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EFFECT OF GAMMA IRRADIATION ON *Salvia officinalis* L. AND *Melissa officinalis* L. *IN VITRO* PLANTS

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Abstract

Salvia officinalis L. (sage) and Melissa officinalis L. (lemon balm) are two valuable medicinal plants from Lamiaceae family. Considering the therapeutic potential of sage and lemon balm extracts, there is currently great interest to increase the production of biological material and the synthesis of bioactive compounds by different methods. Both micropropagation and gamma irradiation represent efficient methods of stimulating the synthesis of bioactive compounds in plants. In order to produce biological material, it is important to establish the doses of gamma radiation that do not have a phytotoxic effect on plants. The aim of the present study was to evaluate the effect of different doses of gamma irradiation (100, 300, and 500 Gy) on sage and lemon balm by quantifying of some biochemical compounds (assimilatory pigments, soluble carbohydrates, and total polyphenols) in in vitro plants. The results obtained revealed that high doses of gamma radiation have phytotoxic effect on in vitro raised plants. However, micropropagation is an efficient method to produce high quality biological material, source for obtaining extracts with therapeutic potential.

Key words*: gamma radiation, in vitro plants, assimilatory pigments, soluble carbohydrates, total polyphenols.*

INTRODUCTION

Salvia officinalis L. (sage) and *Melissa officinalis* L. (lemon balm) are two valuable medicinal plants from *Lamiaceae* family. Sage and lemon balm extracts have various biological properties (antioxidant, antibacterial, antiviral, antifungal, antidiabetic, anti-inflammatory, sedative, spasmolytic, digestive, and cytotoxic properties) due to which they are used in the treatment of many diseases of the nervous, respiratory, digestive, cardio-vascular, and endocrine system (Shakeri et al., 2016; Vosoughi et al., 2018; El Euch et al., 2019).

Considering the therapeutic potential of extracts from these plants, there is currently a great interest to increase the production of biological material by different methods. Micropropagation is an alternative to conventional methods for the production of bioactive compounds from plants (Ravishankar & Ramachandra Rao, 2000; Grzegorczyk et al., 2005). *In vitro* culture has a number of advantages compared to classical multiplication methods: requires a reduced quantity of biological material for the initiation of *in vitro* cultures; is much faster than *in vivo*; ensures the production of free material of pathogens by the initial disinfection of the biological material and its cultivation under aseptic conditions; requires restricted space and is carried out throughout the year; does not require application of pesticides and herbicides; creates the possibility of tracking the production of bioactive compounds under controlled conditions, etc. (Debnarh et al., 2006).

Because the therapeutic potential of the plant extracts is given by bioactive compounds from their composition, current research aims to find different methods to stimulate the synthesis of these compounds. Gamma irradiation is one of the environmental stresses that improving characteristics of plants, produce changes in cellular metabolism stimulating the bioactive compounds synthesis (Charbaji & Nabulsi, 1999; Kovács & Keresztes, 2002; Kim et al., 2004; El-Beltagi et al., 2011; Mali et al., 2011).

Both micropropagation and gamma irradiation represent efficient methods of stimulating the synthesis of bioactive compounds in plants.

Therefore, in order to produce high quality biological material, source of extracts with therapeutic potential, it is important to establish the doses of gamma radiation that do not have a phytotoxic effect on plants.
In this context, the aim of the present study was to evaluate the effect of different doses of gamma irradiation on sage and lemon balm by quantifying of some biochemical compounds (assimilatory pigments, soluble carbohydrates, and total polyphenols) in *in vitro* raised plants.

MATERIALS AND METHODS

Plant material

Apexes and uninodal fragments sampled from actively growing shoots of *S. officinalis* and *M. officinalis* mother stock plants were cultured *in vitro* conditions, on MS medium (Murashige & Skoog, 1962) supplemented with 40 g/l glucose (as carbon source), 32 mg/l NaFeEDTA (as iron source) and 7 g/l agar. No growth regulators were used in the first phase of micropropagation (culture initiation). Subculturing was performed every four weeks, on a similar medium for sage and MS with 0.5 mg/l BAP (benzylaminopurine), for lemon balm. All cultures were transferred in a growth room with controlled conditions (temperature 25±1^{0}C, photoperiod 16 hours light and light intensity 3000-3500 lx).

Gamma irradiation

Two weeks after subculturing on fresh medium, *S. officinalis* and *M. officinalis in vitro* plants were acutely irradiated (0.9 Gy/s) at different average doses (100, 300, and 500 Gy) by using a self-contained, Co-60 research irradiator (GC-5000, B.R.I.T, India). Irradiation temperature, measured in air, varied from 28 to 31°C. All doses are expressed as absorbed dose in water. Dose uniformity ratio, defined as the ratio of maximum dose to the minimum one, was 1.318. Doses were evaluated by means of ethanol-chlorobenzene (ECB) dosimetry system with oscillometric read-out (ISO/ASTM 51538). The uncertainty of the absorbed dose is 3.3% (one standard deviation).

Extraction procedure

Some biochemical compounds (soluble carbohydrates and total phenolic content) were analyzed after the extraction procedure using a MAS-II microwave synthesis and extraction system (Hanon Instruments, Shanghai, China). The fresh plant material (*in vitro* raised plants) was triturated by gradually adding extraction solvent (ethanol 70%, v/v). The plant material:solvent ratio was 1:10 (m/v). The microwave extraction was performed at 40°C for 10 min. The microwave power was controlled and maintained at 250W and magnetic stirring at 200 rpm. The plant extracts were then filtered.

Biochemical parameters evaluation

The chlorophyll and carotenoid pigments from plant material were extracted with 85% acetone (Holm, 1954). 0.1 - 0.2 g green material was cut into small pieces (2-3 mm), milled and mixed with 2-3 ml of 85% acetone. The obtained extract was filtered, and the volume of the extract was then brought to 25 ml, with 85% acetone. Absorbance measurements were read using a spectrophotometer (Thermo Scientific Biomate 5) at 440.5 nm, 644 nm, and 662 nm wavelengths. The content of chlorophyll *a*, chlorophyll *b*, and carotenoid pigments was calculated with the formulas proposed by Holm (1954) and expressed in mg/g of green substance.
Dosing of soluble carbohydrates from the plant material was carried out by a colorimetric method, with anthrone reagent (Pánczél & Eifert, 1960). 0.5 ml of the extract was mixed with 0.5 ml of distilled water and 2 ml of anthrone reagent. The obtained mixture was boiled for 10 minutes, after which it was cooled for 15 minutes. Absorbance was read at 620 nm using a spectrophotometer. The soluble carbohydrates content was expressed as mg/g of green substance.
The total phenolic content was spectrophotometrically evaluated using Folin-Ciocalteu reagent (Singleton & Rossi, 1965). 1 ml of alcoholic extract, 75 ml of distilled water, and 5 ml of Folin-Ciocalteu reagent were placed in a 100 ml flask. After 3 minutes, 10 ml of 20% sodium carbonate was added and filled with distilled water to the mark. The mixture was allowed to stand for 60 minutes at room temperature, in the dark. Thereafter, the absorbance was measured at 765 nm. Tannic acid was used as a standard and the results were

expressed as tannic acid equivalents/fresh weight (mg TAE/g sample).
Evaluation of biochemical parameters in the irradiated plantlets was performed 5 and 8 days after irradiation. Unirradiated plants were treated similarly, constituting the control.

Statistical analysis
The experimental measurements were done in triplicates for each sample. Statistical interpretation of the data was done using SPSS 10 for Windows program. Differences between variants (irradiation doses) compared to the control (non-irradiated) were analyzed with One Way ANOVA - LSD, considering to be significant at $P < 0.05$.

RESULTS AND DISCUSSIONS

The survival capacity of the irradiated plants decreased with increasing the time interval from irradiation and the gamma radiation dose. Phytotoxic effect of the treatment was more pronounced at *M. officinalis* compared to *S. officinalis*, the high doses of gamma radiation (300 and 500 Gy) producing necrosis of *in vitro* plants, which led to the loss of their viability (Figure 1). These results are consistent with those reported by Tangpong et al. (2009), the survival rate of *Anubias congensis* N.E. Brown *in vitro* plants decreasing with increasing gamma radiation dose.
Phytotoxic effect of gamma radiation on explants cultured on sterile medium can be attributed to the reduction in the amount of endogenous growth regulators due to their disturbance or lack of synthesis due to gamma irradiation (Lea, 1946). Also, the activity of exogenous hormones in culture media can be affected by gamma radiation, thereby producing modifications in terms of morphogenetic response of the explants (La Vina et al., 2001).
Before the appearance of effects produced by irradiation at physiological level, they can be detected at metabolic level by quantification of some biochemical parameters (Hasbullah et al., 2012). In the present study, the effect of different doses of gamma irradiation (100, 300 and 500 Gy) on sage and lemon balm was evaluated by quantifying of assimilatory pigments, soluble carbohydrates, and total polyphenols in *in vitro* plants.

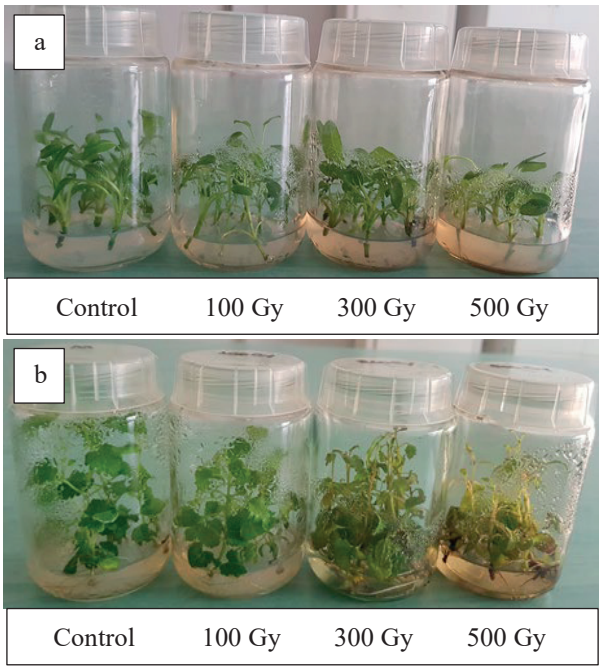

Figure 1. Sage (a) and lemon balm (b) *in vitro* plants, 8 days after the treatment with gamma radiation

Effect of gamma irradiation on the assimilatory pigments content
Photosynthetic pigments are the most sensitive compounds in plants to gamma radiation (Kulandaivelu & Noorudeen, 1983). They can be destroyed by high doses of gamma rays (Strid et al., 1990). For example, high doses of gamma radiation inhibited synthesis of chlorophyll in wheat and pea (Strid et al., 1990; Kovács & Keresztes, 2002). Instead, chlorophyll pigments are practically insensitive at low doses of gamma radiation (Kim et al., 2004).
However, there are studies on tomato, wheat, red pepper, and maize in which low doses of gamma radiation stimulated chlorophyll synthesis. This may be due to the recovery of the plants after exposure to gamma radiation (Zeerak et al., 1994; Osama, 2002; Kim et al., 2004; Ferreira-Castro et al., 2007).
In the present study, the evaluation of chlorophyll pigments content in *S. officinalis* and *M. officinalis in vitro* plants revealed different results, depending on the time interval from the irradiation to their quantification and the radiation dose. After 5 days from irradiation, in all experimental variants, the chlorophyll *a* content in sage and lemon balm did not register significant differences compared to the control at $P<0.05$. In both species studied, 8 days after irradiation, the high doses of gamma radiation inhibited synthesis of chlorophyll *a*, its content

significantly decreasing compared to the control in plants irradiated at 300 and 500 Gy (Figure 2).

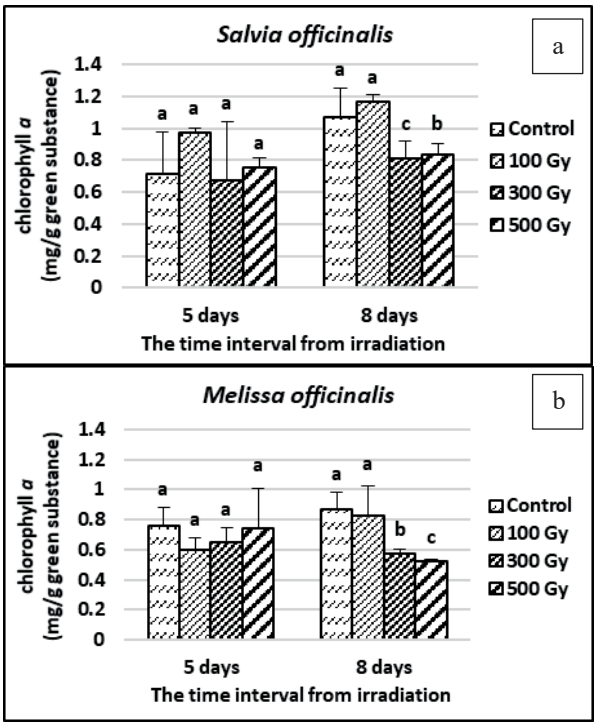

Figure 2. Influence of irradiation dose and time interval from irradiation on the chlorophyll *a* content in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

Regarding chlorophyll *b*, in both species, 5 days after irradiation, its content showed no significant differences compared to the control. In the case of *S. officinalis*, there were not recorded significant differences compared to the control, in term of chlorophyll *b* content, not even at 8 days after irradiation. Instead, at *M. officinalis*, 8 days after irradiation, the chlorophyll *b* content decreased significantly compared to the control in plants irradiated at 300 and 500 Gy (Figure 3). These results are consistent with those reported by Borzouei et al. (2010), who at higher doses of radiation (300 Gy) achieved a decrease in the total chlorophyll *a* and *b* content. The decrease of chlorophyll *b* content is due to a selective destruction of chlorophyll *b* biosynthesis or degradation of chlorophyll *b* precursors (Kiong et al., 2008).

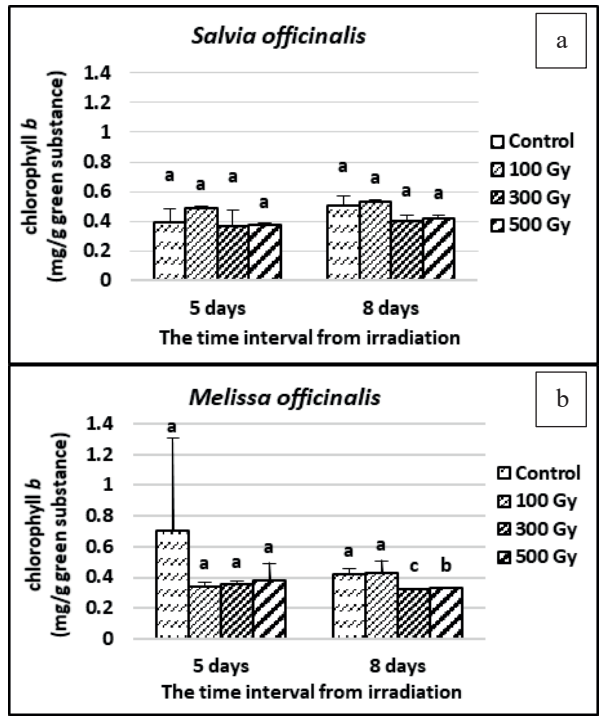

Figure 3. Influence of irradiation dose and time interval from irradiation on the chlorophyll *b* content in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

The main index of chlorophyll assimilation in plants, namely the ratio between chlorophyll *a* and chlorophyll *b*, was calculated. In the present study, this indicator shows the predominance of chlorophyll *a* in plants analyzed (Figure 4). Similar results were reported by Borzouei et al. (2010) for wheat irradiated at 100 Gy, which obtained an increase of chlorophyll *a* content compared to chlorophyll *b*. An important role in effects produced by radiations and release of free radicals is played by carotenoid pigments (Fukuzawa et al., 1998).

In the present study, 5 days after the treatment application, gamma radiation did not significantly influence the carotenoid pigments content in sage and lemon balm *in vitro* plants.

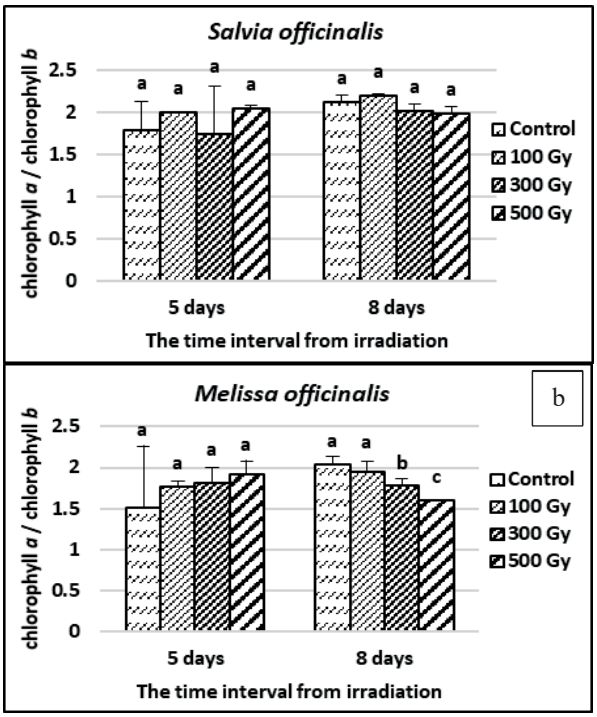

Figure 4. Influence of irradiation dose and time interval from irradiation on the chlorophyll *a*/*b* ratio in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

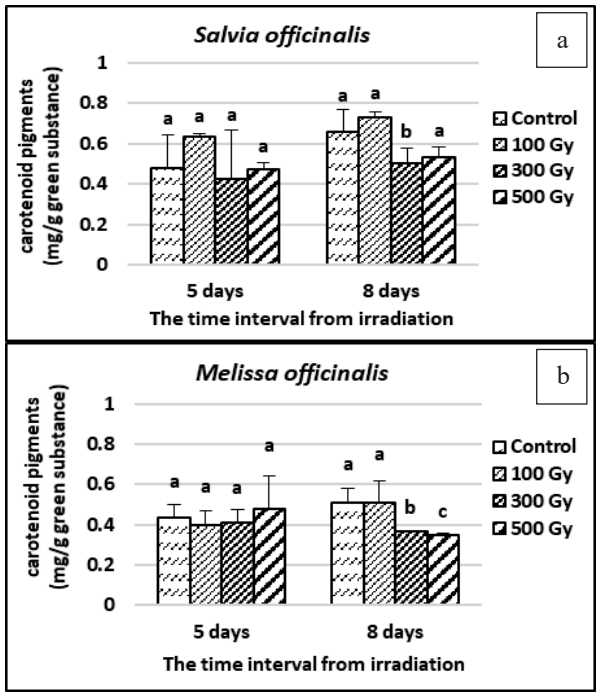

Figure 5. Influence of irradiation dose and time interval from irradiation on the carotenoid pigments content in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

After 8 days from irradiation, the carotenoid pigments content significantly decreased compared to the control in sage irradiated at 300 Gy and in lemon balm irradiated at 300 and 500 Gy (Figure 5). The results are in contradiction with those reported by Kim et al. (2004) who obtained increased amounts of carotenoid pigments with irradiation doses.

However, there are more studies in which the total content of chlorophyll and carotenoid pigments has been reduced in gamma irradiated plants (Borzouei et al., 2010; Pawar et al., 2010). These contradictory results may be due to the different sensitivity to gamma radiation of different types of biological material analyzed.

Effect of gamma irradiation on the soluble carbohydrates content

Research conducted over the time has shown that low doses of gamma irradiation stimulate the synthesis of soluble carbohydrates, the primary products of photosynthesis, while high doses have an inhibitory effect. For example, in the case of green microalgae the carbohydrates concentration decreased with increasing gamma irradiation dose (Farhi et al., 2008; Choi et al., 2014). This phenomenon can be attributed to the repairing mechanisms that require energy for functioning by burning storage compounds for ATP production.

In the present study, 5 days after irradiation, the soluble carbohydrates content decreased significantly compared to the control only in lemon balm irradiated at 300 and 500 Gy. These results can be explained by the fact that *M. officinalis* is more sensitive to high doses of gamma radiation than *S. officinalis*, carbohydrates being used as a source of cellular energy and consumed in higher quantities under stress conditions, in our case gamma irradiation. At 8 days after gamma irradiation, dosing of the soluble carbohydrates in *S. officinalis* and *M. officinalis in vitro* plants did not reveal significant differences compared to the control, regardless of the irradiation dose (Figure 6). The fact that, 8 days after irradiation, the soluble carbohydrates content in lemon balm irradiated to high doses did not reveal significant differences compared to the control can be a consequence of plant recovery after exposure to gamma radiation treatment.

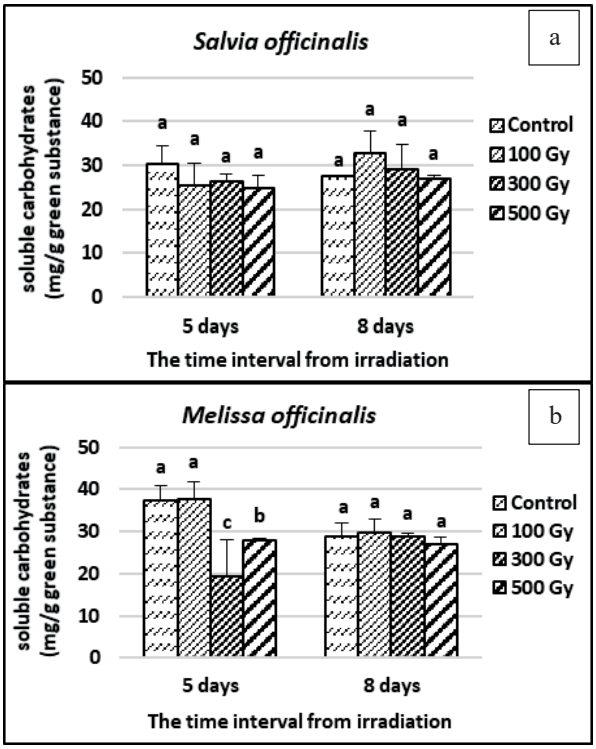

Figure 6. Influence of irradiation dose and time interval from irradiation on the soluble carbohydrates content in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

Effect of gamma irradiation on the total polyphenols content

In addition to the primary metabolites, with a major role in maintaining the viability of the plant are synthesized a number of compounds which belong to the secondary metabolism. From these, polyphenols are known to be a class of compounds that are related to the plant`s response to a stress factor, in our case gamma radiation.

In the present study, quantification of the total polyphenols content in *S. officinalis in vitro* plants revealed its increase in experimental variants compared to the control, but the increase was not significant. At *M. officinalis*, 5 days after treatment, irradiation at 100 Gy induced a significant increase of total polyphenols content compared to the control, while irradiation at 300 and 500 Gy did not show significant differences. Instead, 8 days after gamma irradiation, the evaluated indicator increased significantly compared to the control in all experimental variants (Figure 7).

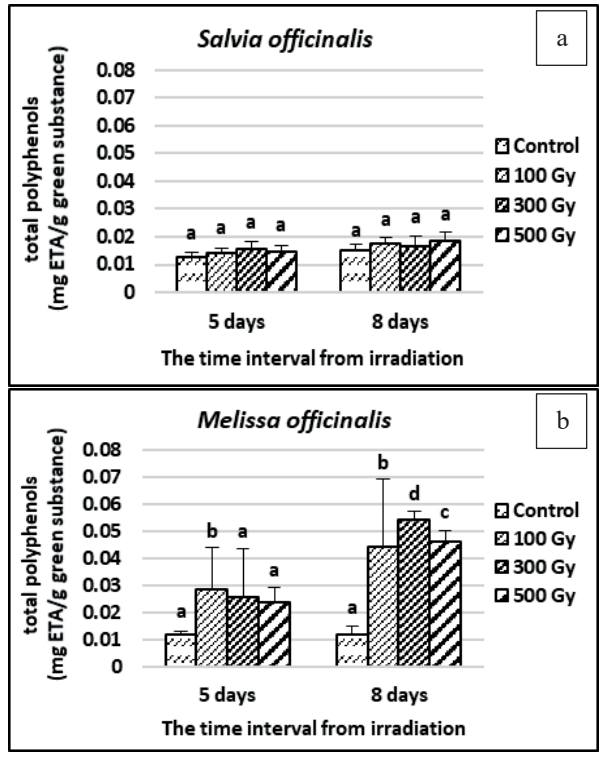

Figure 7. Influence of irradiation dose and time interval from irradiation on the total polyphenols content in *S. officinalis* (a) and *M. officinalis* (b) *in vitro* plants. The mean values are accompanied by their corresponding standard deviations; letters indicate significance of the differences compared to the control at P<0.05

The impact of gamma radiation on sage and lemon balm *in vitro* plants was expressed by higher values of the total polyphenols content, recorded 5 and 8 days after irradiation, in both genotypes studied, but the increase was not significant in all experimental variants. The more or less significant increase of the total polyphenols content in irradiated plants is due to the different sensitivity to gamma irradiation of the two species studied. However, increasing of the total polyphenols content in sage and lemon balm irradiated plants confirms the role of these molecules in the plant defense mechanism against stress factors. Increasing the phenolic compounds content by gamma irradiation can be attributed to the degradation of phenolic compounds with high molecular weight and the release of simple phenolic compounds with lower molecular weight (Harrison & Were, 2007; Kumari et al., 2009).

The results are consistent with several studies showing the stimulatory effect of gamma irradiation on the total polyphenols content in plants (El-Beltagi et al., 2011; Mali et al., 2011). There are also studies in which the phenolic compounds content of the vegetal material has been diminished by gamma irradiation (Bhat & Sridhar, 2008; Alothman et al., 2009). These contradictory results may be due to the sample type, the composition of the phenolic content, the extraction solvent, the extraction method, the gamma irradiation dose, etc. (Khattak, 2008; De Toledo et al., 2007).

In general, the effects produced by gamma irradiation in plants depend on species, genotype, growth stage, size, and type of explants (Banerji & Datta, 1992). Therefore, it is difficult to compare the results with the literature because of the heterogeneity both in the radiation doses used, and the biological material analyzed.

CONCLUSIONS

High doses of gamma radiation had phytotoxic effect on *S. officinalis* and *M. officinalis in vitro* plants. Lemon balm was more sensitive to irradiation compared to sage, the doses of 300 and 500 Gy produced, 8 days after irradiation, necrosis of plants which led to the loss of their viability. However, micropropagation is an efficient method to produce high quality biological material, source for obtaining extracts with therapeutic potential.

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