

Cultivation of Thymus by *In Vitro* And Hydroponics Combined Method

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Abstract—Our results showed that for the growth of qualitative seedling and vegetative raw material of *T. marschallianus* Willd. and *T. serpyllum* L. it is more profitable to use the *in vitro* and hydroponics combined method. In *in vitro* culture it is possible to do micro-propagation whole year with 98-99% rhizogenesis. 30000 micro-plants were obtained from one explant during 9 months. Hydroponic conditions provide the necessary microclimate for microplants where the survival rate without acclimatization was 93.3%. The essential oil content in hydroponic dry herb of both species in vegetative and blossom phase was 1.3% whereas in wild plants it was 1.2%, the content of extractive substances and vitamin C also exceeded wild plants. Our biochemical and radiochemical investigations indicated that the medicinal raw materials obtained from hydroponic and wild plants of Thymus species correspond to the demands of SPh XI, and the content of artificial radionuclides does not exceed the MACL.

Keywords—Hydroponics, *In vitro*, Micro-propagation, Thymus

I. INTRODUCTION

THE preservation of flora is vitally important ecological problem for humanity. By using natural recourses humanity negatively affects the formed ecosystems. As a consequence, the quantity of plant species decreases. During the last decades in Armenia the natural resources of certain individual plant species decreased. This happened as a result of the deforestation, overuse of water and natural resources, and the development of different economic spheres [11]. Therefore, by means of establishing cultured areas, the preservation of natural resources of valuable species of wild medicinal plants has ecological, agricultural, environmental and medical importance.

Thymus, one of the high-priced medicinal plants, belongs to *Lamiaceae* family. The species of Thymus are famous in all over the world with their potential of high-usage. The herb, essential oil and extracts of Thymus were used for thousands of years before and nowadays they are used worldwide particularly in food conservation, pharmaceutical, medicinal and cosmetic industries. It is known that 1g of essential oil is enough for 1kg milled meat to evaporate the specific smell

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from it and to lengthen its retention period. The aerial parts of the plant (fresh or dried) are used as a herb and spice. Thymus species have antibacterial, antifungal, antiviral, spasmolytic and antioxidant activities. The world price of Thymus essential oil is 1.2 million US dollars [3], and its global trade is 15.000ton annually. The major countries that import Thymus are the USA, Germany, Italy and Canada [13]. In the international market the demand for this plant is inclined to grow, and the natural resources of Thymus are not enough to satisfy such increasing requirements, which lead to the decrease of natural resources. Moreover, it becomes necessary to apply new biotechnological methods. The implementation of these methods in aseptic conditions for tissue culture and cell culture of plants allows to obtain genetically identical qualitative seedlings (clons) by induction of morphogenesis and clonal micro-propagation. Further hydroponic cultivation of these seedlings provides high yield [12]. Therefore, the implementation of *in vitro* and hydroponics combined method for cultivation, preservation and propagation of imported and local Thymus species is an actual problem.

II. MATERIALS AND METHOD

A. Collection of plant material

Plant materials for our investigation were the seeds of *T. serpyllum* L. and *T. marschallianus* Willd. The *T. serpyllum* was brought from Czech Republic whereas the *T. marschallianus* was brought from the natural areas of Dilijan, Armenia.

B. Explants preparation and culture condition

Seeds for introduction into aseptic condition were initially washed with water, sterilized in 96% ethyl spirit for 0.5 min followed by 0.1% diacid liquid for 15 min and washed three times with distilled sterile water. Planting of seeds was established in Petri dishes with 6g/l agar medium.

After seeds germination as explants for micro-propagation were served apical segments of plantlets. For clonal micro-propagation Murashige and Skoog (MS) [7] and ½MS mediums with half concentrations of micro- and macro-elements were used, prepared with 30g/l sucrose and solidified with 7g/l agar. For the root formation of cuttings was used Indolebutyric Acid (IBA). All the cultures were incubated in artificial chamber, at 23–27°C temperature, humidity 60-70%, light 5000 lux and with 16hr photoperiod [1], [6].

C. Hydroponic conditions

Micro-plants grown in *in vitro* conditions were planted in open air hydroponic pots with 5m² nutrition area. Volcanic slag was used as a hydroponic substrate. Planting was done by

15 plants per 1m² density. As a nutrient was used G.S.Davtyan's nutrient solution [2].

D. Biochemical and Radiochemical analyses.

Biochemical analyses have been made in dry medicinal raw materials. Essential oil content was determined by hydro-distillation method [4]. Sum of flavonoids was determined by spectrophotometric method [5], [14]. Also there were determined vitamin C [16], extractive substances, ash and water contents [8], [14]. Experiments were made with 4-5 replications.

The contents of ⁹⁰Sr and ¹³⁷Cs in the samples of *Thymus* were determined by the radiochemical method with low background radiometer UMF-1500 [9]. U was determined by means of extraction photometric method by application of Arsenazo III reagent [10]. Obtained data were compared with Maximum Allowed Concentration Limits (MACL) [15]. Statistical analysis was made by GraphPad Prism 5 demo.

III. RESULTS AND DISCUSSIONS

Our researches showed that the main methods during the recovery and rapid micro-propagation of plantlets by *in vitro* and hydroponics combined method are as follows

- selection of seeds or initial materials
- cultivation of plants in *in vitro* conditions
- checking of plants for infections
- rapid propagation of plant-regenerants in *in vitro* culture
- transplantation of micro-plants in open-air hydroponics
- propagation of plants in hydroponic conditions by cuttings and shrub division
- obtainment of medicinal raw materials from hydroponic and soil conditions.

The medium prepared with agar and without micro- and macro-elements was favorable for the germination of seeds. The beginning of germination was recorded after 4-5 days from planting. After 10 days the seeds germination of *T. serpyllum* was 99% and *T. marschallianus* 43%.

For further *in vitro* micro-propagation the explants of 0.4-0.5cm length were planted in 1/2MS medium containing 0.1 mg/l IBA. Root formation was recorded after 7-10 days. Micro-plants were obtained after 28-30 days, multiplication factor of which was 1:5. During the obtainment of the initial plants, the clonal micro-propagation method with activation of axillary buds was used, which was based on the removal of apical domination. The selected test tube plants were further propagated by micro-cuttings. The subculturing was done again in MS medium supplemented with 0.1mg/l IBA. It was found out that the micro-propagation of test tube plants in *in vitro* culture can be held throughout the year. But the best period is from spring to autumn. After 7-10 days rhizogenesis of explants was 98-99%. The length of micro-plants was 7-8cm after 28-30 days and the multiplication factor from one micro-plant was 1:6 (Fig 1).



Fig. 1 Micro-plants of *T. serpyllum* L. on 1/2MS medium containing 0.1mg/l IBA

The subculturing was held after every 30 days. During 9 months, 30 thousand micro-plants were obtained from one explant, which is 15 times more than traditional propagation method provides. In the second decade of April micro-plants were planted in open air hydroponic pots without acclimatization, where plants were fed 2-3 times a day. Hydroponic conditions provide necessary microclimate for micro-plants, giving an opportunity to exclude the acclimatization period due to it the survival rate of micro-plants was 93.3%. According to our biometric research in hydroponic conditions, micro-plants with the length of 2,5cm had plentiful growth from June to the end of September, when the length of the plants reached to 44cm (Fig 2).

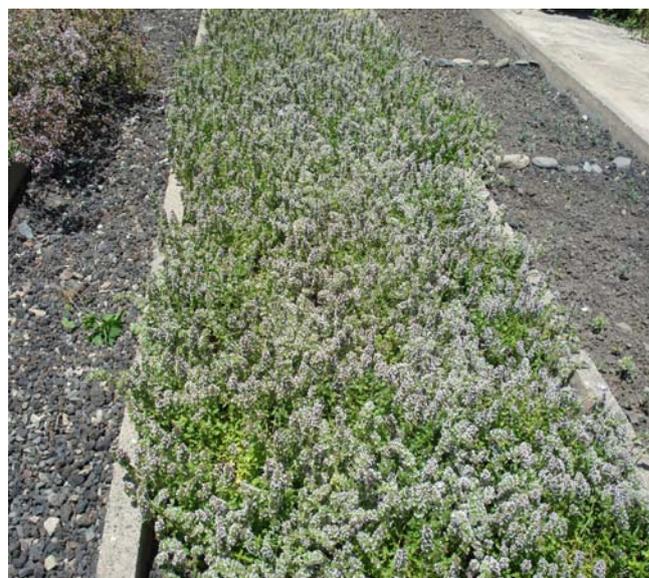


Fig. 2 The hydroponic culture of *T. serpyllum* L. in the first year of cultivation by micro-plants

TABLE I
THE BIOCHEMICAL INDICES OF THYMUS MEDICINAL RAW MATERIAL

± Standart Error of 4 replications

Place of growth and culture conditions	Thymus species	Essential oil, %	Extractive substances, %	Sum flavonoids, %	Vitamin C, mg/%	Total ash, %	Water, %
Yerevan, hydroponics	<i>T. marschallianus</i>	1.3 ± 0.1	40.2 ± 0.14	3.5 ± 0.42	41.0 ± 1.7	12.0 ± 0.28	10.0 ± 0.56
	<i>T. serphyllum</i>	1.3 ± 0.11	36.0 ± 0.14	4.3 ± 0.3	23.0 ± 0.84	6.7 ± 0.28	10.0 ± 0.7
Dilijan (wild), soil	<i>T. marschallianus</i>	1.2 ± 0.07	26.0 ± 0.14	3.6 ± 0.4	31.0 ± 0.6	14.8 ± 0.14	8.2 ± 0.28
	<i>T. serphyllum</i>	1.2 ± 0.08	28.0 ± 2.7	4.0 ± 0.56	20.0 ± 0.3	13.8 ± 1.27	10.0 ± 0.28

The yield of *T. serphyllum* dry vegetative raw material from second year of cultivation was 576.9g/m², which exceeded the yield of *T. marschallianus* 3.6 times. The yield of *T. marschallianus* and *T. serphyllum* dry matter cultured in hydroponics exceeded wild plants grown in soil conditions 1.3 and 2.0 times respectively.

In Table I are presented the biochemical characteristics of investigated species of Thymus. The essential oil content in hydroponic dry matter of *T. marschallianus* and *T. serphyllum*, in vegetative and full blossom phase was 1.3%, and in wild plants it was 1.2% which exceeded fresh matter 3.9 and 3.25 times, respectively. In the seed-formation period of plants the content of essential oil in fresh plants decreased from 0.4% to 0.25%, whereas the primary metabolites are mainly concentrated to the formation of new organs (seeds).

The most important secondary compounds such as extractive substances were high in hydroponic medicinal raw materials. Their content in *T. marschallianus* exceeded wild plants 1.5 times and in *T. serphyllum* exceeded 1.3 times.

As a result of our research it was found out that the sum flavonoids content in Thymus species was different. In *T. serphyllum* their content exceeded *T. marschallianus* 1.2 times. Although between the same species received from hydroponics and wild conditions was not any significant differences.

In hydroponic medicinal raw materials of *T. marschallianus* the vitamin C content exceeded wild plant 1.3 times, it also exceeded *T. serphyllum* hydroponics and wild plants 1.8 and 2.1 times respectively.

Radiochemical analyses were carried out in different radio-ecological stress areas which are as follows:

- Yerevan – Armenian nuclear power station with 30km radius zone.
- Dilijan – Armenian nuclear power station with 90km radius zone.

Our researches indicated an accumulation of artificial and natural radionuclides (RN) with various quantities in the same

radio-ecological stress conditions in different species of Thymus. Thus, in both zones (soil and hydroponic conditions) *T. marschallianus* with its radioactive indices U- 1.4, 1.1; Sr- 1.4, 1.4; Cs- 1.1, 1.5 and β activity- 1.2, 1.1 times, respectively exceeded *T. serphyllum*.

TABLE II
ARTIFICIAL AND NATURAL RN CONTENT IN MEDICINAL RAW MATERIAL OF THYMUS RECEIVED FROM DIFFERENT CONDITIONS

Place of growth and culture conditions	Thymus species	U.10 ⁻⁶ %	⁹⁰ Sr ¹³⁷ Cs	
			Bq/kg	
Yerevan, hydroponics	<i>T. marschallianus</i>	0.9	15.3	14.1
	<i>T. serphyllum</i>	0.8	10.8	9.4
Dilijan (wild), soil	<i>T. marschallianus</i>	1.1	15.5	14.2
	<i>T. serphyllum</i>	0.8	11.3	13.0
MACL		---	100	400

To all appearance it might be associated with biological characteristics of *T. marschallianus* and *T. serphyllum*. We found out that in vegetative raw materials of *T. marschallianus* grown in soil (wild) conditions in Dilijan and cultured in open air hydroponic conditions in Yerevan the artificial RN content was the same, but the natural RN was different. *T. marschallianus* grown in wild conditions accumulated U -1.2 times more compared with the hydroponic plants.

TABLE III
THE PART OF ^{90}Sr , ^{137}Cs IN TOTAL β ACTIVITY IN VEGETATIVE
RAW MATERIALS OF THYMUS

Place of growth and culture conditions	Thymus species	Total β activity Bq/kg	% from amount		Other RN
			^{90}Sr	^{137}Cs	
Yerevan, hydroponics	<i>T. marschallianus</i>	490	3.1	2.9	94.0
	<i>T. serphyllum</i>	430	2.5	2.2	95.3
Dilijan (wild),soil	<i>T. marschallianus</i>	460	3.4	3.1	93.5
	<i>T. serphyllum</i>	380	3.0	3.4	93.6

In vegetative raw material of *T. serphyllum* grown in both zones didn't show any significant differences between U and Sr contents. At the same time *T. serphyllum* grown in Dilijan in wild conditions accumulated ^{137}Cs , which exceeded 1.4 times the hydroponic plants. Data presented in Table III indicate that in vegetative raw materials of *T. marschallianus* and *T. serphyllum* grown in Dilijan wild conditions β radioactive RN content was low 1.1 times than in hydroponic plants. It is related with maximum K content in hydroponic plants compared with soil. Davtyan's nutrient solution which is used in hydroponics contains 300mg/l K. It is known that K due to ^{40}K content has β activity. It was found out that in *T. marschallianus* and *T. serphyllum* grown in hydroponics the total part of Sr and Cs, which are dangerous for human health, in β activity was low 1.1 and 1.4 times respectively compared with wild plants.

Therefore for the first time as a result of the investigation which has been done in Ararat valley in *in vitro* and open air hydroponic culture was established the opportunities and efficacy of Thymus growth by *in vitro* and hydroponics combined method. Hydroponics as a modern biotechnological method for receiving vegetative raw materials is useful and efficient for land poor countries such as Armenia. The intensive development of hydroponics will give an opportunity to involve thousandth hectares of saline, stony areas useless for agriculture.

IV. CONCLUSION

For the first time the investigation which was made in wild conditions of Dilijan and hydroponic conditions of Yerevan approved the opportunities and efficacy of *T. marschallianus* and *T. serphyllum* growth by *in vitro* and hydroponics combined method. Dry medicinal raw materials of *T. marschallianus* and *T. serphyllum* obtained from hydroponics by application of Davtyan's nutrient solution and volcanic slag exceeded wild dry raw materials 1.3 and 2.0 times respectively.

The essential oil content in hydroponic dry herbs of both species in vegetative and blossom phase was 1.3% whereas in wild plants it was 1.2%. In both species of hydroponic raw materials the extractive substances and vitamin C content

exceeded wild plants. The medicinal raw materials of *T. marschallianus* received from hydroponics and soil by the presence of vitamin C exceeded *T. serphyllum*, but by the content of sum flavonoids it does not exceeded.

By taking into consideration the content of artificial RN, medicinal raw materials of *T. serphyllum* cultured in hydroponic conditions in Yerevan were more pure than those grown in wild conditions in Dilijan. The medicinal raw materials of *T. serphyllum* grown in two radio-ecological areas of Yerevan and Dilijan were ecologically safer than those of *T. marschallianus* grown under the same conditions. In the raw materials of *T. marschallianus* and *T. serphyllum* grown in the wild conditions of Dilijan and in the hydroponic conditions of Yerevan the content of artificial RN does not exceed the MACL.

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