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Callus Culture of common thyme (*Thymus vulgaris* L.)

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ABSTRACT

The aim of the study was to investigate the influence of plant growth regulators on callus culture of common thyme (*Thymus vulgaris* L.) and the possibility of using such callus cultures to obtain rosmarinic acid. The explants used to initiate shoot culture were the seeds of common thyme. The seeds were individually placed into test tubes with MS culture media of macro- and micro-element content according to Murashige and Skoog (1962). For the purpose of initiation of cultures, leaves dissected along the vascular bundle were used. The leaves were placed on MS medium with the addition of BAP in combination with NAA in a concentration of 3 and 5 $mg \cdot dm^{-3}$. Propagation of callus cultures was conducted with the use of fragments of callus tissue placed on MS media supplemented with BAP used separately in a concentration of 3 $mg \cdot dm^{-3}$, and in combination with NAA (1, 2, 3 $mg \cdot dm^{-3}$) and 2,4-D in a concentration of 0.5, 1 or 1.5 $mg \cdot dm^{-3}$ respectively. In each stage of the experiment, explants placed on MS media without the addition of plant growth regulators were the control. It was found that initiation of common thyme cultures should be conducted on MS media without plant growth regulators. For the purpose of initiation of callus culture of common thyme, the optimal culture medium was MS with the addition of 3 $mg \cdot dm^{-3}$ BAP and NAA. Propagation of callus tissue of common thyme should be conducted on culture media supplemented with 3 $mg \cdot dm^{-3}$ BAP used in combination with 1 $mg \cdot dm^{-3}$ NAA. It was observed that the mass of the propagated callus tissue decreased with an increase of NAA content.

***Keywords*:** growth regulator, micropropagation, *in vitro* culture, secondary metabolite

1. INTRODUCTION

Air pollution, everyday stress, poor dietary habits may result in formation of reactive oxygen species (ROS) which damage DNA and cause severe mutations (Czajka 2006). Various serious diseases are attributed to ROS, e.g. arthritis, atherosclerosis, hypertension and neoplasms. In order to prevent the aforementioned diseases, antioxidants should be ingested in appropriate amounts (Łukaszewska 2007). However, synthetic antioxidants are not absorbed as well as their natural counterparts. Because of the rising costs of treatment with synthetic medicine, there is a tendency to use cheaper substances extracted from plants (Szajdek and Borowska 2004). One of the most valuable antioxidants is rosmarinic acid which exhibits strong antioxidant properties (Parus 2013). Rosmarinic acid is synthesized by various plant species (sage, common thyme, rosemary) which are widespread in Europe (Szajdek and Borowska 2004).

Common thyme (*Thymus vulgaris* L.) belongs to *Lamiaceae* family (Shabnum and Wagay 2011). It is also called garden thyme or German thyme (Mikołajczyk and Wierzbicki 1987). Thyme grows to a height of 10 – 30cm and is native to the Mediterranean region (Goodner and Mahattanatawee 2006).

Stalks of thyme (Photo.4) grow out of shallow-rooted and poorly branched rhizomes and are quadrangular along the entire length (up to the base of inflorescence) (Szafer and Kulczyński 1986). The flowers are lavender-pink, bisexual (larger) or female (smaller) and compiled into stems. The leaves of thyme are distributed in a crosswise manner, are small (4-8 mm in length), narrow lanceolate and short-stemmed, have curled leaf margin and are slightly hairy and brighter underside. The fruits are four-chambered schizocarps containing nutlets. Common thyme gives off the scent of thymol and has a bitter and spicy taste. Thyme is harvested in the flowering season (the upper section of the green shoots) and dried at the temperature of 35 °C (Kohlmuzer 2007). It is commonly sold in ground form, as dried stalks, essential oil and fresh plant (Goodner and Mahattanatawee 2006).

The plant was used by the ancient Greeks as incense in temples, and by ancient Romans as seasoning (Shabnum and Wagay 2011). Ancient Egyptians recognised the antiseptic and preservative effects of thyme and used it for embalming (Ciaciura and Umiatowska 2006).

The plant material contains more than 1% of essential oils. The main components of the essential oil are phenolic compounds, flavonoids, carvacrol, caffeic acid, tannins, pinene, linalool and thymol. Thyme has numerous beneficial properties, just to mention the antiseptic, carminative antimicrobial and antioxidant effects (Shabnum and Wagay 2011).

Extract of thyme aids the mechanisms of self-purification of airways as it liquefies viscous bronchial secretion enabling its removal (Danysz and Kwieciński 2007). Flavonoids (thymonin, cirsilineol, and 8-metoksycirsilineol) present in thyme leaf showed spasmolytic effects in *in vitro* studies (Van den Broucke 1983, Kania and Baraniak 2014). It was found that extract of thyme has antispasmodic effects with regard to smooth muscles of trachea and ileum of guinea pig (Kania and Baraniak 2014).

In vivo studies by Christoffel (1998) showed that the ethanol extract of thyme leaves in the dose of 162 mg/kg exhibited antiedematous effect comparable to that of phenylbutazone in the dose of 123 mg/kg of body weight.

Thyme oil contains phenolic compounds and has antimicrobial properties against *Klebsiella* and *Escherichia* and flu virus type A and RSV. Ethanol extract contains thymol and carvacrol and is known to inactivate development of pathogenic bacteria affecting the

airways *Moraxella catarrhalis,Klebsiella pneumoniae* and *Diphlococcus pneumoniae*, whereas acetone extract inhibits *Mycobacterium tuberculosis* (Nowak and Nawrot 2009). In terms of microbiological quality, the most biologically active compounds of *Thymus vulgaris* are caffeic acid, thymol, tannins and flavonoids.

Moreover, thyme oil shows fungostatic effects against yeast-like fungi *C. albicans* and *C. krusei*, and mould fungi: *Aspergillus flavus, A. ochraceus, A. parasiticus* and *Fussarium moniliforme* (Kania and Baraniak 2014).

Thyme is commonly used as a seasoning for various dishes. It is used to complete the taste of bouillon, marinade, stuffing, sauces, soups, salads and dishes slow-cooked in wine – poultry, shrimps, and game meat. Also, herbal liqueur Benedictine contains thyme as the key ingredient. Thyme is additionally used as an ornamental plant, grown as hedges and used as decoration of summer bouquet (Ciaciura and Umiatowska 2006).

Thyme oil shows high antioxidant activity which is stronger than that of BHT (butylated hydroxytoulene, E321) (Szajdek and Borkowska 2004). When added to meat, thyme preserves the colour of meat by inhibiting degradation of heme pigments, delays the formation of metmyoglobin, slows down protein oxidation, and inhibits the growth of bacteria and maliondialdehyde formation (Wereńska 2013).

There are some contraindications to the use of thyme essential oils, such as skin reactions, pregnancy and breastfeeding, age of less than 6, epilepsy, hypertension (Zdrojewicz and Minczakowska 2014).

Callus cultures are used for the purpose of secondary metabolite production (Nawrot-Chorabik 2015). Plant callus is a mass of undifferentiated cells developed from a particular tissueas a result of cell division of the primary explant. The whole seeds, fragments of organs (leaves, roots, stalks), cells and protoplasts can be used to initiate callus culture. Callus is rarely formed on the whole surface of an explant and it is formed mainly on the site of the cut. Development of callus depends on the species which was used to initiate the culture, the type of tissue used (preferably young tissue), the composition of the culture medium, time of cultivation and physical conditions.

Callus tissue may vary in terms of the structure (smooth/ granular), hardness (loose/ compact), colour (brown, green or white), ability to produce a given substance and the number of chromosomes (Nadolska-Orczyk 1990). Zia et al (2010) conducted a study in which a callus culture of common thyme was established. 10 days old explants were placed on basic MS medium supplemented with 3% sucrose and various concentrations of growth regulators (combinations with 2,4-D, NAA, Kin and BAP). The best results in terms of induction of callus culture were found for MS medium containing NAA and BAP in a 1:1 ratio.

For the purpose of callus culture initiation, Tamura et al. (1993) used MS culture medium supplemented with 3 g of sucrose and 0.9 g Difco Bacto Agar with the addition of growth regulators in various concentrations (NAA, IAA, 2,4-D, kinetin, auxin). The best effects were obtained with the use of a culture medium containing 1ppm NAA and 1ppm kinetin and 1ppm kinetin and 1 ppm 2,4-D.

The aim of the present paper was determination, based on the literature on the subject, of the possibility of using *in vitro* cultures for secondary metabolite production, particularly antioxidants, and identification of the content of plant growth regulators which is most appropriate for initiation and propagation of callus culture of common thyme (*Thymus vulgaris* L.) in *in vitro* cultures.

2. MATERIALS AND METHODS

2. 1. Initiation of cultures

The seeds of common thyme were used as explants for initiation of cultures. Prior to being transferred to media, the seeds were decontaminated by soaking for 10 sec in 70% ethanol solution, followed by 10 min in 10% sodium hypochlorite solution. Following decontamination, the seeds were rinsed three times with sterile distilled water under sterile conditions in a laminar flow cabinet.

The seeds which were prepared in the aforementioned way were then placed individually into 10 ml test tubes containing 5ml medium of macro- and micro-elements content according to Murashige and Skoog (1962), hereinafter referred to as MS medium. The initiated cultures were transferred to phytotron at the temperature of 25 °C. Half of the test tubes with seeds were lighted with the intensity of quantum irradiance of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 16 hours, and the other half was cultured in the dark. Observations were conducted each week for a period of one month and the number of germinating and contaminated seeds was determined.

In all phases of the experiment, the culture media were prepared using redistilled water. The pH of the media was adjusted to 5.7 (using 0.1 M HCl or NaOH solution), following the addition of plant growth regulators. The media were supplemented with 30 $g \cdot dm^{-3}$ sucrose, 7 $g \cdot dm^{-3}$ agar and 100 $mg \cdot dm^{-3}$ inositol and heated to boiling point for agar to dissolve. The test tubes or jars with the media (depending on the phase of the experiment) were autoclaved at the temperature of 121 °C for 20 min. The experiment was repeated twice.

The effect of growth regulators on initiation of callus tissue of common thyme

The second experiment was conducted using the leaves dissected along the vascular bundle and placed on MS culture media supplemented with 6-bezylaminopurine (BAP) combined with 1-naphthaleneacetic acid (NAA) in a concentration of 3 and 3 $mg \cdot dm^{-3}$, 3 and 5 $mg \cdot dm^{-3}$, 5 and 3 $mg \cdot dm^{-3}$, 5 and 5 $mg \cdot dm^{-3}$, respectively. The control sample was leaves placed on culture media without the addition of incretins. In a 330 ml jar containing 30ml of the culture media, 10 halves of leaves were placed. The jars were put into the phytotron at the temperature of 25 °C and lighted with the intensity of quantum irradiance of 50 $\mu mol \cdot m^{-2} \cdot s^{-1}$ for 16 hours. After a month, the calli were weighted to determine the optimal composition of plant hormones needed for callus initiation. The experiment was repeated twice.

The effect on growth regulators on propagation of callus tissue of common thyme

In the last stage of the research, the study material was callus tissue initiated on medium which was considered superior in the previous stage of the research, i.e. MS with the addition of $mg \cdot dm^{-3}$ BAP combined with 3 $mg \cdot dm^{-3}$ NAA. The fragments of callus tissue of a size of 2-3 mm and total weight of 0.5g were placed on media of macro- and micro-elements content according to Murashige and Skoog (1962), supplemented with 3 $mg \cdot dm^{-3}$ BAP used separately and in combination with NAA in a concentration of 1, 2 and 3 $mg \cdot dm^{-3}$ or 2,4-dichlorophenoxyacetic acid (2,4-D) in a concentration of 0.5, 1 and 1,5 $mg \cdot dm^{-3}$. After a month, the mass of explants of the propagated callus culture was determined [g]. The experiment was repeated twice.

3. RESULTS

3. 1. Seed germination in *in vitro* cultures

The seeds placed on MS medium started germinating as soon as in the first week, regardless of the applied lighting conditions (Fig. 1). The seeds in lighted cultures germinated gradually, up to the 4th week of the experiment. Germination under such conditions amounted to 16 seeds in total – 64% of all the seeds plated on medium (Photo 1). The seeds which were cultured in the dark stopped germinating after 2 weeks of the experiment and only 10% of the seeds germinated (40% of all the seeds plated on medium). Contamination of cultures was found as early as in the first week of the experiment, and the number of contaminated seeds was small (4%).

3. 2. The effect of growth regulators on initiation of callus tissue of common thyme

The highest mass (0.066 g) was found for callus propagated on medium supplemented with 3 mg·dm^{-3} BAP and NAA applied in combination (Fig. 2). The mass of callus tissue decreased with the increase of the content of plant growth regulators in medium. The mass of callus cultured on medium with 3 mg·dm^{-3} BAP and 5 mg·dm^{-3} NAA amounted to 0.058 g, and on medium supplemented with 5 mg·dm^{-3} BAP and 3 mg·dm^{-3} NAA – 0.041 g. The lowest mass (0.026) was found for callus cultured on medium with the addition of 5 mg·dm^{-3} BAP and NAA.

3. 3. The effect of growth regulators on propagation of callus tissue of common thyme

The highest mass (0.066 g) was determined for callus propagated on medium with 3 mg·dm^{-3} BAP + 1 mg·dm^{-3} NAA (Fig. 3, Photo 2). The mass of callus cultured on medium with 3 mg·dm^{-3} BAP and NAA was 0.055 g. In the case of medium containing 3 mg·dm^{-3} BAP, the mass of the callus amounted to 0.059 g. The lowest mass (0.024 g) was found for callus cultured on medium with the addition of 3 mg·dm^{-3} BAP + 1.5 mg·dm^{-3} 2,4-D (Photo 3).

4. DISCUSSION

Thyme is an aromatic and medicinal plant naturally occurring in America, North Africa and Europe. It is commercially cultivated on a large scale in many countries and therefore it is of high economic value. Thyme shows antimicrobial and antioxidant properties and is used in the cosmetics and food industry (Al-Ramamneh 2009). Due to growing interest in cultivation of herbaceous and medicinal plants, new methods are being investigated for the purpose of obtaining large amounts of high-quality seedlings showing genetic identity. The method which allows to obtain such seedlings quickly is *in vitro* culture method. Callus culture is a modification of the classic method of obtaining seedlings in *in vitro* cultures, and it can constitute an alternative to shoot cultures and open-field cultivation.

The presented research allowed to determine the appropriate content of plant growth regulators in medium for the best possible development of shoot and callus culture of common thyme. The study was divided into the following stages: growth initiation and propagation of callus culture.

Almost all fragments of plants can serve as initial explants, however apical meristems or seeds are used most frequently for the purpose of establishing a culture. Following appropriate decontamination, they are placed on media often containing only macro- and micro-elements. Such method of culture initiation was used, among others, in the case of *Campanula velebitica* (Stamenković et al. 2012), *Desmodium gangeticum* (Puhan and Rath 2012), *Helleborus niger* (Seyring 2002) and *Ocimum basilicum* (Dode et al. 2003). Similarly, own research employed the method of culture initiation by transferring the seeds on the medium of micro- and macro-elements according to Murashige and Skoog (1962).

Callus cultures were initiated on medium with the addition of 3 or 5 mg·dm^{-3} BAP applied in combination with NAA in the same concentration. The highest mass of developed callus was found for leaf explants placed on medium with 3 mg·dm^{-3} BAP and NAA. With the increase in concentration of both regulators the mass of the callus decreased – the lowest mass was determined for callus initiated on medium with the addition of 5 mg·dm^{-3} BAP and NAA. For the purpose of propagation of the previously initiated callus tissue, media supplemented with 3 mg·dm^{-3} BAP applied in combination with 1, 2 and 3 mg·dm^{-3} NAA or 0.5, 1 and 1.5 mg·dm^{-3} 2,4-D were used. Primary increase in mass was recorded for the medium with 3 mg·dm^{-3} BAP + 1 mg·dm^{-3} NAA, which constituted 97% of the control. The medium with 3 mg·dm^{-3} BAP + 1.5 mg·dm^{-3} 2,4-D proved to be least valuable – 34% of the control. The mass of the callus decreased with an increase in concentration of growth regulators (NAA and 2,4D).

For the purpose of establishing callus culture of eggplant Bari et al. (2007) used culture media supplemented with 2,4-D (1-3 mg·dm^{-3}) and BAP (0.05-0.5 mg·dm^{-3}). It was found that the medium with the addition of 3 mg·dm^{-3} 2,4-D and 0.15 mg·dm^{-3} BAP was the optimal medium for callus culture. The difference between the highest and the lowest mass amounted to 114%. In this case, the best effects were achieved using almost maximum concentration of growth regulators. In comparison to media with BAP and NAA, the highest increase (by 29%) was recorded for the medium with the addition of BAP and 2,4-D.

The experiments conducted as a part of the research allowed to determine the appropriate composition of the media for the purpose of the maximum propagation and rooting of common thyme. Due to its properties, thyme is a prospectively promising plant. A considerable advantage of thyme essential oil is the fact that it interacts well with synthetic medicine, and in most cases does not cause any side effects (Pawlik 2005). With adequate development of plants (higher mass obtained from a single plant),production costs could be reduced and, due to appropriate rooting, the plant would be more resistant to drought (Olszewska 2004).

5. CONCLUSIONS

On the basis of the conducted research, the following conclusions can be formulated:

Initiation of callus culture of common thyme (*Thymus vulgaris* L.) should be conducted on culture media of mineral composition according to Murashige and Skoog [1962] without the addition of plant growth regulators.

The optimal culture media for initiation of callus culture of thyme is that of a mineral composition according to Murashige and Skoog [1962] with the addition of 3 mg·dm^{-3} BAP and NAA.

Propagation of callus tissue of common thyme should be conducted on media supplemented with 3 mg·dm^{-3} BAP used in combination with 1 mg·dm^{-3} NAA. The mass of the propagated callus tissue decreases with an increase of NAA content.

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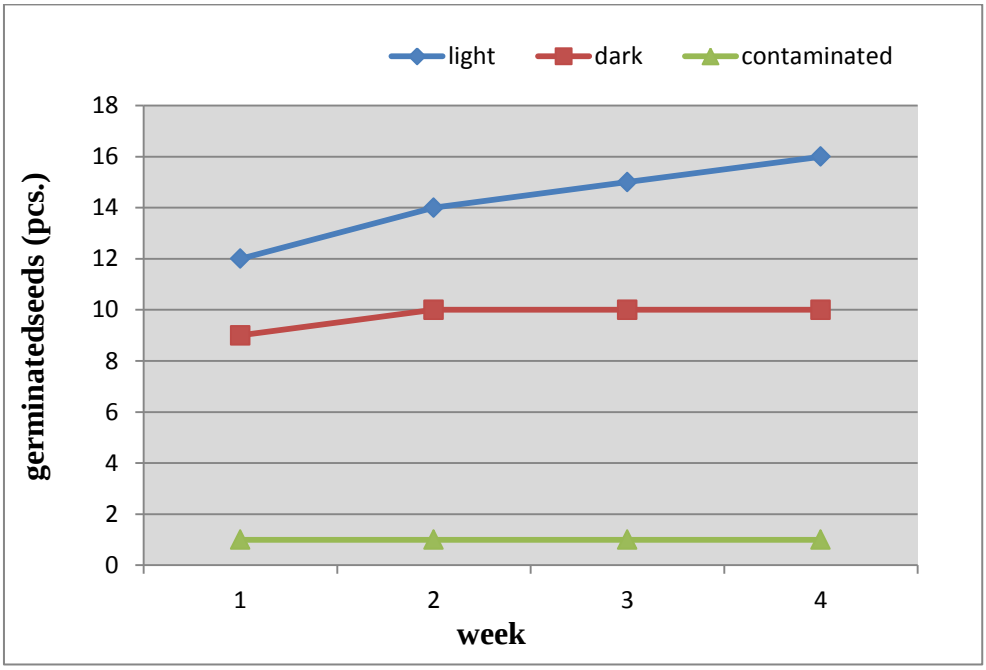

Figure 1. The number of germinated and contaminated seeds of common thyme, depending on the lighting conditions.

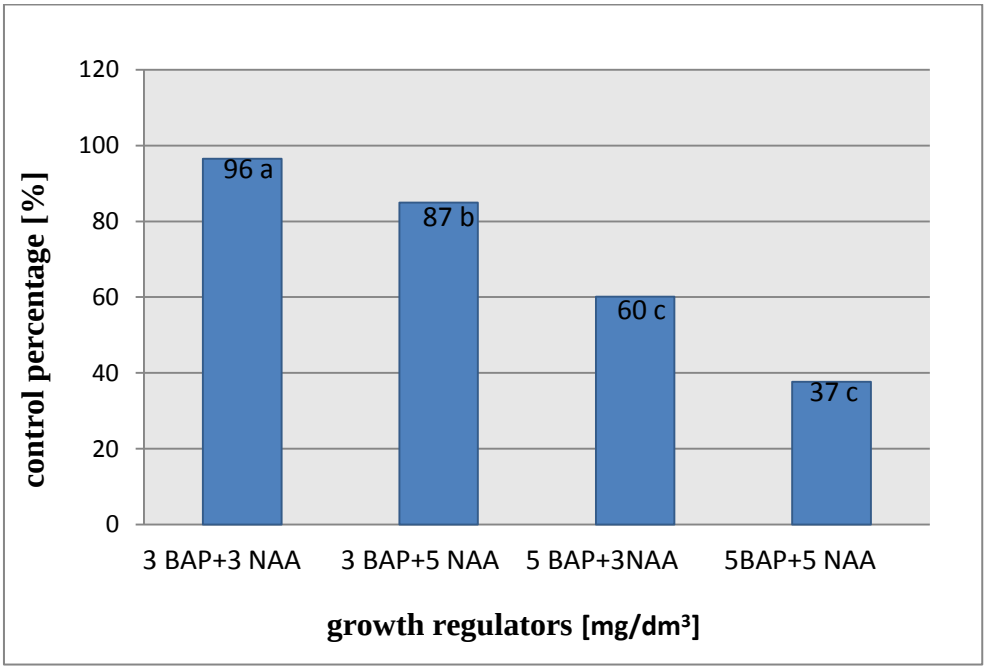

Figure 2. Mass of callus tissue induced from leaf explants of common thyme placed on medium with plant growth regulators.

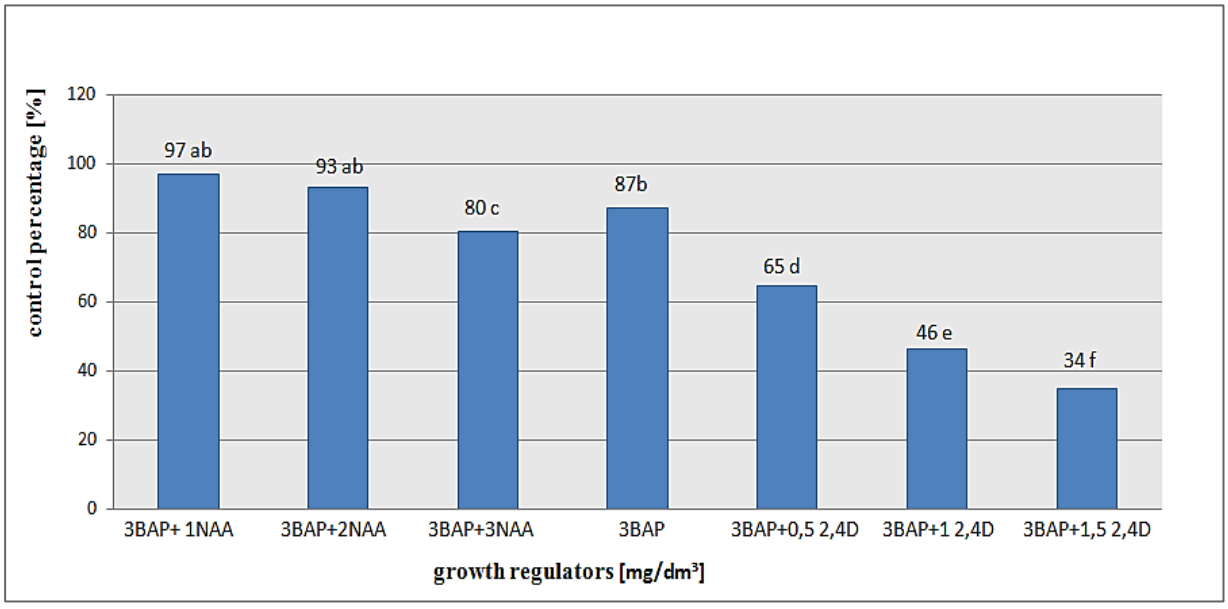

Figure 3. Mass of callus tissue of common thyme propagated on control medium and medium with plant growth regulators.

Photo 1. Seedlings of common thyme germinating in sterile cultures lighted 4 weeks after being placed on medium.

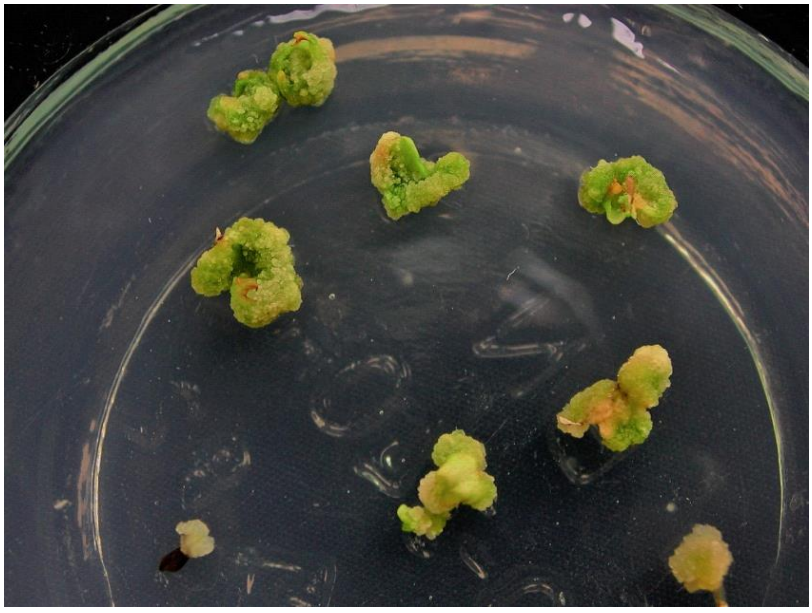

Photo 2. Callus tissue of common thyme propagated on medium supplemented with 3 mg·dm^{-3} BAP and 1 mg·dm^{-3} NAA

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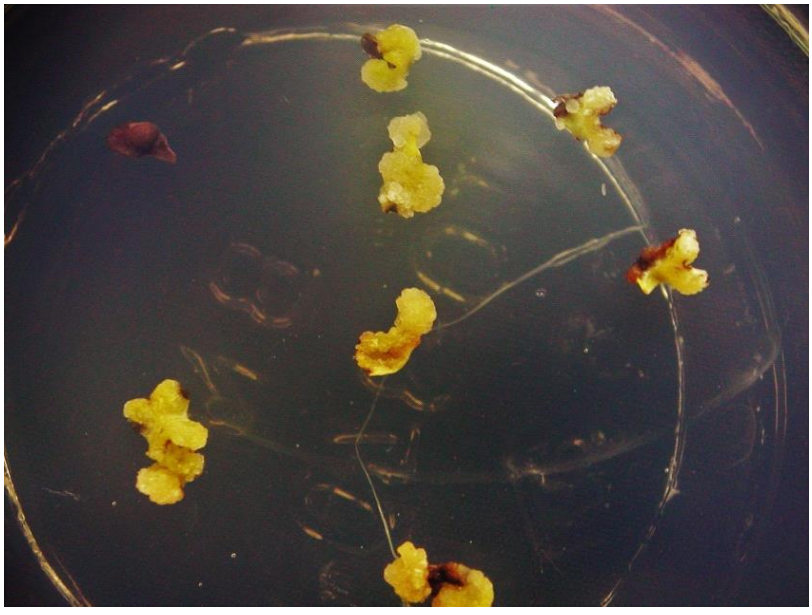

Photo 3. Callus tissue of common thyme propagated on medium supplemented with 3 mg·dm^{-3} BAP and 1.5 mg·dm^{-3} 2,4D

Photo 4. *Thymus vulgaris* L. (30)