

ACKNOWLEDGEMENTS

This research work was partially financed by project number 5PCCDI/2018, within PNCDI III and growth dynamic experiments by Romanian Academy in project number RO1567-IBB06/2019 from the Institute of Biology Bucharest.

REFERENCES

Almeida, J. R., D'Amico, E., Preuss, A., Carbone, F., de Vos, C. R., Deiml, B., Mourgues, F., Perrotta, G., Fischer, T. C., Bovy, A. G., Martens, S., Rosati, C. (2007). Characterization of major enzymes and genes involved in flavonoid and proanthocyanidin biosynthesis during fruit development in strawberry (*Fragaria x ananassa*). *Archives of Biochemistry and Biophysics*, 465(1), 61-71.

Busso, C., Torres, Y., Ithurrart, L., Richards, J. H. (2015). The TTC-technique might not appropriately test the physiological stage of plant tissues. *Russian Journal of Plant Physiology*, 62(4), 551-556.

Chaves, V. C., Calvete, E., Reginatto, F. H. (2017). Quality properties and antioxidant activity of seven strawberry (*Fragaria x ananassa* Duch.) cultivars. *Scientia Horticulturae*, 225, 293-298.

Cogălniceanu, G. C., Mitoi, M. E., Ciocan A. G., Holobiuc, M. I., Maximilian, R. C., Helepciuc F. E., Moroșanu, A. M. Procedeu biotehnologic de inițiere și obținere de masă celulară proliferativă înalt producătoare de compuși bioactivi la *Fragaria X ananassa* Duch. (căpșun) și a extractului brut. OSIM Romania Patent Application A/00808 from 4.12.2020.

Giusti, M. M., Wrolstad, R. E. (2001). Characterization and measurement of anthocyanins by UV-visible spectroscopy. *Current Protocols in Food Analytical Chemistry*, 1, 1-13.

Lindsey, K., Yeoman, M. M. (1983). The relationship between growth rate, differentiation and alkaloid accumulation in cell cultures. *Journal of Experimental Botany*, 34(8), 1055-1065.

Marxen, K., Vanselow, K., Lippemeier, S., Hintze, R., Ruser, A., Hansen, U. P. (2007). Determination of DPPH radical oxidation caused by methanolic extracts of some microalgal species by linear regression analysis of spectrophotometric measurements. *Sensors*, 7(10), 2080-2095.

Mihailović, V., Matić, S., Mišić, D., Solujić, S., Stanić, S., Katanić, J., Stanković, N. (2013). Chemical composition, antioxidant and antigenotoxic activities of different fractions of *Gentiana asclepiadea* L. roots extract. *EXCLI Journal 2013*, 12, 807-823.

Murashige, T., Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15(3), 473-497.

Nowicka, A., Kucharska, A.Z., Sokół-Łętowska, A., Fecka, I. (2019). Comparison of polyphenol content and antioxidant capacity of strawberry fruit from 90 cultivars of *Fragaria x ananassa* Duch. *Food Chemistry*, 270, 32-46.

Schaart, J. G., Dubos, C., De La Fuente, I. R., Van Houwelingen, A. L. M., De Vos, R. C., Jonker, H. H., Xu. W., Routaboul, J., Lepiniec, L. C., Bovy, A. G. (2013). Identification and characterization of MYB-b HLH-WD 40 regulatory complexes controlling proanthocyanidin biosynthesis in strawberry (*Fragaria x ananassa*) fruits. *New Phytologist*, 197(2), 454-467.

Towill, L. E., Mazur, P. (1975). Studies on the reduction of 2, 3, 5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. *Canadian Journal of Botany*, 53(11), 1097-1102.

Yuan, H., Yu, H., Huang, T., Shen, X., Xia, J., Pang, F., Wang, J., Zhao, M. (2019). The complexity of the *Fragaria x ananassa* (octoploid) transcriptome by single-molecule long-read sequencing. *Horticulture Research*, 6(1), 46-56.

Zhang, J., Wang, X., Yu, O., Tang, J., Gu, X., Wan, X., Fang, C. (2010). Metabolic profiling of strawberry (*Fragaria x ananassa* Duch.) during fruit development and maturation. *Journal of Experimental Botany*, 62(3), 1103-1118.

Zhishen, J., Mengcheng, T., Jianming, W. (1999). The determination of flavonoid content in mulberry and their scavenging effects on superoxide radicals. *Food Chem*, 64, 555-559.