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OBSERVATIONS ON THE MORPHOGENETIC REACTION OF *THYMUS VULGARIS* EXPLANTS CULTIVATED *IN VITRO*

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***Key words**: Thymus vulgaris, in vitro culture; caulogenesis, micropropagation*

INTRODUCTION

From ancient times until today, the leading provider of plant materials for medical purposes has been the spontaneous flora (Saleh Hosseinzadeh, 2015). Gradually, however, along with the formation and development of the pharmaceutical industry, there also increased the demand for raw materials to produce medicines of plant origin. The efficiency of harvesting some species of medicinal and aromatic plants may be reduced due to their low density in various natural basins and because they grow on inaccessible terrain, but also because of the influence of pedoclimatic conditions that are different, uncontrollable. Therefore, some species of medicinal plants began to be grown in farms and improved. Cultivating medicinal and aromatic plants imply certain limits, because not all species can be transferred from the spontaneous flora to cultures, due to their ecological demands, which, in some cases, cannot be reproduced in farms. Thus, the number of cultivated medicinal plants is much lower than the number of those required on the market. Also, transferring some medicinal plants to farms sometimes leads to loss or reduced biosynthesis of specific active principles, which requires serious efforts to improve them. For these reasons, efforts are made to develop technologies for micropropagation and production of seedling material, isolation of new genotypes by speculation of the somaclonal variability, obtaining active principles by unconventional routes (Ghiorghiță G, 1992).

Thymus vulgaris L. (thyme), belonging to the family Lamiaceae (Labiate), is a species originating in the Mediterranean region, having been grown as medicinal and spice plant since antiquity. In the spontaneous flora of Romania there were identified 15 species belonging to the genus *Thymus*, most of them with therapeutic value (Ciocârlan V., 2000). From this species, the aerial green or dry parts are used, *Tymi herba* containing volatile oil in different proportions (0.2-0.9%) (Hina Javed, 2013). The bioactive compounds identified in thyme plants are represented by flavonoids, thymol, carvacrol, eugenol, phenols, luteolin, tymol, terpenoids that cause such effects as antispasmodic, bactericide, antiseptic, antioxidant, having anthelmintic properties and having been lately recommended as substitute for cancer prevention agents (Monira et al., 2012; Ciulei I. et al., 1993; Fachini-Queiroz F. C. et al., 2012).

MATERIAL AND METHOD

The biological material used in our research consisted of *Thymus vulgaris* seeds, purchased from S.C. SEM-LUCA SRL, Timişoara, România.

The seeds were washed with 70% ethanol for 2 minutes, then subjected to sterilization, carried out in the laminar air flow cabinet, SPACE PBI. Sterilization consisted in treating the seeds with 5% chloramine T solution for 20 minutes, followed by rinsing them with sterile distilled water 3 times.

After sterilization, the seeds were inoculated on a basic Murashige and Skoog (MS) medium without hormones and the seeded pots were placed in the growth chamber *LEEC* at 22.5° C temperature, in the dark, for the purpose of seed germination. 4 days later, the culture vessels with the germinated seeds were transferred to the growth chamber *SANYO*, under light conditions (2500 lux) at a temperature of 22.5 ° C, photoperiod of 16 h. Throughout the period of incubation, the seeded containers were monitored periodically to remove any infections.

After about 4 weeks from *in vitro* cultivation, the plants obtained from the seeds inoculated on Murashige-Skoog (MS) without hormones were used as a source of explants (phytoinocul), represented by shoot apexes and nodes. These were inoculated into nutrient media supplemented with the phytohormones from the category of auxins and cytokinins in different combinations and concentrations (Table 1).

RESULTS AND DISCUSSIONS

The initiation of the *in vitro* culture at the species *Thymus vulgaris* did not raise problems in obtaining sterile plants. Thus, 90% of the inoculated seeds on the MS medium (Murashige-Skoog) without hormones yielded in vitro plants, which were subsequently the source for sterile explants. Also, we did not experience the phenomenon of crop infection, which shows that all the conditions of sterility were

ensured during the stages of handling the *in vitro* technology. The apex and node explants, inoculated on the different hormonal versions tested showed a good morphogenetic reaction, manifesting both organogenetic and callusogenetic phenomena. The best reaction highlighted was caulogenesis, followed by rhizogenesis and, to a small percentage by callusogenesis. The type of the morphogenetic reaction and the intensity of its manifestation varied depending on the interaction of internal and external factors that characterize this type of cultivation: genotype seeds, type of explant used, the physiological state of the in vitro plants used as a source of explants, the culture medium (in particular, the hormonal formula) and the *in vitro* cultivation conditions (Table 2).

Table 1. The hormonal versions used to highlight the morphogenetic reaction of *Tymus vulgaris* explants

No.	Hormonal formula	GROWTH REGULATORS (ml/l)						
		IAA	IBA	NAA	2,4-D	BBAP	KIN	ZEA
1	MS	-	-	-	-	-	-	-
2	IAA_2	2	-	-	-	-	-	-
3	IBA1	-	1	-	-	-	-	-
4	IBA_2	-	2	-	-	-	-	-
5	NAA_1	-	-	1	-	-	-	-
6	$2,4\text{-}D_2$	-	-	-	2	-	-	-
7	BA_1	0,5	-	-	-	1	-	-
8	BA_2	0,1	-	-	-	1	-	-
9	BB_1	-	0,5	-	-	1	-	-
10	BB_2	-	0,1	-	-	1	-	-
11	BN_1	-	-	0,5	-	1	-	-
12	BK_1	-	-	-	-	1	0,5	-
13	BZ					1		1

Table 2. The morphogenetic reaction of the species *Thymus vulgaris* L.

No.	Hormonal formula	Type of inoculated explant	The morphogenetic reaction manifested
1	MS	Seeds	Growth of neoplantlets from germinated seeds (+++).
2	IAA_2	Nodes, shoot apexes	Percentage of reacting explants - 62,5%. Regeneration of shoots (+); Rhizogenesis absent (-)
3	NAA_1	Nodes, shoot apexes	Percentage of reacting explants -92,8% Caulogenesis (+); Indirect caulogenesis (+); Rhizogenesis (+) from callus.
4	$2,4\text{-}D_2$	Nodes, shoot apexes	Percentage of reacting explants - 92,85% Caulogenesis (++). Rhizogenesis (++)
5	BB_1	Nodes, shoot apexes	Percentage of reacting explants 100%; Caulogenesis (+++); Rhizogenesis (++).
6	BB_2	Nodes, shoot apexes	Percentage of reacting explants 100%, Caulogenesis (++++); Rhizogenesis (++).
7	BA_1	Nodes, shoot apexes	Percentage of reacting explants 84,5%, Caulogenesis (++++); Rhizogenesis (++).
8	BA_2	Nodes, shoot apexes	Percentage of reacting explants 100%, Caulogenesis (+++); Rhizogenesis (+++).
9	BN_1	Nodes, shoot apexes	Percentage of reacting explants *50%;* Caulogenesis (+); Rhizogenesis (+);
10	BK_1	Nodes, shoot apexes	Percentage of reacting explants *100%*; Caulogenesis (++); Rhizogenesis (+)
11	BZ	Nodes, shoot apexes	Percentage of reacting explants 25,7%; Caulogenesis (+); Rhizogenesis (+++)

Legend: ++++ very good morphogenetic reaction, shoots with vigorous aspect;
+++ very good caulogenetic intensity, but the shoots had lower growth;
++ medium intensity morphogenetic reaction;
+ low intensity morphogenetic reaction.

The morphogenetic reaction of the apex and node explants inoculated on the nutritive versions tested in our research have shown that the presence of auxins along with cytokinins in the nutrient medium, at a ratio in favour of cytokinins, induced the phenomenon of regeneration of shoots, with a high efficiency. Thus, the hormonal combinations BA_1, BB_1, BB_2 and BA_2 determined the best caulogenetic response, the shoots showing rapid development. The presence, in nutrient media, only of auxins or cytokinins, regardless of concentration, did not produce a satisfactory evolution of phytoinoculi, the regeneration of new shoots occurring with little or no intensity. Keeping for 1.5 - 2 months the shoots regenerated from the initial explants in the same culture dish determined the formation of secondary and tertiary shoots from their nodes. The phenomenon of secondary caulogenesis was accompanied by the emergence of adventitious roots. To prevent adventitious root development, the biological material newly formed *in vitro* must be pricked out at intervals of 1 - 1,2 months.

On the hormonal version supplemented with 1 ml/l NAA, there was highlighted the callusogenesis phenomenon. The callus showed average proliferation intensity and organogenetic capacity, generating both shoots and roots. The transfer of callus fragments on the nutritional versions BA_1 and BB_1 led to the regeneration of new shoots, but the caulogenetic capacity was reduced.

Regarding the forming of roots, rhizogenesis was highlighted on all the nutritional versions tested, the intensity of this phenomenon varying depending on the concentration and combination of phytohormones in the nutrient media tested. Thus, the best rhizogenetic intensity (+++) was observed at the nutritional versions BA_2 and BZ.

Some thyme in vitro plants were accommodated to the *ex vitro* environment through hydroponic cultivation. The accommodation was carried out over 10 days, with no loss of plant material.

CONCLUSIONS

- Initiation of *in vitro* culture of the species *Thymus vulgaris* L. was performed without difficulties, using in vitro plants obtained from seeds, as a source of sterile explants.
- For the germination of seeds it is recommended to use the MS medium (Murashige Skoog) without the addition of phytohormones, the germination percentage being, on average, 90%.
- The main morphogenetic reaction highlighted at the shoot apexes and nodes explants was caulogenesis, followed by rhizogenesis, whose intensity varied depending on the concentration and combination of growth regulators in the nutrient media.
- Intense rhizogenesis was recorded for the nutritive versions BA_2 and BZ, and adventitious root formation was highlighted on the media BA_1, BA_2, BB_1 and BB_2, under conditions of extending the *in vitro* development period in the same culture dish.
- *Thyme* callus formation was highlighted in the medium version NAA_1, the callus showing low proliferation and organogenetic capacity.
- At the species *Tymus vulgaris* the media BA_1 and BB_2 proved to be the most favourable to the regeneration of shoots, followed by the versions BA_2 and BB_1, where the shooting phenomenon was intense, but the shoots were frail, with smaller leaves and thinner stems.
- Accommodation of the in vitro plants to the *ex vitro* environment was achieved without loss of plant material, all the in vitro plants having survived this step.
- To obtain plant material destined for exploitation in the pharmaceutical industry, it is recommended to use the *in vitro* multiplication technology on the nutrient media BA_1 and BB_2 and to complete harvesting within a maximum of 1.2 months from incubation to avoid adventitious root formation.

ABSTRACT

Thymus vulgaris L. (thyme), belonging to the family Lamiaceae (Labiate), is a species originating in the Mediterranean region, having been grown as medicinal and spice plant since antiquity. The bioactive compounds identified in thyme plants are represented by flavonoids, thymol, carvacrol, eugenol, phenols, luteolin, tymol, terpenoids that cause such effects as antispasmodic, bactericide, antiseptic, antioxidant, having anthelmintic properties and having been lately recommended as substitute for cancer prevention agents. Due to its economic importance, the purpose of our research was to highlight the *in vitro* reaction of this species by observations on the morphogenetic response of a series of explants on various nutritive variants.

In view of initiating the *in vitro* cultures, the biological material used in our research consisted of *Thymus vulgaris* seeds, disinfested with chloramine-T 5% solution, for 20 minutes. After the removal of disinfesting solutions (by rinsing in distilled water), the seeds were inoculated on Murashige-Skoog (MS) without hormones. The plants obtained from the seeds were used as a source of explants (phytoinocul), represented by shoot apexes and nodes, were inoculated into nutrient media supplemented with the phytohormones from the category of auxins and cytokinins in different combinations and concentrations.

Our observations led to the following conclusions: the best reaction highlighted was caulogenesis, followed by rhizogenesis and, to a small percentage by callusogenesis. The media BA_1 -

1mg/l BAP (benzylaminopurine) + 0.5 mg/l IAA (indole-3-acetic acid), and BB_2 - 1mg/l BAP (benzylaminopurine) + 0.1 mg/l IBA (indole-3-butyric acid) proved to be the most favourable to the regeneration of shoots, followed by the versions BA_2 - 1mg/l BAP (benzylaminopurine) + 0.1 mg/l IAA (indole-3-acetic acid) and BB_1- 1mg/l BAP (benzylaminopurine) + 0.5 mg/l IBA (indole-3-butyric acid), where the shooting phenomenon was intense, but the shoots were frail, with smaller leaves and thinner stems. *Thyme* callus formation was highlighted in the medium version NAA_1, the callus showing low proliferation and organogenetic capacity.

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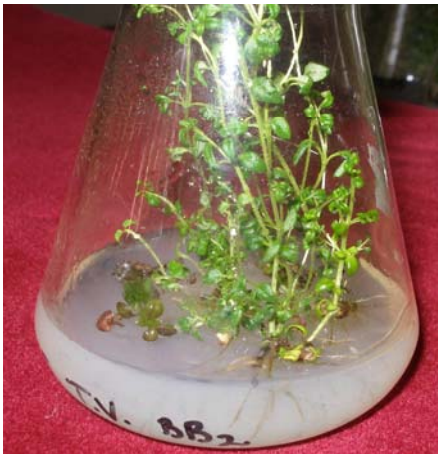
Caulogenesis on BA2



Shoots and roots on BB2

Multiple shoots on BA1

Caulogenesis and rootedness on 2,4-D

Shoots regenerated on BB1

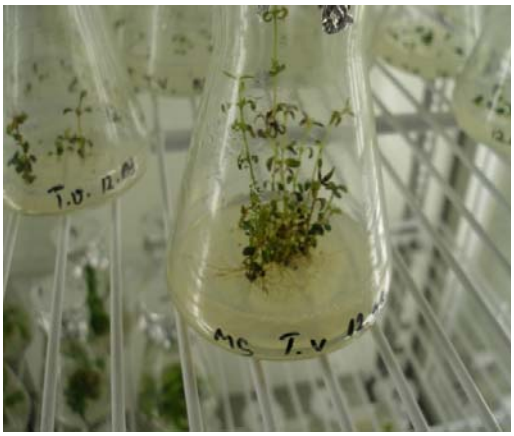
Vigorous shoots on BA1

Thyme vitroplants on BA1

Shoots regenerated on IBA1

Node Explants on BK1

Node Explants on BN1



Vitro plants grown from seeds on MS