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Evaluation Role of Activated Charcoal and Growth Regulators on Enhancement of Thymus Capitatus Micropropagation

Elmundr Abughnia[1], Abdulhamid Saleh[1], Sadeq Alzubik Belkair[1], Mohamed Salem[2] ,Kheri Lazrag[1] and Khaled Alhejaji[1]

[1]-Libyan Biotechnology Research Center, Department tissue culture plant

2- Faculty of Veterinary Medicine and Agricultural Sciences – University of Zawia

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ABSTRACT

Due to the presence of bioactive compounds that have medicinal uses, the thyme plant (*Thymus capitatus*) is under increasing pressure. The population has continuously declined due to this pressure, which puts it at risk of depletion and extinction. The aim of this study is to identify effective ways to preserve this plant *in vitro* using plant tissue culture technique, as well as to pinpoint the key factors that contributed to its successful vegetative propagation, rooting, and acclimatization. Plant materials were collected from the Al-Shaafiyin region, followed by surface sterilization. They were then cultured on a medium supplemented with a combination of the growth regulators BA and NAA along with activated charcoal for vegetative propagation, another combination of IBA, NAA, and activated charcoal for rooting, and a separate formulation for plant acclimatization. The results showed that the best treatment for obtaining vegetative growth was the use of activated charcoal, which significantly outperformed all other treatments in terms of plant height, number of leaves, and number of branches, recording values of 3.03 cm, 76.4 leaves, and 5.5 branches, respectively.

This is attributed to its ability to absorb growth-inhibiting substances. The results also indicated that activated charcoal was the most effective treatment for root development, showing a significant superiority over the other treatments, with an average of 5 roots and a root length of 6.2 cm. Activated charcoal improved the properties of the growth medium by adsorbing inhibitory compounds and providing a phenol-free environment conducive to root development. Furthermore, the experiment demonstrated that a 1:1 (v/v) mixture of soil and peat moss resulted in the highest plant survival rate (80%), which is likely due to the balanced physical and chemical properties of the mixture. The use of activated charcoal in micropropagation programs is recommended due to its effectiveness in enhancing both shoot and root development, thereby creating a more favorable environment for plant tissue growth. It is also advisable to avoid culture media lacking activated charcoal. For the acclimatization stage, a 1:1 (v/v) mixture of soil and peat moss is recommended, as it provides adequate aeration, moisture retention, and essential nutrients necessary for plant survival.

Introduction

Thyme is an important medicinal and aromatic plant widely found across various regions of Libya. It belongs to the genus *Thymus*, which comprises over 200 species globally (Jamshidi & Cohen, 2017). The most common species in Libya is wild thyme (*Thymus capitatus*), also known as *Thymbra capitata*. This species naturally grows in coastal and mountainous regions such as the Nafusa Mountains, Western Mountains, and the al Jafara

Corresponding author: E-mail: nme755@yahoo.co.uk

region. It thrives in rocky limestone soils and dry climates, exhibiting notable tolerance to drought and salinity (El-Moghraby, 2005). Thyme is a perennial herbaceous plant, reaching heights of 30–60 cm, with small leathery leaves that emit a strong aroma due to volatile oils rich in the antimicrobial compound carvacrol (Bounatirou et al., 2007). Local studies indicate that Libyan thyme contains higher concentrations of active compounds than other varieties, supporting its traditional use as a flavoring agent and in folk medicine to treat digestive and respiratory ailments (Ben Miri et al., 2023).

Given the increasing demand for thyme's medicinal compounds and its declining wild populations, preservation and propagation efforts are essential. Plant tissue culture technology offers a promising solution for conserving *Thymus capitatus* by enabling rapid, large-scale, and disease-free propagation. This method is particularly valuable for producing high-quality plants free of viruses and bacteria, which can affect plants propagated through conventional means (Boukef et al., 2021). Additionally, tissue culture supports the production of plants in limited space and time, addressing resource scarcity in harsh agricultural environments such as parts of Libya.

Beyond propagation, tissue culture aids in conserving genetic resources of thyme species threatened by overharvesting and environmental changes. Clonal propagation ensures the preservation of desirable traits such as drought resistance and high volatile oil content. Furthermore, tissue culture techniques facilitate the development of suspended cell cultures capable of producing secondary metabolites industrially, bypassing the need for traditional cultivation (Rahman et al., 2022).

In light of the medicinal and economic significance of thyme, this study aims to propagate *Thymus capitatus* using plant tissue culture. It focuses on identifying factors that enhance vegetative growth and root development in vitro, with the goal of producing and preserving this valuable genetic resource under controlled laboratory conditions.

Materials and Methods

This experiment was carried out at the Plant Tissue Culture Laboratory of the Libyan Center for Biotechnology Research. Study aimed to evaluate the effects of activated charcoal and varying concentrations of growth regulators Benzyladenine (BA) and kinetin (Kin) on vegetative propagation, on the other hand indole-3-butyric acid (IBA) and naphthaleneacetic acid (NAA) Were used for *in vitro* rooting of *Thymus capitatus*

Plant Materials

Plant samples were collected in April 2024 from the Al-Shaafin Reserve in Masalata. The precise geographical coordinates of the collection site were recorded using a GPS device and verified with Google Earth Pro (Figure 1).

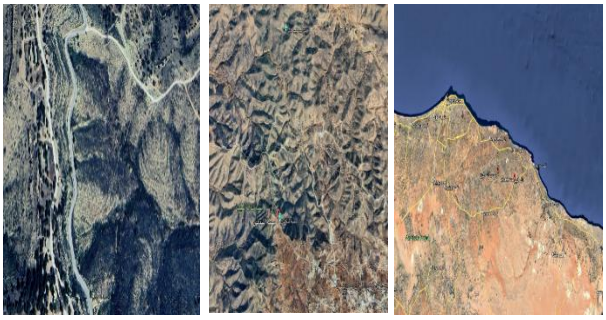

Figure (1): An aerial image of the collection site of thyme (Thymus spp.) explants, located at 32°43'25.36" N and 13°22'50.05" E.

Plant sterilization

Upon arrival at the laboratory, single-node explants of *Thymus capitatus* were thoroughly washed under running tap water for 30 minutes. Surface sterilization was then carried out in an isolation room. The explants were first immersed in 70% ethyl alcohol for two minutes with continuous stirring, followed by immersion in 20% sodium hypochlorite solution containing one drop of Tween 20 per 100 mL for 20 minutes, also with continuous stirring. To remove residual sterilizing agents, the explants were rinsed three times with sterile distilled water for five minutes each. This sterilization protocol achieved an 80% germination rate and a 20% contamination rate, demonstrating its effectiveness in providing a sufficient number of viable explants for subsequent culture. Previous studies have similarly reported the use of 2–3% sodium hypochlorite for sterilizing single cuttings of Thymus vulgaris without compromising tissue vitality.

Figure (2): Thyme plant growing in its natural environment

Sterilization of the culture medium (MS)

Plant samples were cultured in Murashige and Skoog (MS) medium supplemented with 3% sucrose and 7% agar, with the pH adjusted to 5.7-5.8. Twenty milliliters of the prepared medium were then placed in 200-ml jars and autoclaved at 121°C and 1.02 bar for 20 minutes before inoculation.

Establishment of Aseptic Tissue Cultures

To ensure sufficient, contamination-free plant material for subsequent stages, single-node, 1-centimeter-tall plants were sterilized and defoliated before being placed in culture pots inside an isolation cabinet. Two plants were grown in each pot. The inoculated pots were then transferred to a germination chamber maintained at 25°C, with LED lamps operating at 16 hours on and 8 hours off, for six weeks.

Plant Propagation (Multiplication Stage)

After four weeks of initial growth and successful establishment of contamination-free cultures, the explants were subcultured onto fresh MS media for an additional four weeks. For this propagation phase, the MS culture medium was supplemented with activated charcoal at a concentration of 3 g/L, and various concentrations of growth regulators: BA (1, 1.5, and 2 mg/L) and Kin (1, 1.5, and 2 mg/L). A control treatment (without activated charcoal or growth regulators) was also included. Each treatment consisted of 10 jars, with two single-node explants (1 cm in length) planted per jar. The planted jars were maintained under the same environmental conditions as the previous planting. After 8 weeks of culture, data were recorded for the following characteristics: length of vegetative growths, number of branches, and number of leaves.

Root Formation

Following four weeks in the multiplication phase, explants were subcultured onto MS medium for an additional four weeks to induce rooting. This medium was supplemented with activated charcoal at the concentration of 3 g/L and the growth regulators IBA and NAA at concentrations of 1, 1.5, and 2 mg/L. A control treatment was also included. Each treatment consisted of 10 replicates, with each replicate containing two 1 cm long explants (single nodes). The planted jars were maintained under the same conditions as the previous stages. After 8 weeks of culture, data were collected on the length of vegetative growth, number of branches, number of leaves, and specifically, root length and number of roots.

Plant acclimatization

After obtaining plants with well-developed vegetative and root systems from the *in vitro* stages, the plants were carefully removed from the jars, and residual culture medium was washed from their roots with water. Peat moss and sand were sterilized in an autoclave. Subsequently, pots were prepared with different volume/volume (v/v) ratios of peat moss and sand: 1:1, 2:1, 1:2, 1:0 (soil only), and 0:1 (peat moss only). The pots were then covered with a plastic cover for two weeks, after which the cover was gradually removed. After three weeks, the plants were transferred to a greenhouse, maintaining them in the same peat moss and sand media. The survival percentage of the well-growing plants was then estimated during the acclimatization process.

Data analysis

Data recorded included plantlet length, number of branches, number of leaves for vegetative growths, and root length and number of roots. The experiment was designed with 10 replicates at each stage. Statistical analysis of the results was performed using R software (version 4.0.3; R Core Team, 2020).

Results and Discussion

Plant Sterilization

The sterilization protocol employing a 2% sterilizing solution yielded an 80% germination rate and 20% contamination. This outcome indicated a successful sterilization process, providing a sufficient number of viable explants for subsequent experimental stages. These findings align with previous studies that demonstrate the effectiveness of sodium hypochlorite (NaOCl) at concentrations ranging from 2-3% for sterilizing single cuttings of Thymus vulgaris without adversely affecting tissue vitality (El-Bakry et al., 2018).

Evaluation of Vegetative Growth

The study revealed that the addition of activated charcoal to the MS medium at a concentration of 3 mg/L significantly improved the average plant height (Figure 3). The highest average plant height (3.03 cm), number of leaves (76.4), and number of branches (5.5) were recorded with the activated charcoal treatment, outperforming all other treatments. This significant superiority is attributed to activated charcoal's ability to absorb inhibitory phenolic compounds secreted from the plant tissues of thyme plants during cultivation, which can hinder plant growth. Additionally, activated charcoal purifies the culture media from toxic substances and reduces the concentration of excess auxins, creating a suitable environment for plant growth (Sani et al., 2022). Activated charcoal also purifies the culture media from toxic substances and reduces the concentration of excess auxins, making it a suitable environment for plant growth (Thomas, 2008). The results showed a gradual increase in average plant length with rising BA concentration, from 2.13 cm at 1 mg/L to 2.69 cm at 2 mg/L. These findings are consistent with results reported by Kalidass et al., 2023). For the growth regulator kinetin, a concentration of 2 mg/L yielded the highest length of 2.75 cm, compared to a relative decrease at 1.5 mg/L (1.95 cm). The activated charcoal treatment demonstrated significant superiority in plant length, reaching 3.03 cm. This is attributed to its ability to absorb growth-inhibiting substances such as phenols (Pan & van Staden, 1998; Khalilsaraie et al., 2015).

As shown in Figures 4 and 5, the activated charcoal treatment significantly outperformed the other treatments in terms of both the number of leaves and branches. It recorded the highest leaf count (76.4), highlighting its effectiveness in enhancing the growth environment by absorbing inhibitory compounds, purifying the medium, and improving nutrient uptake. Regarding the number of leaves and branches, as illustrated in Figures 5 and 6, the results demonstrated that the activated charcoal treatment significantly outperformed the other treatments, producing the highest number of leaves (76.4).

This suggests that, beyond its ability to enhance nutrient absorption, activated charcoal effectively improves the growth environment by absorbing inhibitory compounds and purifying the medium. These findings align with the observations reported by Khalil Saraei et al. (2015). However, the optimal treatment was found to be 1 mg/L Kin, which produced 64.4 leaves. This indicates that the growth regulator Kin effectively promotes cell division and adventitious bud formation. In comparison, BA at 2 mg/L resulted in 56.8 leaves. Notably, the different concentrations of BA and Kin did not demonstrate the same level of efficiency.

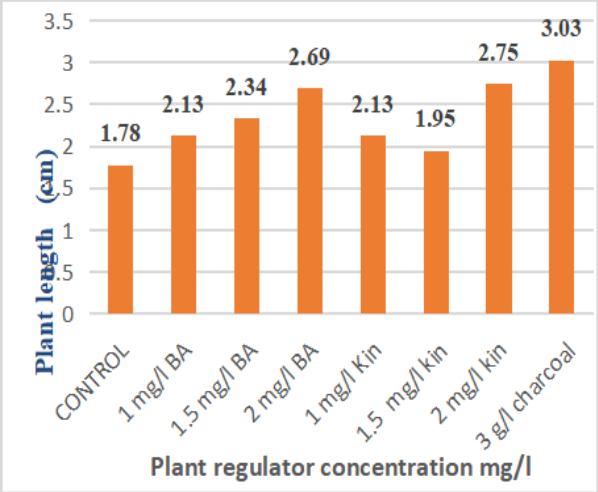

Figure (3): Effect of growth regulators and activated charcoal on the mean plant height of *thymus capitatus*

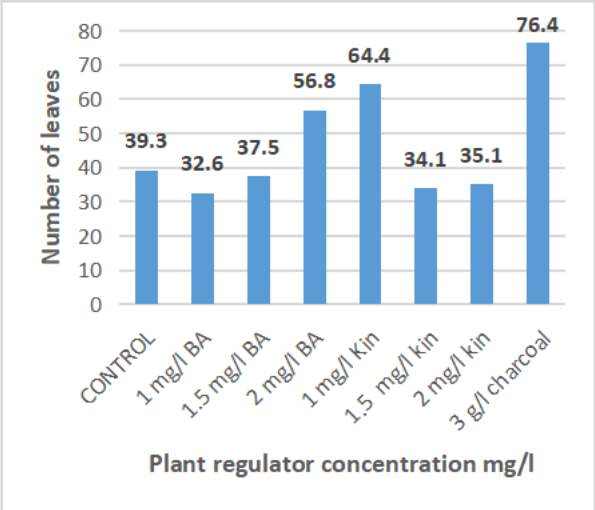

Figure (4): Effect of growth regulators and activated charcoal on the average leaves number of *thymus capitatus*

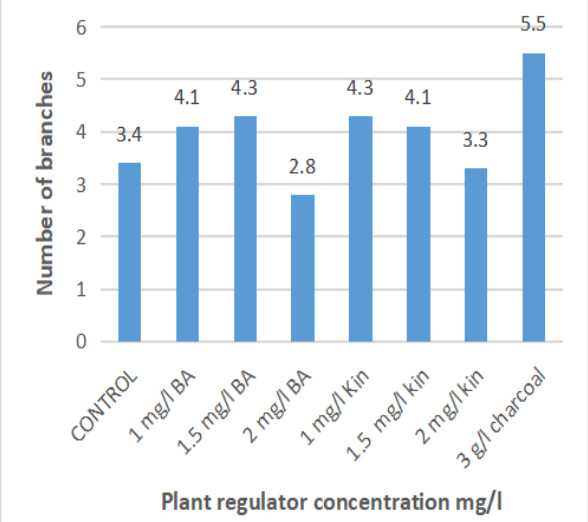

Figure (5): The effect of growth regulators and activated charcoal on the mean branches number of *thymus capitatus*

The results revealed variations in the average root length of thyme plants (Figures 6, 7, and 8). The activated charcoal treatment achieved the greatest root length at 6.2 cm, significantly outperforming the other treatments. This was followed by the 1.5 mg/L IBA treatment with a root length of 5.3 cm, while the 2 mg/L NAA treatment recorded the shortest roots at 2.8 cm.

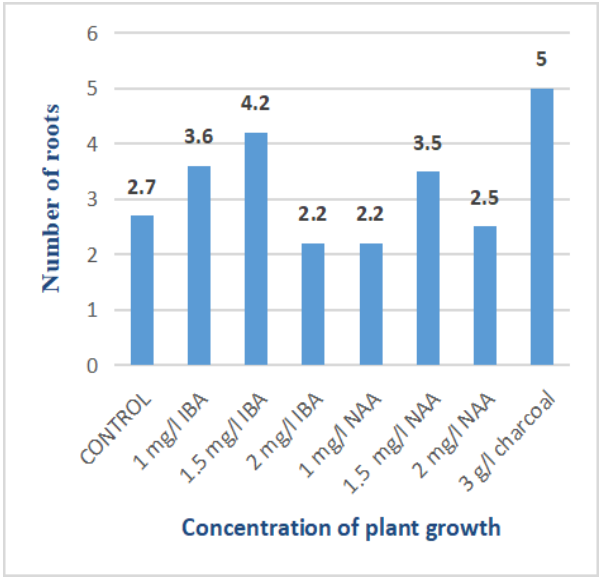

Figure (6): Effect of growth regulators and activated charcoal on the mean number of roots in *Thymus capitatus*

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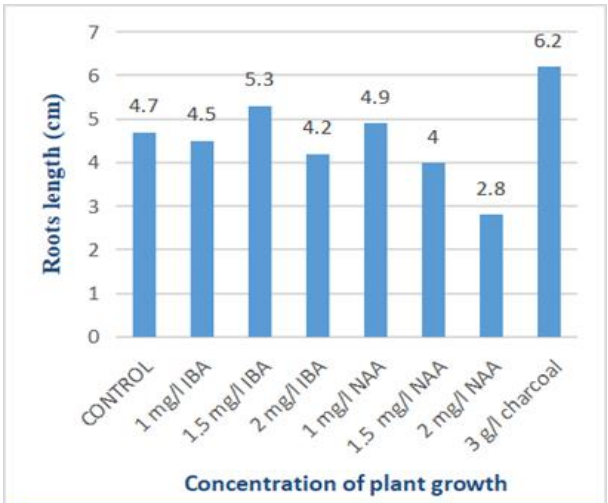

Figure (7): Effect of growth regulators and activated charcoal on the mean root length of *Thymus capitatus*

Ask ChatGPT

Figure (8): Growths of explants grown in MS medium supplemented with activated charcoal.

The superiority of the activated charcoal treatment is attributed to its ability to improve the growth medium by absorbing inhibitory compounds and creating an environment free of phenols and toxic ions that can impede root development, as noted by Pan & van Staden (1998). Additionally, activated charcoal helps stabilize the pH, which indirectly promotes root growth. Regarding the growth regulator IBA, treatments particularly at 1.5 mg/L demonstrated a positive effect in stimulating root elongation. This aligns with numerous studies that highlight IBA's effectiveness among auxins in promoting adventitious root formation, due to its slow decomposition and efficient absorption by plant tissues.

In contrast, increasing the NAA concentration to 2 mg/L inhibited root growth, consistent with George et al. (2008), who highlighted that certain auxins like NAA can become toxic or ineffective at high doses. Based on these findings, activated charcoal and IBA at 1.5 mg/L are the optimal treatments for promoting root growth in thyme. The results for root count showed that adding activated charcoal at a concentration of 3 g/L produced the highest average number of roots, significantly outperforming all other treatments. This beneficial effect is attributed to activated charcoal's ability to absorb rooting-inhibiting compounds such as excess phenols and ethylene (Yaseen et al., 2020). Additionally, activated charcoal may indirectly enhance auxin uptake, further stimulating root development (Ali et al., 2023).

Plant Acclimatization

The experiment evaluated plant acclimatization using different ratios of peat moss and sand: (1:1), (2:1), (1:2), (1:0), and (0:1) v/v. The results showed that the medium with a 1:1 v/v ratio of soil to peat moss outperformed the others, achieving an 80% survival rate of thyme (*Thymus capitatus*) plants derived from tissue culture. This medium also recorded the highest average success rate, root growth, and number of leaves compared to using soil or peat moss alone.

The superiority of this mixture is attributed to its balanced physical and chemical properties: peat moss provides excellent moisture retention and aeration, while soil supplies essential mineral nutrients necessary for plant growth (Khodadadi et al., 2021).

The study results indicated that the addition of activated charcoal at a concentration of 3 mg/L was the most effective treatment in promoting both vegetative and root growth of thyme, recording the highest average plant length (3.03 cm), number of leaves (76), and number of shoots (5.5) compared to all other treatments. This superiority is attributed to the ability of activated charcoal to absorb inhibitory phenolic compounds secreted by plant tissues during cultivation, detoxify the medium of harmful substances, and reduce excess auxin levels, in addition to its role in stabilizing pH and enhancing nutrient uptake (He, 2024; Permadi, 2024). Recent studies have also reported that activated charcoal improves root length and dry root biomass in soybeans (Barbosa, 2024), and its presence has been associated with stimulating gene expression related to the phenylpropanoid pathway, thus supporting root growth (Ebrahimi, 2024). Regarding plant growth regulators, cytokinins have shown a clear effect; BA at 2 mg/L produced the highest plant height (2.69 cm), while Kin at 1 mg/L resulted in the highest number of leaves (64.4), confirming the role of cytokinins in stimulating cell division and axillary bud formation. In contrast, auxins exhibited differential effects on root growth: IBA at 1.5 mg/L achieved the greatest root length (5.3 cm), due to its slow degradation and efficient uptake by plant tissues, making it the most effective auxin for stimulating root growth. Conversely, NAA at 2 mg/L reduced root length (2.8 cm) due to its toxic effects and ethylene stimulation, which inhibits rooting. Therefore, combining activated charcoal with IBA at 1.5 mg/L represents the optimal strategy for promoting root growth, while activated charcoal with Kin at 1 mg/L was the most efficient at improving vegetative growth. This suggests that activated charcoal acts as an indirect promoter, balancing the activities of auxin and cytokinin and providing a favorable environment for integrated plant growth (Barbosa, 2024; Ebrahimi, 2024; He, 2024).

Conclusion

This study successfully demonstrated the significant role of activated charcoal and specific growth regulators in enhancing the *in vitro* propagation of *Thymus capitatus*. The results unequivocally showed that the inclusion of activated charcoal in the micropropagation program led to superior shoot and root development. Its effectiveness is primarily attributed to its ability to adsorb inhibitory phenolic compounds and purify the growth medium, thereby creating a more favorable environment for plant tissue growth. Based on these findings, it is highly recommended to incorporate activated charcoal into micropropagation protocols for *Thymus capitatus*, and to avoid culture media lacking this component. For successful acclimatization, a 1:1 (v/v) mixture of soil and peat moss proved to be the most effective substrate, resulting in the highest plant survival rate (80%). This is likely due to the balanced physical and chemical properties of the mixture, which provide adequate aeration, moisture retention, and essential nutrients crucial for plant survival during the transfer from *in vitro* to *ex vitro* conditions. Overall, these findings provide valuable insights and practical recommendations for optimizing the *in vitro* propagation and conservation efforts of *Thymus capitatus*, a species facing increasing pressure due to its medicinal value.

Conflict of interest: The authors declare that there are no conflicts of interest

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