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ORIGINAL RESEARCH PAPER

COMPARATIVE STUDY OF PIGMENTS ON REGENERANTS FROM *IN VITRO* AND SPONTANEOUS FLORA OF *THYMUS sp.*

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Abstract: This paper presents the quantitative determination of pigments extract of *Thymus sp.* from *in vitro* culture and spontaneous flora. For *in vitro* culture were used phytohormones (indoleacetic acid-IAA, indole-3-butyric acid-IBA and benzylaminopurine-BAP) in order to obtain a high yield in development of *Thymus sp.* plants. The source of spontaneous flora of *Thymus sp.* was Bacau (Magura), Romania. The biochemical investigations realised on *Thymus sp*. using Thin Layer Chromatography and the UV-Vis spectrophotometry showed that the production of assimilating pigments is influenced by growth and lighting conditions of plants.

Keywords: *hormones, medicinal applications, pigments analyze, Soxhlet extraction, Thymus sp*

INTRODUCTION

It is well-know from the oldies time, that plants from spontaneous flora are a rich source of therapeutically active compounds [1]. These compounds can be antioxidants, dyes, vitamins, amino acids and other substances of particular importance to human life [2]. An important role in therapeutically application is due by assimilating pigments from different plants. Assimilating pigments of plants are framed based on their structure particularities in 3 groups: chlorophylls pigments, carotenoids pigments and phycobilins pigments [3, 4]. Chlorophylls pigments are a specifically pigments of green plants with an important role in photosynthesis. They are represented by chlorophyll "a" (plants, algae, and cyanobacteria which photosynthesize), chlorophyll "b" (green algae and in plants) and chlorophyll "c" (photosynthetic members of the *Chromista* as well as the dinoflagellates). Carotenoids pigments cannot transfer sunlight energy directly but must pass their absorbed energy to chlorophyll. That is the reason they are also called accessory pigments. Phycobilins pigments are water-soluble pigments, and are found in the cytoplasm, or in the stroma of the chloroplast.

There is a great interest for high revaluation of bioactive compounds from different plant materials, perfecting separation and purification techniques from plants, for their use in cosmetics, food and biochemical industries [4]. Gradually, as the formation and development of the pharmaceutical industry have increased requests for production of pharmaceutical materials of plant.

Thymus sp. is a species of flowering plant in the mint family Lamiaceae. It is useful in the garden as groundcover, as an ingredient in cooking and as an herbal medicine. Studies about *Thymus sp.* demonstrate that this plant has the ability to be used in pharmaceutical industry were the extracts may have anti-inflammatory, antiseptic and antimycotic properties for dermatological usage [5 – 7]. Adapting plants to specific climatic conditions becomes to be a problem extensively studied and we are focused to start this study by *in vitro* culture of plants from spontaneous flora.

The aim of this article is to highlight the content of bioactive compounds (chlorophylls (chlorophyll a and chlorophyll b) and carotenes (carotenes and xanthophyll) pigments) from *in vitro* cultures versus spontaneous flora of *Thymus sp.* extracts.

MATERIALS AND METHODS

Preparation of *in vitro* cultures

The researches were realized using as biological material the selected seeds of *Thymus sp.* by SC SEM-LUCA SRL, Timisoara city, Romania for *in vitro* culture. The seeds were sterilized and inoculated on the nutritive Murashige and Skoog (MS) medium without hormones, in order to obtain plantlets, carried out in the laminar air flow cabinet, SPACE PBI [8]. The plants obtained from the seeds inoculated on Murashige-Skoog (MS) without hormones were used as a source of explants, represented by shoot apexes and nodes. These were inoculated into nutrient media supplemented with the phytohormones in different combinations and concentrations, presented in Table 1.

Table 1. *The phytohormones concentrations used for in vitro culture of Thymus sp.*

Medium	Concentration of phytohormones [mg·L^{-1}]		
	IAA	IBA	BAP
MS	-	-	-
A_2	2	-	-
BA_1	0.5	-	1
BA_2	1	-	1
BB_1	-	0.5	1

The inoculated pots were incubated in a growth chamber PBI SPACE 120 at a temperature of 22.5 °C, 16 hours photoperiod and 2,500 lux intensity of light.
All samples obtained in *in vitro* cultures are compared with plants from spontaneous flora - Bacau (Magura), in order to determine the content of pigments from each sample of *Thymus sp*. We also tested the morphogenetic reaction of each sample developed on nutritive variants of medium tested. Each experiment was performed in duplicate for more accuracy.

Determination of humidity

The determination of humidity was realized for biological material from *in vitro* culture and spontaneous flora (stems and leaves). This determination has been due by classical method with hot air using an oven Froilabo Air Perfomance. The temperature used was 105 °C for 3 hours. Comparatively, the determination of humidity was realized using a programmable domestic microwave oven with an intensity of 340 W (digital easy CIATRONIC - MWG 729) for 10 minutes, for each sample. When the sample has constant mass, the weight was noted. The humidity calculation was achieved with the formula (1):

$$Humidity\ (U)\ \% = (M_1\text{-}M_2)/M_1 \cdot 100 \quad (1)$$

where:
M_1 - weight of the sample before drying, [g]
M_2 - weight of the sample after drying, [g]

Extraction of pigments

The extraction was realized with Soxhlet extractor, using as solvent ethanol and water mixture in 7 : 3 ratio (v/v). The purity of ethanol solvent is 98 %. Dried vegetable material by microwave was used in this extraction. Six extraction cycles was used for each sample of dried material resulting 5 samples of pigment extracts.

Analyze of assimilating pigments

The first analyze of pigment extracts was realized by the Thin Layer Chromatography (TLC), followed by spectrophotometric analysis using a Spectrophotometer Beckmann UV-DU640. The TLC was realized with plastic sheets silica gel $60F_{254}$ pre-coated

(Merck) and has the aim to determinate retention factor. The solvent used for chromatography separation of pigments was ethanol 98 %.
Then, the absorbance of extracted pigments was spectrophotometrically measured at 470 nm, 646 nm and 663 nm wavelength. The UV-Vis spectrophotometric method helps us to highlight the chlorophylls, carotenoids and xanthophyll content in *Thymus sp*. According to McKinney-Arron relationship chlorophyll content was measured as [9]:

$$Chl\ a = 12.21 \cdot (A_{663}) - 2.81 \cdot (A_{646}) \quad (2)$$

$$Chl\ b = 20.13 \cdot (A_{646}) - 5.03 \cdot (A_{663}) \quad (3)$$

$$Chl_{total} = 17.32 \cdot (A_{646}) + 7.18 \cdot (A_{663}) \quad (4)$$

where:
Chl a - chlorophyll a [mg·L^{-1}]
Chl b - chlorophyll b [mg·L^{-1}]
Chl_{total} - total chlorophyll content [mg·L^{-1}]
A_{663} - sample absorbance at 663 nm
A_{646} - sample absorbance at 646 nm
For xanthophyll and carotenoids content was use the relationship [10]:

$$Carotenes\ and\ xanthophyll = [(1000\ A_{470}) - (3.27\ Chl\ a) - (1.04\ Chl\ b)] / 229 \quad (5)$$

where:
Chl a - chlorophyll a [mg·L^{-1}]
Chl b - chlorophyll b [mg·L^{-1}]
A_{470} - sample absorbance at 470 nm
Determination concerning the content of pigments was made on extract of stems and leaves of culture *in vitro Thymus sp*. versus stems and leaves of *Thymus sp*. from spontaneous flora.

RESULTS AND DISCUSSION

The morphogenetic response of explants at *Thymus sp.*

According to the researchers for *in vitro* culture we identify the nutritive medium to increase the yield on the multiplication of biological material [11].
Neoplantlets from seeds of *Thymus sp*. on MS medium without phitohormones has been developed after 30 days. The explants represented by apex and nodes, inoculated on nutritive medium adding phytohormones, have reacted 100 %.
The morphogenetic reaction and the intensity of its manifestation were depending on the interaction between internal and external factors: genotype of seeds, growing medium (particularly hormonal formula) and *in vitro* culture conditions.
The most important morphogenetic reaction was caulogenesis. Nod explants developed on nutritional medium has generated between 2 at 4 shoots / explant. In the case of apexes they grow up and the number of shoots regenerated was between 1 at 2 shoots /apex. The concentration of phytohormones in nutritive medium has influence on the development of secondary and tertiary shoots on nodes level of primary regenerates. That's why we have obtained an important quantity of biological material for each sample that is presented in originals photos (Figures 1 - 4).

Figure 1. *In vitro regenerated plants of Thymus sp. on A_2 medium*

Figure 2. *In vitro regenerated plants of Thymus sp. on BB_1 medium*

Figure 3. *In vitro regenerated plants of Thymus sp. on BA_2 medium*

Figure 4. *In vitro regenerated plants of Thymus sp. on BA_1 medium*

Determination of humidity

By calculating the humidity we have obtained the elevated amount of water from plants. In our study important water content exist in biological material developed on the A2 medium. This quantity of evaporate water it has been compared with other samples from *in vitro* culture, including spontaneous flora. In the case of drying method, the most efficient is the method using microwaves (Figure 5).

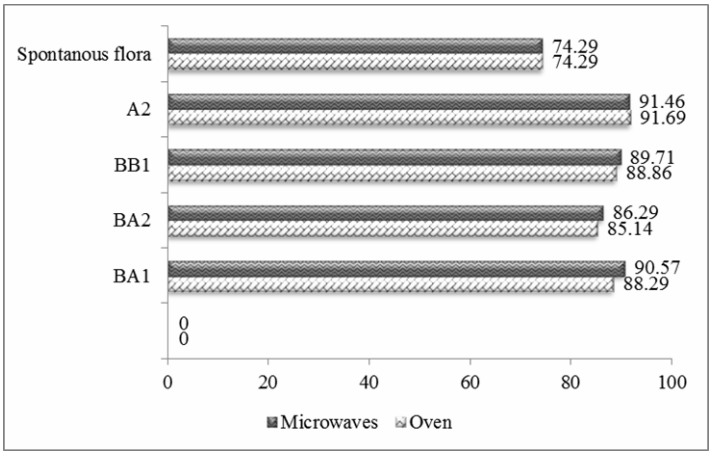


Figure 5. *Water percent for the two drying methods used for plant material samples*

The efficiency of microwaves drying was evidenced by the percentage of evaporated water and time to drying the plant material (10 minutes). For this reason we reserve the dried material by microwaves methods for the following analysis.

Analyze of assimilating pigments by TLC

Plant material of *Thymus sp.* obtained by microwaves drying was extract by Soxhlet extraction using ethanol-water solvent. We obtained 5 samples to be analyzed regarding pigments compounds (one sample from spontaneous flora - Bacau (Magura), and 4 samples from *in vitro* culture on nutritive medium with phytohormones: A2, BA1, BA2 and BB1). The preliminary analyze, TLC, confirm the presence of the most important pigments from plant material of *Thymus sp.* due to the *Rf* value (0.79). The use of ethanol for extraction and like mobile phase has economics and toxicity reasons, in the case of industrial transfer of this biotechnology.

Analyze of chlorophylls (chlorophyll a and chlorophyll b) and carotenes (carotenes and xanthophyll) pigments

Quantitative analysis of the plant pigments (chlorophylls, carotenoids and xanthophylls) was realized using UV-Vis spectrophotometric determination. The values of absorbance registered for each sample are presented in Table 2.

Table 2. *UV-Vis Absorbance for analyses samples*

Absorbance	BA1	A2	BA2	BB1	Spontaneous flora
A_{470}	0.2498	0.2794	0.312	0.1666	0.4193
A_{646}	0.1195	0.0904	0.0835	0.0808	0.1409
A_{663}	0.2203	0.1628	0.1305	0.1592	0.2012

With values from Table 2 and using McKinney-Arron relationship we calculate the assimilating pigments content for each analyzed samples presented in Table 3.

Table 3. *Content of specific pigments for analyzed samples [mg·L^{-1}]*

Assimilating pigments	Source of plant material				
	In vitro culture				Spontaneous flora
	BA1	A2	BA2	BB1	Bacau (Magura)
Chlorophyll a [mg·L^{-1}]	2.35	1.73	1.36	1.72	2.06
Chlorophyll b [mg·L^{-1}]	1.30	1.00	1.02	0.83	1.82
Chlorophylls Pigments [mg·L^{-1}]	3.65	2.73	2.38	2.54	3.89
Carotenes and xanthophyll pigments [mg·L^{-1}]	1.05	1.19	1.34	0.70	1.79

From experimental data, we can observe that plants from *in vitro* culture, specifically nutritive medium with BA1, have highest content of chlorophylls pigments. In this

medium the cytokinins concentration (1 mg·L^{-1}of benzylaminopurine) was double as against auxin (0.5 mg·L^{-1}of indole acetic acid).
In the case of plant material from spontaneous flora the content of chlorophylls pigments is 3.89 mg·L^{-1}, a value similar to that from BA1 nutritive medium (3.65 mg·L^{-1}), (Figure 6).

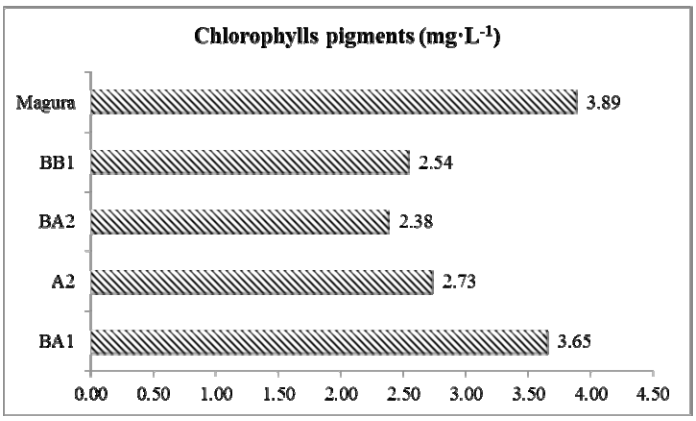
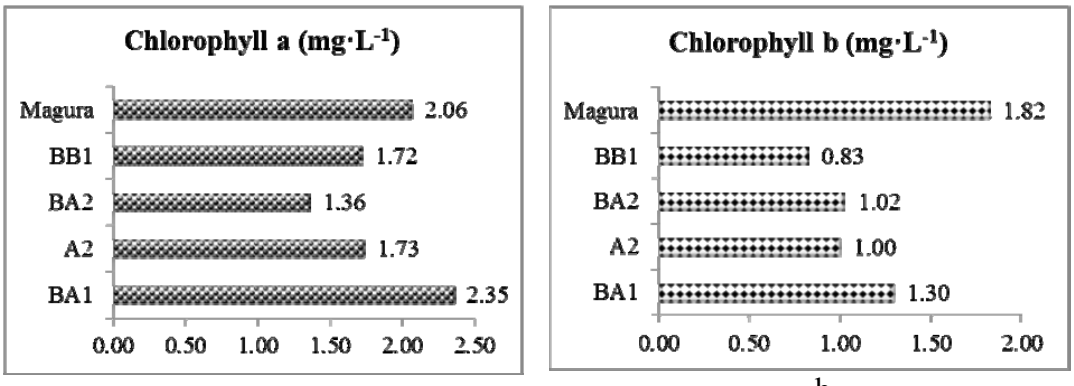


Figure 6. *Content of chlorophylls pigments for analysed samples [mg·L^{-1}]*

Based on researches of extraction and separation from green leaves of chlorophylls pigments, it was demonstrated that chlorophyll a and chlorophyll b are concomitantly presented. The ratio between chlorophyll a and chlorophyll b is always constant (3 : 1) with small variation in function of lighting conditions [12]. Our researches differences are due to the developing conditions, representative factor is the light (intensity and photoperiod). If in spontaneous flora, the plants growing is influenced by solar spectrum visible and invisible, with multiples variations, in the case of *in vitro* culture, the light is white, being controlled both intensity (2500 lx) as well as photoperiod (16 hours light/ 8 hours darkness), (Figure 7). Among chlorophylls pigments identifies in plant material analyzed, chlorophyll a is quantitatively representative, which confirmed the 3:1 ratio (chlorophyll a: chlorophyll b), (Figure 7).



a b

Figure 7. *Comparative content of chlorophylls pigments for analyzed samples [mg·L^{-1}] (a)- chlorophyll a content; b)-chlorophyll b content)*

Carotenes pigments are represented by carotenes (α-carotene, β-carotene and γ-carotene) and carotenols which has alcoholic groups, named xanthophyll (luteolin, fucoxanthin and violaxanthin) [13]. Carotenes pigments accompany chlorophyll, having the role of protection for chlorophylls and enzymes, especially for the action of ultraviolet radiations.

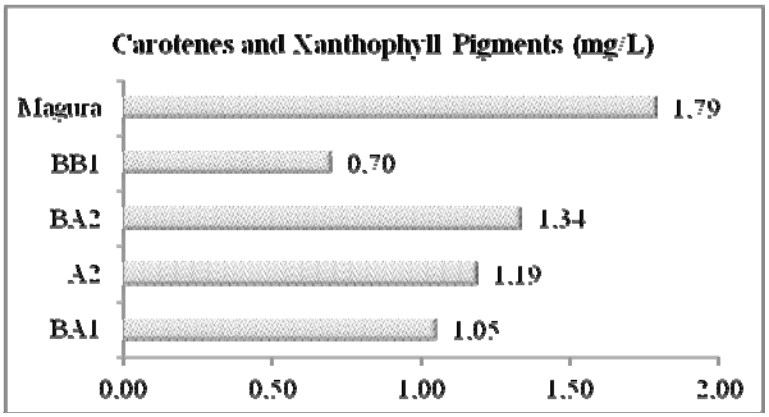


Figure 8. *Content of carotenes and xanthophyll pigments for analyzed samples [mg·L^{-1}]*

From Figure 8 can be observed a high content of carotenes pigments in the case of plants grown on nutritive medium BA2 (1.34 mg·L^{-1}), justifying the importance of indole acetic acid in medium culture. Combination of phytohormones like auxin-cytokinins stimulate the secretion of this category of pigments, such as the auxin-cytokinins ratio being essential in the determination and adjusting vital processes of explants [14, 15]. As in the case of chlorophylls pigments, a key role in their syntheses is light radiation. *Thymus sp.* plants from spontaneous flora, Bacau (Magura), are determinate to synthesis a high content of carotenes pigments following of adaptation in the growing medium.

CONCLUSIONS

The results of this research showed that multiplication *in vitro* of *Thymus sp.* on MS medium supplemented with phytohormones has a great success with the selected concentration in auxine and cytokinins stimulating the phenomenon of caulogenesis.
The content of water was high in the case of *Thymus sp.* plants from spontaneous flora, Bacau (Magura), by microwaves drying. This method is efficient to economizing time in the process of drying vegetal material.
The phytochemical analyses show an important content of assimilating pigments represented by chlorophylls and carotenes pigments. In the case of chlorophylls pigments from biological material, the 3 : 1 ratio of chlorophyll a: chlorophyll b is confirmed and the high content of pigments was identified for regenerative plant of BA1 nutritive medium. An important content of carotenes pigments was obtained in the case of *Thymus sp.* regenerated plant on BA2 nutritive medium.

The most evident result is that phytochemical analysis confirms that the production of pigments in analyzed samples is influenced by the growing and lighting conditions of plants.

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