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BIOTECHNOLOGICAL PROPAGATION OF LOCAL MEDITERRANEAN VARIETY OF *THYMUS VULGARIS* L. FOR THE PURPOSE OF SECONDARY METABOLITES PRODUCTION

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Abstract: In the present work, the potential of a local Mediterranean cultivar of *Thymus vulgaris* L. (ThEg) for biotechnological development was investigated. Starting material for the experiment were *Thymus vulgaris* L. seeds kindly provided by Sekem Company, Egypt. An experiment for comparison of the germination rate of *T. vulgaris* L. ThEg seeds with a commercial sample of seeds from the Bulgarian market (ThBg) was conducted in the conditions of sterile *in vitro* culture, as well as in commercial peat substrate propagator. *In vitro* shoot cultures of *T. vulgaris* L. of both ThEg and ThBg seeds were developed in media with modification of the auxin applied (1-Naphthaleneacetic acid vs. indole-3-butyric acid) in the presence of the cytokinin benzyl adenine. The effect of cultivation conditions on the plant's physiological status and polyphenolics productivity was compared showing the supplementation of 1-Naphthaleneacetic acid to be more favorable in terms of biomass formation, physiological status and polyphenolics productivity.

INTRODUCTION

Thymus vulgaris L. (common thyme, Lamiaceae) is an essential oil-bearing plant natively distributed throughout Southwestern and Southeastern Europe, the Mediterranean region and neighboring countries, Northern Africa and parts of Asia. In Africa, the plant is being cultivated in Egypt, Morocco, Algeria, Tunisia,

Libya, Cameroon, Nigeria and South Africa. In addition to its traditional flavoring application as a culinary herb, its application as a tea infusion for conditions such as pulmonary infections, diabetes, digestive disorders is well known in the indigenous medicinal traditions in the countries of origin of the plant (Kuete, 2017 and references cited within).

Scientific research has provided evidence of the phytochemical composition of the species determining its biological activities. The essential oil of the plant is mainly dominated by monoterpenes and monoterpene hydrocarbons, thymol and its phenol isomer carvacrol being the most abundant amongst them (Niksic, 2021). Thyme essential oil has been shown to possess beneficial activity in acute and chronic respiratory conditions as well as a potent antibacterial agent. Its anti-inflammatory properties have been proven in mice as well as cell models of THP-1 (human acute monocytic leukemia cell line), human polymorphonuclear neutrophils, and RAW 264.7 (murine macrophage cell line) (Horváth et al., 2021 and references cited within). Recently, Horváth et al. (2021) have also demonstrated its potent activity against neuro-inflammation. Non-volatile components found in *T. vulgaris* L. extracts have also been shown to possess marked phytopharmacological potential. Thus, the ethanol-water extract of T. *vulgaris* was shown to possess anti-inflammatory activity through NO-inhibition in murine macrophage cell line J774A.1 (Vigo et al., 2004). The 70 % ethanol extract of the plant aerial parts was found to possess marked protective activity against $NaNO_2$-induced hepatic injury in a pretreatment model of application (Soliman et al., 2021). The activity of *Thymus ssp*. non-volatile preparations have been explained by the abundance of phenolic and flavonoid derivatives found in them (Schött et al., 2017) making these species prospective candidates in the search of secondary metabolites with antioxidant potential (El-Boshy et al., 2019).

Exploration of tissue culture techniques for elucidation of T. vulgaris potential for the *in vitro* production of phenolic compounds date back as far as 25 years ago when Shetty et al. (1996) utilized Pseudomonas sp. treatment of *T. vulgaris* shoot cultures in order to induce phenolics-rich in vitro lines. Up to 315 % increase in thymol production was achieved by Affonso et al. (2009) by means of using 1.0 μM indole-3-acetic acid. Micropropagation and in vitro essential oil production were studied in *Thymus vulgaris* L. "Słoneczko" cultivar with the most abundant components being thymol, ϒ - terpinene, p-cymene, carvacrol, carvacrol methyl ether and linalool (Kulpa et al., 2018). The effect of PEG-induced osmotic stress on physiological and morphological features of *T. vulgaris* L. *in vitro* was studied, showing that mild stress impact was able to stimulate phenolics and essential oil components production, at the expense of oxidative stress and growth retardation (Razavizadeh et al., 2019).

Previous studies on the organic farming approach for *T. vulgaris* L. cultivation in Egypt have shown that high organic fertilization stimulated the accumulation of oxygenated components in thyme oil especially thymol (51.1 %), as well as carvacrol, and γ-terpinene (Edris et al., 2009).

The aim of the present work was (i) to compare the germination capacity in vitro and in commercial peat substrate composition of seeds of *T. vulgaris* L. kindly provided by the Sekem company, Egypt, with the one of seeds of the species obtained at the Bulgarian market and (ii) to assess the biosynthetic capacity of different *in vitro* lines of the two origins and compare it with the one of commercially obtained *T. vulgaris* L. herbal tea, obtained at the Bulgarian market.

MATERIALS AND METHODS

Plant material

Seeds of local variety of *T. vulgaris* L. from Egypt were kindly provided by the Sekem Company (https://www.sekem.com/en/index/), origin abbreviated further on as ThEg. Seeds of Bulgarian origin were purchased by Lactofol Botanica Company (Lot. 127705), origin abbreviated further on as ThBg. Herbal tea preparation of the Bilec Company was purchased from a Bulgarian pharmacy (LO 10318).

Germination experiments

In vitro germination experiment was performed in the following medium formulation (abbreviated as SGM): Murashige and Skoog (1962) full strength micro- and half strength macrosalts, supplemented with Gamborg vitamins (Gamborg et al. 1968), 20 g/l sucrose and 6 g/l agar. Seeds of the two *T. vulgaris* L. origins were surface sterilized by 30 seconds immersion in 70 % ethanol, followed by 8 minutes rinsing in 10 % $NaOCl_2$. Then, seeds were washed in triplicate in sterile distilled water, dried on sterile filter paper, inoculated into the SGM media and kept in the dark at 25 °C. Germination rate was recorded on the 8^{th} day after inoculation.

Peat substrate germination was performed with ThEg and ThBg in a commercial propagator (ROOT!T Large Value Propagator, HydroGarden Brand, Binley, Coventry, UK, http://www.propagateplants.com) in Natural rooting sponge, Root!t substrate. Germination was recorded on the 8th day after inoculation

In vitro culture experiments

Four plant growth regulators (PGR) treatments were applied as the effect of benzyl adenine (BA) as a cytokinin, was experimented in combination with either 1-Naphthylacetic acid (NAA) or indole-3-butyric acid (IBA) as auxins (Table 1). The germinated *T. vulgaris* L. ThEg and ThBg plantlets were placed in the respective experimental media and cultivated at a 16/8 h photoperiod at 25 °C.

Table 1 Plant growth regulators supplementation to the four media compositions

	BA [mg/l]	NAA [mg/l]	IBA [mg/l]
ThEg/Bg_0	-	-	-
ThEg/Bg _1	0.2	-	-
ThEg/Bg _2	0.2	0.1	-
ThEg/Bg _3	0.2	-	0.1

Lipid peroxidation levels *in vitro*

Lipid peroxidation in the experimented PGR treatments was estimated as the levels of malondialdehyde in the in vitro *T. vulgaris* L. samples. The assays were performed spectrophotometrically after the method of Dhindsa et al. (1981). MDA was calculated using its extinction coefficient of 155 mM^{-1} cm^{-1} (Heath and Packer 1968).

Oxidative stress *in vitro*

Oxidative stress in the different PGR treatments was estimated by the spectrophotometric assay for endogenous hydrogen peroxide analysis after the method of Jessup et al. (1994).

Total flavonoid levels *in vitro*

The levels of total flavonoids were estimated in 80 % ethanol extracts *in vitro*, as well as in the commercial herbal tea samples by the colorimetric assay of Zhishen et al. (1999). The absorption at 510 nm was measured and the concentration was calculated using a calibration curve of (+)catechin (in the range of 2 μg/ml to 80 μg/ml). The results were expressed as milligrams of (+) catechin equivalent per gram of DW of the sample.

Total phenolic levels *in vitro*

The levels of total phenolics were estimated in the 80 % ethanol extracts of the *in vitro* samples, as well as in the commercial herbal tea samples using the colorimetric assay of Singleton et al. (1999). The absorption was measured at 730nm and the total phenolics were calculated by means of a calibration curve of chlorogenic acid (in the range of 10 μg/ml to 100 μg/ml). The results were expressed as milligrams of chlorogenic acid equivalent per gram of DW of the sample.

Statistical processing of data

Samples, collected for the analyses consisted of the homogenized plant material of three individual plant vessels, containing up to three plant individuals each. Analyses were performed twice with three technical repetitions for each. The means were compared by t-test of unequal variances at $P \leq 0.05$.

RESULTS

Seed germination

On the 8th day after the *in vitro* inoculation of seeds in the SGM medium, the following germination rates were estimated for the two *T.vulgaris* L. origins (ThEg - 68 % and ThBg - 74 %), (Fig. 1 A and B).

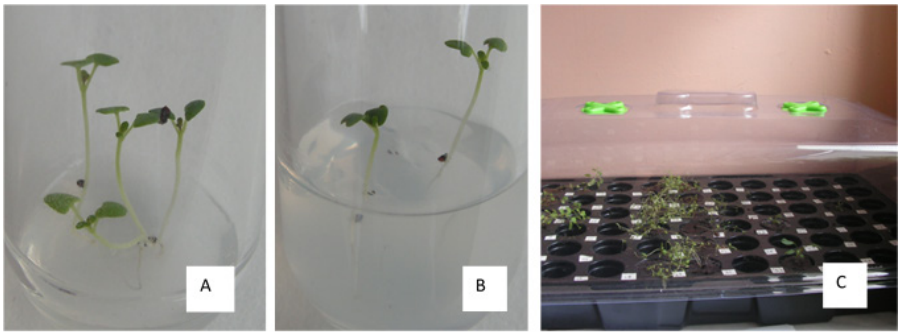

Figure 1. Germinated plantlets of ThEg (A) and ThBg (B), eight days after inoculation in the SGM germination medium as well as germination of the two species in commercial peat substrate ROOT!t system (C)

Eight days after the inoculation of seeds in the peat substrate the following germination rates were recorded: ThEg - 74 % and ThBg - 85 % (Fig. 1C).

Plant growth regulator treatments

Four months after placement of the germinated plantlets into the four media compositions, the propagated biomass (Fig. 2) was collected for further analyses. The observations showed that NAA (illustrated by ThEg_2 in Fig. 2 C) was superior in terms of biomass formation as compared to IBA (illustrated by ThEg_3 in Fig. 2 D). Interestingly, for the ThBg origin, the 0.2 mg/l BA supplementation medium (ThBg_1, Fig. 2 E) led to abundant biomass formation, whereas for ThEg this medium supplementation was shown to be unfavorable for multiplication (ThEg_1 in Fig. 2 B).

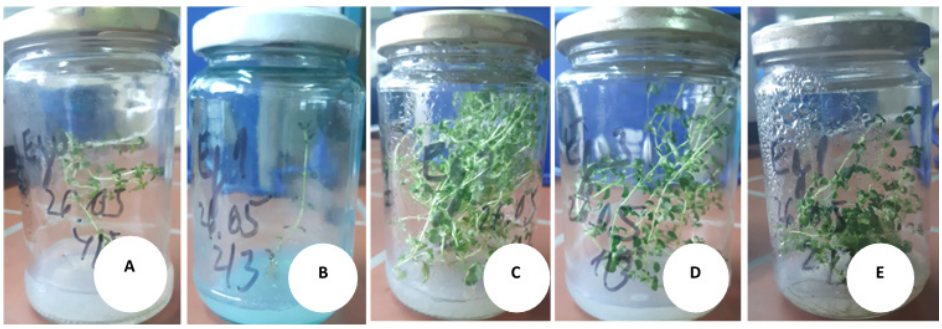

Figure 2. Effect of plant growth regulators on in vitro growth of *T. vulgaris* L.: ThEg in the four media modifications: ThEg_0 (A), ThEg_1 (B), ThEg_2 (C), ThEg_3 (D) and ThBg_1 (E)

Lipid peroxidation

The PGR-free Th_0 cultivated plants showed higher MDA constitutive levels for ThBg_0 as compared with ThEg_0 (Fig. 3 A and B). The levels of MDA obtained for the two *T. vulgaris* L. origins *in vitro* were commensurable for the Th_2 and Th_3 PGR modification showing that for both *Thymus* origins NAA supplementation was related to lower lipid peroxide production as compared with IBA. Interestingly, ThEg_1 expressed significantly higher MDA levels as compared both with ThBg_1, as well as with all other PGR treatments for both *T. vulgaris* origins (Fig. 3 A and B).

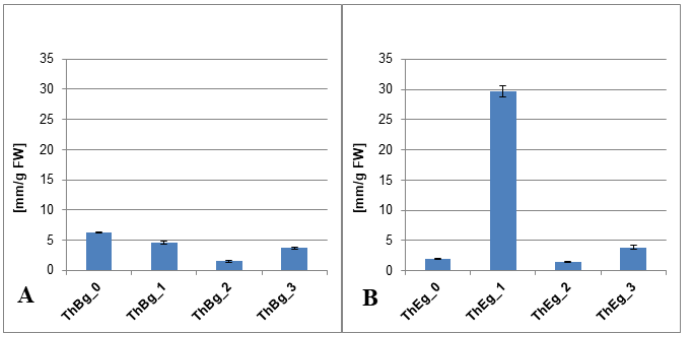

Figure 3. Effect of plant growth regulators on malondialdehyde (MDA) levels in ThBg (A) and ThEg (B)

Oxidative stress

The observations of the oxidative stress (estimated as levels of endogenous hydrogen peroxide) follow the same dependencies as the ones, discussed for MDA above, in the PGR-free (Th_0) and BA (Th_1) treatments for both *Thymus* origins (Fig. 4 A and B).

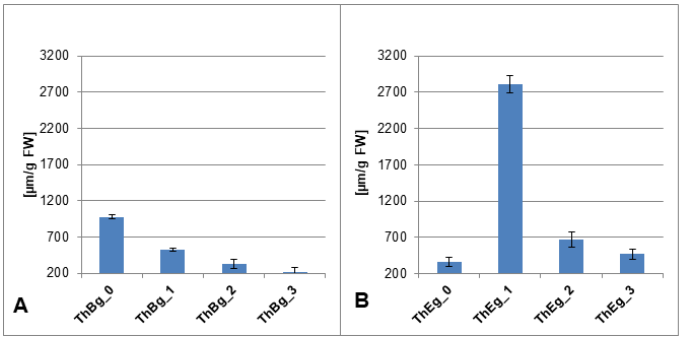

Figure 4. Effect of plant growth regulators on hydrogen peroxide (H_2O_2) levels in ThBg (A) and ThEg (B)

Interestingly, however, for both T. *vulgaris* L. cultivars NAA treatment stimulated endogenous hydrogen peroxide generation to a higher extent as compared to IBA.

Flavonoid and phenolic levels

It was established that the levels of both total flavonoid (Fig. 5 A) and phenolic (Fig. 5 B) compounds were significantly higher in PGR-free and BA supplemented, as compared to cytokinin + auxin treatments for both *T. vulgaris* origins. In addition, for the PGR-free and BA supplemented media, the ThEg origin of *T. vulgaris* L. was superior as compared with the ThBg one.

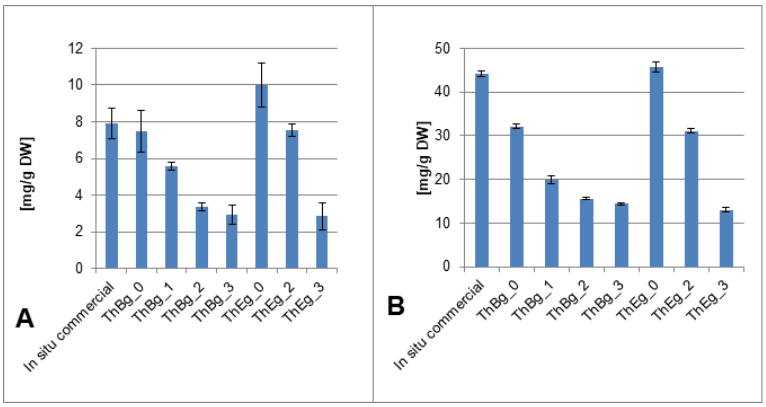

Figure 5. Total flavonoid (A) and phenolic (B) levels in the commercial *T. vulgaris* L. sample, as well as in the *in vitro* cultivated ThEg and ThBg. Same letters denote statistically non-significant differences of the means at P≤0.05

Moreover, levels of flavonoid and phenolic compounds in the ThEg_0 treatment significantly exceeded not only the rest of PGR-treatments, but also the ones of the sample of commercial herbal tea.

DISCUSSION

Noteworthy, the comparison of germination rate between the two *T. vulgaris* L. origins was analogical for both approaches showing slightly higher values for ThBg as compared to ThEg. Interestingly, peat substrate germination resulted in slightly higher results as compared to the *in vitro* protocol. In a research work of Ozudogru et al. (2011), the comparison germination of *T. vulgaris* seeds i*n vitro* was significantly higher as compared to the one in soil (79 % vs. 0.80 %, respectively, recorded 45 days after sowing). In the experiment presented in the present work, germination in vitro varied from 68 % to 74 %, however results

were recorded eight days after inoculation. The higher results obtained for the peat substrate experiment (74 % to 85 %) could be explained by the lack of sterilization procedure which could itself cause slight inhibition on germination and the specificity of the formulation of the commercially obtained substrate and the favorable conditions of the germination propagator system.

The biomass formation response to PGR treatment of the two origins was commensurable except the 0.2 mg/l BA treatment which led to markedly suppressed growth in ThEg_2 (Fig. 2 B) as compared to ThBg_2 (Fig. 2 E). Interestingly, this effect was also correlated to markedly elevated lipid peroxidation (Fig. 3 B) and oxidative stress (Fig. 4 B) in the ThEg_1 treatment. The comparison of 0.2 mg/l BA + 0.1 mg/l NAA (ThEg/Bg_2) with 0.2 mg/l BA + 0.1 mg/l IBA (ThEg/Bg_3) showed that NAA supplementation stimulated biomass formation to a greater extent as compared with IBA for both *T. vulgaris* L. origins (Fig. 2 C and D, respectively). The obtained results are confirmatory to the ones obtained by Ozudogru et al. (2011) who established that NAA supplementation was generally favorable as compared to IBA in terms of several biomass formation parameters in four different concentrations of the two auxins, although the experiments were performed in a combination of NAA and IBA with kinetin.

In our experiment, the lower lipid peroxidation in the NAA supplemented ThEg/Bg_2 as compared to one for IBA (ThEg/Bg_3) also contributes to the observation that NAA supplementation is more favorable for the physiological adaptiveness of *T. vulgaris* L. towards tissue culture development. This was also accompanied by slightly higher hydrogen peroxide (Fig. 4 A and B), flavonoid and phenolic (Fig. 5 A and B) levels in the NAA treatment as compared to the IBA one, this tendency being better expressed for the ThEg origin of *T. vulgaris* L. These dependencies could find their physiological explanation in the roles of hydrogen peroxide and phenolic compounds as indigenous mechanisms of the plant organism in coping with environmental stress. It is known that hydrogen peroxide is produced by the detoxification of the superoxide radical by the enzymatic activity of the enzyme superoxide dismutase (SOD, EC 1.15.1.1). The process occurs by means of the electron transport in chloroplasts and mitochondria, plasma membrane NADPH oxidases, peroxisomal oxidases, type III peroxidases and other apoplastic oxidases. The apoplastic H_2O_2 plays role in cell expansion, development and defense and mediates acclimation to stress and pathogen defense. The role of hydrogen peroxide is realized through signaling which affects protein functions and gene expression (Smirnoff and Arnaud, 2019 and references cited within). In a model system of stationary liquid cultures of sugarcane, Arencibia et al. (2012) have studied the relations between oxidative burst and phenylpropanoid pathways showing that hydrogen peroxide induces the production of phenylpropanoid metabolites under PEG-induced stress. Thus, the reverse relations between H_2O_2 and lipid peroxidation (MDA levels), in the comparison between NAA and IBA treated ***T. vulgaris*** L. in our work, as well

as the elevation of phenolic levels in NAA treated plants is confirmatory to the concept of the role of hydrogen peroxide messenger of as oxidative stress alleviation through phenolics production stimulation.

The results of the present work also outline *T. vulgaris* L. from Mediterranean origin to be favorable as an in vitro producer of secondary metabolites with flavonoid and phenolic structure.

CONCLUSION

The interplay between oxidative stress alleviation and secondary metabolites production in vitro is an important factor, and its understanding and correct interpretation are of great importance for the successful utilization of plant cell tissue and organ culture for the production of compounds with phytotherapeutic potential.

T. vulgaris L. of Mediterranean origin is prospective for biotechnological development for the production of extractable plant biomass with future practical application.

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