Growth and GC/MS based metabolic profile of the hydroponically propagated species of genus *Thymus* (*Lamiaceae*)

Boryanka D. Traykova*, Milena T. Nikolova, Marina I. Stanilova & Ina Y. Aneva

Institute of Biodiversity and Ecosystem Research, Department of Plant and Fungal Diversity and Resources, Bulgarian Academy of Sciences, 23, Acad. G. Bonchev Str., 1113 Sofia, Bulgaria, e-mail: borianka_traikova@abv.bg* (corresponding author), mtihomirova@gmail.com, marina.stanilova@gmail.com, ina.aneva@abv.bg

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Abstract. The present study evaluates the feasibility of hydroponic cultivation of three *Thymus* species native to Bulgaria: *T. longedentatus, T. pannonicus* and *T. zygioides*. The use of a Flood & Drain hydroponic system has produced a significant increase in the growth and development rates of the target species, with *T. pannonicus* reaching the flowering stage in just three weeks. The metabolic profiles of hydroponically grown plants correspond to those of the parent plants from the matching natural populations. In short, hydroponic cultivation of the studied Thyme species could be a viable alternative to conventional agricultural practices, accelerating plant growth and preserving their medicinal and aromatic properties.

Key words: hydroponic technologies, medicinal and aromatic plants, secondary metabolites, Thyme

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Introduction

The genus *Thymus*, which comprises numerous species, draws attention with the remarkable bioactive properties of its essential oils: antiseptic, antifungal, antispasmodic, sedative, and diuretic. They make it valuable in the pharmaceutical, cosmetic and food industries. However, despite their outstanding potential, commercial use of the *Thymus* essential oils has been impeded by challenges posed by the quest for sustainable raw material supply and complexity of the species' identification.

Twenty-one of more than 250 species of the genus are distributed in Bulgaria. An *ex situ* collection representative of the genus *Thymus* has been created, with plants collected from the Bulgarian populations of these species, as part of the project "Metabolic profile and genetic diversity of the *Thymus* species in Bulgaria – scientific basis for phytotherapy and potential for pharmacy".

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The present study focuses on three *Thymus* species: *T. longedentatus* (Degen & Urum.) Ronniger, *T. pannonicus* All. and *T. zygioides* Griseb., which are native to Bulgaria and differ by their phytochemical profiles and essential oil composition. The reasons for selecting these three species are that they share the same locality, often intertwine their tufts, bloom at the same time, but still retain their distinctive characteristics. Their habitat is located in the Eastern Rhodopes, which explains a relatively earlier onset of the growing season.

Thymus longedentatus is a Balkan endemic, which has been the subject of several phytochemical and biological studies in recent years (Aneva & al. 2019; Nikolova & al. 2021, 2023, 2024; Georgiev & al. 2022). A rich content (above 60%) of citral isomers - neral and geranial - in the essential oil (EO) profile of the species determines its citrus aroma and its biological activity (Aneva & al. 2019). The high herbicidal potential of EO found in the species has been associated with inhibition of seed germination of some grass species, such as *Lolium perenne* and *Trifolium pratense* (Nikolova & al. 2021). Also, a high acetylcholinesterase inhibitory activity has been recognized for the EO of the species (Georgiev & al. 2022). Important bioactive compounds, such as triterpene acids - ursolic, oleanolic and several phenolic acids - flavonoids and monoterpenes have been detected in the different extracts of the species (Nikolova & al. 2023, 2024). All that outlines the species as promising for researching its breeding and cultivation potential.

Thymus zygioides is distributed in Southeast and Southwest Europe. The strong antioxidant properties and antihelminthic and cytotoxic effects revealed by various extracts of the aerial parts of *T. zygioides* imply the potential use of that species in the food industry and medicine (Kaska & al. 2018). Four chemotypes have been identified in *T. zygioides* growing in Türkiye, with geraniol, carvacrol, α-terpinyl acetate and thymol as major components, respectively (Baser & al. 1996). Bulgarian populations of the species belong to the thymol chemotype, and the aromatic compounds (61.2%) form the most abundant group in its EO, with thymol (51.2%) as the principal component, followed by borneol, rcymene, *cis*-sabinene hydrate, and γ-terpinene (Trendafilova & al., 2021). This makes *T. zygioides* a potential species for mass propagation, considering the fact how pharmaceutically important molecule is thymol.

Thymus pannonicus has a wide distribution across Europe. In the Bulgarian populations of the species, EO is characteristically the richest in components, with 69 compounds in a concentration above 0.1% (Trendafilova & al. 2019). It also has the highest content of sesquiterpene hydrocarbons (74.2%). Its major components, germacrene D (42.2%) and β-caryophyllene (12.3%), define the oil chemotype of *T. pannonicus* as a germacrene D/β-caryophyllene.

Considering the pressing need in sustainable Thyme cultivation practices and the inherent phytochemical diversity of the three target *Thymus* species, introduction of innovative methods for their mass propagation is encouraged. The present study describes a pioneering cultivation of these *Thymus* species in a hydroponic system. Soilless cultivation, especially hydroponics, offers a viable alternative to traditional soil-based growing methods by facilitation of the controlled nutrient management, reduction of the risk of pests and diseases, and allocation of potentially higher yields and quality of the plant secondary metabolites (Jones 2016). Initially used in vegetable production, hydroponic technologies are presently applied for growing an extensively wider range of plant species. In recent decades, soilless cultivation has also been applied for medicinal plants, including species of the genera *Mentha, Stevia, Arnica, Ocimum* (Giurgiu & al. 2014). The main advantages of hydroponic technologies are: accelerated growth, high yields, water and area reduction, and environmental control (Texier 2014). However, most studies have been concerned with the feasibility of hydroponic cultivation of various species rather than by their cost-effectiveness (Atherton & Li 2023). Dorais & al. (2001) have evaluated profitability of a floating raft growing system in a greenhouse for some medicinal plant species with a short vegetative cycle and strong market demand, such as *Achillea millefolium*, *Taraxacum officinalis,* and *Valeriana officinalis*, and have found that their shoot and root yields increase several times. The possibility of using automated computer control of the production process featuring dozens of input parameters and of maintaining optimum environmental conditions in the greenhouses permits a scaling-up of soilless plant propagation and harvesting directly for the market (Jensen 2002). When hydroponically propagated plants are intended for introduction in agriculture, they need to be adapted to the soil substrate and environmental conditions.

The advantage of the Flood & Drain hydroponic systerm (F&D system) is that it lowers slowly the water level and provides excellent oxygenation of the plants. It is important to optimize the watering cycle according to the requirements of the cultivated species, so as to avoid possible waterlogging or drying in the root zone. Likewise, a F&D hydroponic system can be used in multi-storeyed constructions suitable for cultivation of plant species with small habitus, such as Thyme. Integration of vertical farming with hydroponics is considered a very effective and advanced mode to grow food crops and medicinal plants in urban settings, because of ensuring fresh plants across the year, grown in a small area, which practically reduces the production and transportation costs (Kumar 2019).

Thymes are insect-pollinated plants; they reproduce sexually by seeds, but also vegetatively by rooting at the nodes of the repent stems, which makes them suitable for propagation by cuttings. The present study deals with a feasibility test and application of hydroponic technologies starting from shoots, in order to accelerate the growth and reproduction of three Thyme species from the Bulgarian flora (*T. longedentatus, T. pannonicus* and *T. zygioides*) and identification of the metabolic profiles of the hydroponically propagated plants.

Material and methods

Plant material

Plants belonging to three Thyme species (*T. longedentatus, T. pannonicus* and *T. zygioides*) were collected early in March 2019 from the same habitat in the Eastern Rhodope Mts, Bulgaria, close to the Gorni Glavanak village.

Soilless cultivation

Fresh whole stems of the Thyme plants with removed roots, about 10 cm long, leafy and with several nodes, were used as cuttings for both hydroponic propagation and control cultivation in soil. Forty cuttings per each of the three studied species were used, half of them pretreated with auxin for rooting stimulation by smearing the base of the cuttings with 0.25% powder of indole-3-butyric acid (IBA) (Rhizopon BV, The Netherlands) before their placing in the substrate. Four variants were tested, each consisting of 10 cuttings per species: hydroponic Flood & Drain system (F&D system) cultivation with untreated and IBA-pretreated cuttings, and cultivation in soil terrines with untreated and IBA-pretreated cuttings. The F&D system measured 60 cm × 60 cm × 5 cm, with 20 L perlite as substrate, and nutrient solution in a tank (Flora Micro®, Flora Grow® and Flora Bloom®, GHE, in a ratio 1:1:1, diluted in water; pH 5.5-6.5; EC from 0.4 $mS.cm^{-1}$ in the rooting stage to 1.2 $mS.cm^{-1}$ during blooming). The substrate was flooded with the solution for 15 min four times a day using a water pump, thereby ensuring multiple water reuses. A Light mix Biobizz® soil mixture was used in the control variants. Cultivation was carried out in a room phytotron at 16/8h light/dark regime (metal halide lamp MH superveg 250 W with reflectors), temperature of 22±3 °C, and relative air humidity varying around-the-clock between 36% and 72% using a room humidifier.

In June 2019, three months after the beginning of cultivation, all obtained plants were transferred to containers (12 cm diameter) filled with Light mix Biobizz® soil mixture, and placed on the shelves in a phytotron, with mixed natural and artificial (LED-strips) light, for adaptation to the soil substrate and/or growth acceleration. In September 2019, they were transferred to the unheated greenhouse of IBER for further acclimatization.

Also, in November 2019, 120 IBA-treated cuttings per species, originating from the same populations, were planted in soil, at a distance of 10 cm from each other, on a shelf in the unheated greenhouse of IBER. They were used as another control variant.

Phytochemical analyses

Sample preparation: Dry powdered plant material (100 mg of hydroponically grown shoots during mass flowering from each studied species) was extracted with methanol (1 mL) by maceration at room temperature for 24 hours, with added 50 µg of 3,4 dichloro-4-hydroxybenzoic acid (50 µL from 1mg/mL solution) as internal standard at the beginning of extraction. After filtration of the aliquot into a vial, the extract was evaporated and derivatized by 100 µL of *N,O*-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) in 100 µL pyridine at 70 °C for two hours. After cooling, 300 µL of chloroform was added and the samples were analyzed by GC/MS.

GC/MS analysis: GC/MS analysis was performed with a Thermo Scientific Focus GC, coupled with a Thermo Scientific DSQ mass detector, operating in EI mode at 70 eV. Chromatographic conditions were described by Berkov & al. (2021). Metabolites were identified as TMSi derivatives by comparing their mass to the authentic standards from the National Institute of Standards and Technology (NIST) spectra. The amounts of the identified compounds have been presented as response ratios calculated for each compound, relative to the internal standard using the calculated areas for both components.

Results

Soilless cultivation

Differences in plant growth and development were noticed depending on both plant species and cultivation conditions. In the F&D hydroponic system, most cuttings of all three species rooted in a short time, somewhere within 20 days, and ramified. *T. pannonicus* reached flowering stage in only three weeks, while *T. zygioides* and *T. longedentatus* needed four and six weeks, respectively (Plate I, Figs 1, 2). Mass flowering was observed one week after the beginning of flowering. No difference was stated between IBA-pretreated and untreated cuttings of the same species in terms of their rooting. The length of the roots in all variants ranged from 5 cm to 20 cm (Plate I, Figs 3, 4). However, in *T. zygioides* the effectiveness of plant propagation was the highest, because the plant stems tended to creep and formed roots and new shoots in the nodes, while the other two species were more upright (Plate I, Figs 3b, 4b). Although some of the cuttings did not root and died, the final plant number showed an increase due to formation of roots and new shoots growing from the nodes, followed by separation of the new plants by cutting the internodes. At the end of the three-month soilless cultivation, the plants have multiplied up to 120% for *T. zygioides*, as compared to the initial number of cuttings, and up to 105% for *T. pannonicus* and *T. longedentatus*.

In the soil control variants (Plate I, Figs 5, 6), survival of cuttings was significantly lower and formation of new plants was seldom observed, which resulted in the development of fewer flowering plants: 65% for *T. zygioides* and 40% for the other two species, as compared to the original number of cuttings. The plants of the control variants were smaller in size when compared with the hydroponically grown plants, which was explained by their less developed root system. Similarly to the soilless cultivation variants, flowering was observed first in the *T. pannonicus* plants.

All surviving plants from all variants were placed on the shelves in the phytotron room (June, 2019) (Plate I, Fig. 7) and then transferred to the unheated greenhouse (September, 2019). *Thymus pannonicus* proved to be more sensitive to waterlogging, so it was attacked by powdery mildew during cultivation in the phytotron, and treated with the Topaz fungicide to overcome the disease. All plants successfully overwintered in the greenhouse (Fig. 8, A-C). In April 2020, the plants of *T. pannonicus* bloomed first, followed by the plants from the other two species (Fig. 8, D). All plants were ready to be planted in the *ex situ* collection of IBER, and several pots with *T. zygioides* were offered to the Borika Botanic Garden located in the Borika village, Ihtiman Municipality, for the establishment of a plant nursery.

Concerning those cuttings of the three Thyme species, which were planted directly in soil on the greenhouse shelf in November, 2019, only some of them

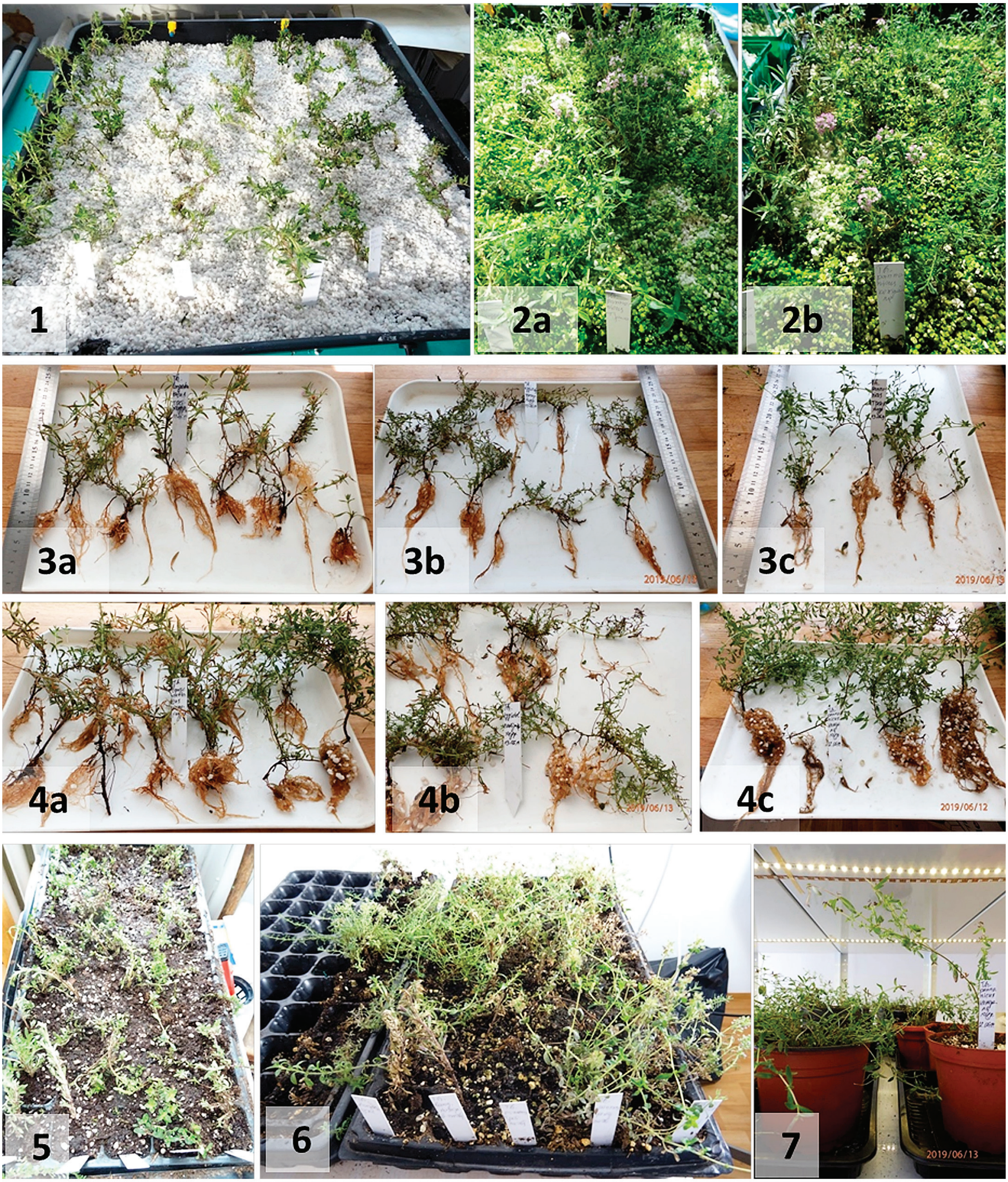

Plate I: Fig. 1. Cuttings of the three *Thymus* species in Flood & Drain hydroponic system at the beginning of the experiment. **Fig. 2.** The three species at the end of soilless cultivation: **2a**, from IBA-pretreated cuttings; **2b**, from untreated cuttings. *T. pannonicus* is in flowers. **Fig. 3.** Roots of the plants of the three species grown from IBA-pretreated cuttings: **3a,** *T. longidentatus*; **3b**, *T. zygioides*; **3c**, *T. pannonicus*. **Fig. 4.** Roots of the plants of the three species grown from untreated cuttings: **4a**, *T. longidentatus*; **4b**, *T. zygioides*; **4c**. *T. pannonicus*. **Fig. 5.** Cuttings of the three *Thymus* species in soil-filled terrine (control variant) at the beginning of the experiment. **Fig. 6.** The three species at the end of cultivation in a soil-filled terrine. **Fig. 7.** Plants transferred to containers on the shelves of a rack in the phytotron.

Fig. 8. Hydroponically grown plants from cuttings, acclimatized in an unheated greenhouse: **A**, *T longedentatus*; **B**, *T. zygioides*; **C**, *T. pannonicus*; **D**, The three species in their flowering phase.

have rooted and overwintered successfully due to inappropriate conditions in the greenhouse. Plants grew, developed new stems and ramified. By the spring of 2021, they covered the entire area of approximately one square meter designated for each of the species. A dense cover was observed for *T. longedentatus*, *T. zygioides* formed dense tufts, while *T. pannonicus* shaped loosely spaced tufts (Fig. 9).

Phytochemical analyses

Chemical composition of the methanolic extracts of *T. longedentatus* and *T. zygioides* was analyzed by GC/MS. Isomers thymol and carvacrol and triterpene acids (ursolic and oleanolic) were found as main components in the profiles of the studied extracts (Table 1). Several phenolic acids were identified and among them the rosmarinic, chlorogenic, caffeic, hydroxycinnamic, and hydrohybenzoic were most abundant. Compounds belonging to fatty acids, organic acids, sterols, polyols, and sugar acids (quinic) were also identified. The extract of *T. zygioides* differed from that of *T. longedentatus* by the high content of thymol and carvacrol and the presence of some specific compounds, such as geranic acid and hydroquinone (Fig. 10).

Discussion

These were the first trials of hydroponic propagation of the Thyme species *T. pannonicus*, *T. zygioides* and *T. longedentatus*. Feasibility of the process was ascertained and the plants' growth and development were accelerated: they bloomed somewhere three to six weeks after the beginning of the experiment, but mass flowering lasted only two weeks. In natural populations, the flowering stage was longer and lasted three to five months: April to August for *T. zygioides*, May to July for *T. longedentatus*, and May to September for *T. pannonicus* (Markova 1989). Therefore, harvesting of hydroponically grown Thymes would be easier, following a shorter growing period. The three studied *Thymus* species differ in their stem morphology (Markova 1989), which could explain the difference observed in their reproductive efficiency. Only in *T. zygioides* stems are creeping and forming roots and shoots in almost all nodes, which explains the highest percentage of obtained plants at the end of soilless cultivation. In *T. longedentatus,* the stems are also creeping, and in *T. pannonicus* they are falsely creeping but both species have formed less roots in the nodes. Despite the identical cultivation conditions in the F&D hydroponic system, the plants of *T. pannonicus* flowered before those of the other two species,

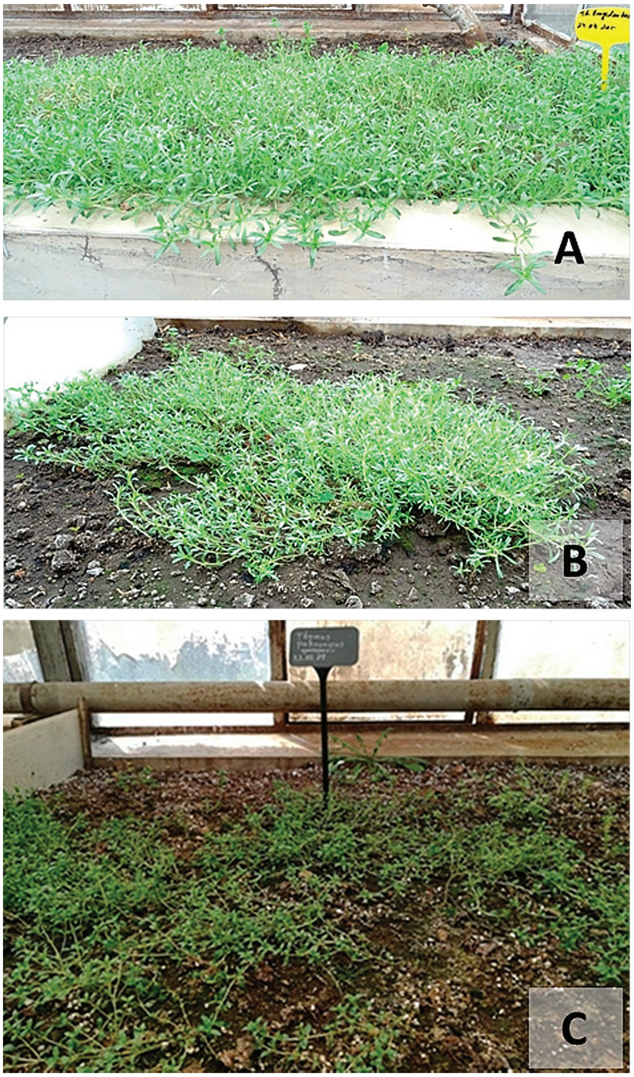

Fig. 9. Plants from the three species obtained from cuttings planted directly in soil on a shelf in the unheated greenhouse: **A**, *T. longedentatus*; **B**, *T. zygioides*; **C**, *T. pannonicus*.

which was likewise observed in the *ex situ* collection of IBER, as well as in their natural locality (*in situ*).

Pretreatment with auxin did not produce better rooting either in the F&D system or in the control soil variant. However, it proved worthwhile to investigate experimentally the effect of IBA on each species of interest, considering that the authors' earlier studies have shown opposite results for the different plant species. For instance, their study on *Vaccinium vitis-idaea* has shown that the effect of the same pretreatment depended on the type of cutting: 100% of the IBA-treated and only 60% of untreated semi-woody cuttings have rooted, and IBA-pretreatment did not influence the rooting of the young green or woody cuttings (Stanilova & al. 2022). IBA lacked any rooting-promotion effect in the leaves of *Haberlea rhodopensis* cultivated on a vertical aero-hydroponic system, but pretreatment of petioles with IBA has significantly enhanced small rosette formation (Traykova & Stanilova 2020).

Literature data vary on the effect of hydroponic cultivation on the biomass yield of different medicinal plants and on the concentration of their secondary metabolites (Atherton & Li 2023). In an extensive review, the authors have compared the hydroponic cultivation of many medicinal species from different organs, including flowers and stems, and have stated that effectiveness of the cultivation depended on both the plant species and the hydroponic method. A good example of successful hydroponic cultivation, suitable for urban and peri-urban areas, has been set by *Mentha spicata,* which has shown that its yield, content of active compounds, and antioxidant effect have been much higher in hydroponically grown than in soil-grown plants (Surendran & al. 2017). The Nutrient Film Technique (NFT) has proved very appropriate for *Hypericum perforatum* and the plants grown in that system have manifested greater concentrations of secondary metabolites than the field-grown plants. However, the same method applied to *Crocus sativus* has led to biomass reduction and lesser flowering, as compared with the plants grown in soil or in aeroponics. The F&D system has proved effective for both St John's Wort and Saffron. Two other species from the *Lamiaceae* family, *Mentha piperita* and *Nepeta cataria,* also grew very well in a F&D system (known as Ebb and Flow System as well), even if the aeroponic cultivation was better and produced higher yields (Hayden 2006).

The metabolic profiles of hydroponically grown plants of *T. longedentatus* and *T. zygioides* corresponded to the profiles of the already studied parental plants (Nikolova & al. 2023; Nikolova & Aneva, unpubl.). The high contents of thymol and carvacrol found in the methanolic extract of *T. zygoides* in the present study was consistent with the reported high content of these isomers in the essential oil of the same species (Trendafilova & al. 2021).

Table 1. Metabolite profiles of hydroponically grown plants of *T. longedentatus* and *T. zygioides*.

Metabolite*	RT	*T. longedentatus*	*T. zygioides*
Phenolic acids			
Benzoic acid	8.02	4.2	13.7
4(p)-Hydroxybenzoic acid	12.64	2.2	7.7
Vanilic acid	14.71	3.8	4.1
Protocatechuic acid	15.70	1.0	2.6
Quinic acid	16.30	274.8	774.1
Gentisic acid	17.96	0.2	0.3
Hydroxycinnamic acid *trans*	18.07	3.0	41.3
Ferulic acid	20.57	1.7	27.2
Caffeic acid	24.74	14.5	21.5
Taxifolin	34.38		2.4
Chlorgenic acid	36.93	72.1	53.5
Rosmarinic acid	44.76	20.8	15.4
Fatty acids			
Octanoic acid	11.83	5.6	80.9
Hexadecanoic acid C16:0	20.01	62.2	297.7
Octadecanoic acid C18:0	23.43	8.1	50.0
Fatty alcohols			
Glycerol	8.21	396.2	1032.4
Octadecanol	21.73	0.2	1.5
Monoterpenes			
Thymol	8.67	34.8	473.5
Carvacrol	8.85	18.2	28.7
Hydroquinone	9.54	0.8	
Geranic acid	10.18	14.3	
Sterols and Triterpenes			
Stigmasterol	40.46	15.0	1.8
β-Sitosterol	42.06	8.4	37.5
Oleanolic acid	49.30	79.4	48.9
Ursolic acid	51.47	98.8	132.9
Organic acids			
Succinic acid	8.71	179.2	340.1
Glyceric acid	8.81	8.6	281.7
Malic acid	10.61	290.7	587.2
Polyols			
meso-Erythritol	11.00	8.3	147.7
Myo-Inositol	20.46	195.9	891.6
Saccharides			
Fructose 1	15.38	644.7	604.5
Fructose2	15.58	1083.8	1500.6
Glucose	17.09	580.5	329.8
Monosaccharide	18.50	1325.7	6573.9
Sucrose	29.68	1458.2	1239.1

*he values (μg) are mean of three independent experiments and represent the response ratios calculated for each compound relative to the internal standard.

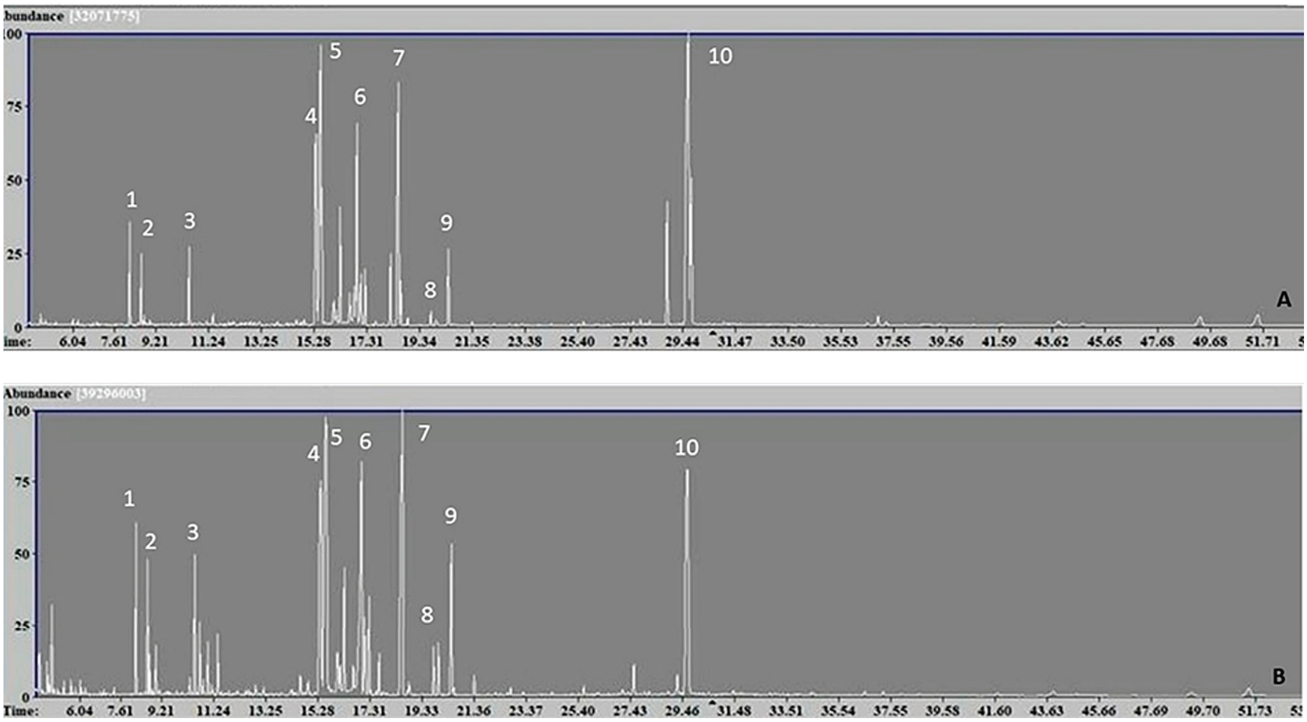

Fig. 10. GC/MS chromatograms of the hydroponically grown plants: **A**, *T. longedentatus;* **B**, *T. zygioides*: **1**. Glycerol; **2**. Thymol; **3**. Malic acid; **4**, Fructose 1; **5,** Fructose 2; **6**, Glucose; **7**, Monosaccharide; **8**, Hexadecanoic acid; **9**, Myo Inositol; **10**, Sucrose.

Conclusions

In spite of the species characteristics, rooting, survival and propagation of cuttings in all three target Thyme species have been much better in the tested hydroponic Flood & Drain system than in a soil substrate. Soilless cultivation of the target *Thymus* species has led to accelerated propagation, with minimum water, space, and labor consumption. It proved to be very appropriate for these species, given the difficult and slow rooting of cuttings by the conventional method. The metabolic profiles of hydroponically grown plants corresponded to those of the parental plants. The efficiency of hydroponic Thyme cultivation can be further improved and scaled up by using a vertical multistorey Flood & Drain system.

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