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Assessment of callus induction and micropropagation of two medicinal plants

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Abstract

This study investigated optimized protocols for callus induction and micropropagation of two Algerian medicinal plants, *Origanum* sp. and *Mentha* sp., evaluating synthetic plant growth regulators (PGRs) and natural extracts (*Pistacia atlantica* galls and *Cinnamomum* spp.). Results demonstrated that kinetin (0.5–1.0 mg/L) and *P. atlantica* galls extract (50 mg/L) significantly enhanced shoot proliferation in *Origanum* sp., while *Cinnamomum* extract (50 mg/L) promoted early bud initiation, though field-derived *Mentha* sp. explants exhibited high contamination rates, emphasizing the superiority of sterile *in vitro*-propagated material. Optimal callus induction occurred with 2,4-D (0.5–1 mg/L) and kinetin (0.5–1 mg/L) in *Origanum* sp., although oxidative browning after four weeks indicated the need for antioxidant supplementation. The study validated micropropagated explants as contamination-resistant alternatives to field-collected material and highlighted natural extracts as viable biostimulants, providing a foundation for sustainable conservation and pharmaceutical utilization of Algeria's medicinal flora through integrated traditional and biotechnological approaches.

Keywords: medicinal plants, tissue culture, plant growth regulators, natural biostimulants, sterile propagation.

Résumé

Cette étude a développé des protocoles optimisés pour l'induction de cals et la micropropagation de deux plantes médicinales algériennes, *Origanum* sp. et *Mentha* sp., en évaluant des régulateurs de croissance synthétiques (PGR) et des extraits naturels (galles de *Pistacia atlantica* et *Cinnamomum* spp.). Les résultats ont démontré que la kinétine (0,5-1,0 mg/L) et l'extrait de galles de *P. atlantica* (50 mg/L) améliorent significativement la prolifération des pousses chez *Origanum* sp., tandis que l'extrait de *Cinnamomum* (50 mg/L) favorise l'initiation précoce des bourgeons. Cependant, les explants de *Mentha* sp. prélevés en milieu naturel ont présenté des taux de contamination élevés, soulignant la supériorité du matériel stérile propagé *in vitro*. L'induction optimale de cals a été obtenue avec du 2,4-D (0,5-1 mg/L) et de la kinétine (0,5-1 mg/L) chez *Origanum* sp., bien qu'un brunissement oxydatif après quatre semaines ait indiqué la nécessité d'une supplémentation en antioxydants. L'étude a validé les explants micropropagés comme alternative résistante à la contamination par rapport au matériel collecté sur le terrain et a mis en évidence le potentiel des extraits naturels comme biostimulants viables. Ces travaux établissent une base pour la conservation durable et l'utilisation pharmaceutique de la flore médicinale algérienne grâce à une approche intégrant les savoirs traditionnels et les biotechnologies.

Mots-clés : plantes médicinales, culture tissulaire, régulateurs de croissance, biostimulants naturels, propagation stérile.

الملخص الأكاديمي

هدفت هذه الدراسة إلى تطوير بروتوكولات مثلى لتحريض الكالوس والإكثار الدقيق لنباتين طبيين جزائريين، *الأوريجانوم والنعناع*، من خلال تقييم منظمات النمو الصناعية والمستخلصات الطبيعية (أورام نبات *البتيسيا* ومستخلصات *القرفة* .) أظهرت النتائج أن استخدام الكاينتين (0.5-1.0 ملغ/لتر) ومستخلص أورام *البتيسيا* 50)ملغ/لتر) عزز بشكل ملحوظ تكاثر البراعم في نبات *الأوريجانوم*، بينما ساعد مستخلص *القرفة* 50)ملغ/لتر) على تحفيز تكوين البراعم المبكر. ومع ذلك، لوحظت معدلات تلوث عالية في عينات *النعناع* المأخوذة من البيئة الطبيعية، مما يؤكد تفوق المواد المعقمة المكاثرة معملياً. تم تحقيق أفضل نتائج لتحريض الكالوس باستخدام 2،4-د (0.5-1 ملغ/لتر) مع الكاينتين (0.5-1 ملغ/لتر) في *الأوريجانوم*، رغم ظهور اسمرار تأكسدي بعد أربعة أسابيع يستدعي إضافة مضادات أكسدة. أكدت الدراسة جدوى استخدام الأنسجة المكاثرة معملياً كمصادر مقاومة للتلوث مقارنة بالعينات الميدانية، كما أبرزت إمكانية استخدام المستخلصات الطبيعية كمنشطات حيوية فعالة. توفر هذه النتائج أساساً علمياً لحفظ الموارد النباتية الطبية الجزائرية واستغلالها المستدام في المجال الصيدلاني من خلال دمج المعارف التقليدية مع التقنيات الحيوية الحديثة.

الكلمات المفتاحية :النباتات الطبية، زراعة الأنسجة، منظمات النمو، المنشطات الحيوية الطبيعية، الاسمرار التأكسدي، الإكثار المعقم

Dedication

To myself...

To the one born from the patience of prophets, the defiance of mountains, and the longing of the heavens.

To the one who walked alone through nights with no moon, becoming her own light, her own guide.

To the soul that was exhausted, yet never shattered — the more the path hardened, the more she bloomed.

To the heart that witnessed betrayal yet never sealed shut, that embraced pain yet never abandoned its glow.

To me...

Daughter of determination, writer of glory, and disciple of destiny.

You, who knelt in secret before God in the darkness of fear, raising your hands in tears, asking for light — and He sent you peace like a gentle rain.

This work is not merely the fruit of intellect, but a prayer hidden deep in the night — and it was answered.

To my father...

The first pillar of manhood, the bearer of heavy words, the one whose back never falters, no matter how fierce the storm.

To my mother…

The wellspring of unwavering light, the harbor of safety in the tempests of my days.

To my sister…

The light flowing through my veins when the world dims around me.

To my little brother…

The star of my childhood and companion of my heart — the innocent dreamer who taught me that simplicity is the purest form of joy.

To my friends…

Those who came to my joy like a song, and to my sorrow like a prayer.

Those who understood my silence better than my words, who planted seeds of hope in a field nearly gone dry.

You were answered prayers in human form — who read the grief between my lines, and held my weakness as if it were treasure.

Those who laughed sincerely, cried honestly, and remained through every season.

And to my professors…

Who lent me the light of their minds, and drew from the river of knowledge with hands of patience, grace, and dignity.

You were the bridge, the compass, and the first spark that lit the fire of discovery in my heart.

To my family...

You are the sap that nourished my roots, and the echo that never betrays the voice.

And finally...

To everyone who passed through my world — fleeting or lasting — you are all pieces in the painting of triumph I have drawn.

But I… I was the first color... and the final shadow.

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I would also like to take a moment to acknowledge myself—for the perseverance, the late nights, the quiet strength in the face of difficulty. To the version of me who never gave up, who carried the weight of this endeavor with grace: thank you.

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To all who lit the path, thank you—from the bottom of my heart

Table of Contents

List of Figures :

List of Tables :

I) Introduction :

Conventional medicine, rooted in ancient healing practices, represents humanity's earliest reliance on nature for therapeutic solutions. Medicinal plants have been integral to this heritage, with global communities employing them for centuries based on ethnobotanical knowledge transmitted across generations (Heinrich et al., 2018; Jain et al., 2016). Scientific advancements have validated the efficacy of bioactive compounds in plants, sustaining interest in their applications for both traditional medicine and modern pharmaceuticals (Fabricant & Farnsworth, 2001; Patra et al., 2018). Notably, the World Health Organization (WHO, 2019) estimates that over 80% of populations in developing countries depend on medicinal plants for primary healthcare, attributing this to their accessibility, affordability, and cultural significance.

Algeria's traditional medicine exemplifies this reliance, serving as a cornerstone of its cultural identity and supported by a diverse flora of over 3,164 species, including endemic and halophytic plants adapted to arid and Saharan climates (Miara et al., 2019; Quézel & Santa, 1962). Regions like Adrar and Biskra highlight this biodiversity, where species such as *Artemisia herba-alba* and *Marrubium vulgare* (with 100% fidelity in treating ailments) are used to address conditions ranging from digestive disorders to cancer (Bendif et al., 2019; Miara et al., 2018). Ethnobotanical studies further emphasize the prevalence of the Lamiaceae and Asteraceae families, with leaves (29% usage) and decoctions (35%) as dominant preparation methods (Benarba et al., 2015; Bouasla & Bouasla, 2017). Despite this rich heritage, systematic documentation remains limited, risking the erosion of ancestral practices (Miara et al., 2018; WHO, 2019).

The scientific potential of Algerian medicinal plants is underscored by their phytochemical richness, including polyphenols, flavonoids, and sesquiterpene lactones, which exhibit antioxidant, anti-inflammatory, and anticancer properties (Miara et al., 2019). For instance, *Lantana camara* demonstrates antimicrobial and insecticidal activities (Sharma et al., 2007), while *Achyranthes aspera* callus cultures yield high phenolic content under optimized growth regulators like BAP and NAA (Pandey et al., 2016). However, habitat destruction and overharvesting threaten species such as *Nepeta binaloudensis*, necessitating conservation strategies like *in vitro* micropropagation.

Callus induction represents a critical step in plant micropropagation, enabling the mass production of undifferentiated cell masses with totipotency the ability to regenerate into whole plants or synthesize bioactive compounds. This process hinges on the interplay of explant type, culture medium, and plant growth regulators (PGRs). For example, leaf explants of *Nepeta binaloudensis* (Lamiaceae) demonstrated superior callogenic potential when cultured on half-strength MS (½ MS) medium supplemented with 2 mg/L BAP (cytokinin) and 2 mg/L NAA (auxin), yielding high fresh/dry weight and phenolic content (Ahmadian et al., 2017). Similarly, *Achyranthes aspera* callus induction was optimized using leaf explants on MS medium with 2,4-D (2.0 mg/L) and NAA (0.5 mg/L), highlighting the role of auxin-cytokinin balance in dedifferentiation (Pandey et al., 2016).

Micropropagation, a tissue culture-based method, extends these benefits by achieving large-scale, rapid multiplication of plants *in vitro* (George et al., 2008). This technique is particularly valuable for medicinal plants, as it produces genetically identical clones without losing desired biochemical traits, circumventing challenges of sexual reproduction (e.g., genetic variation) (Rout et al., 2000). The process involves explant sterilization, culture initiation, shoot propagation, rooting, and acclimatization (Bhojwani & Dantu, 2013). Beyond conservation, micropropagation enables mass production of secondary metabolites through cell suspension cultures (Verpoorte et al., 2002) and generates pathogen-free plants, critical for endangered species (Engelmann, 2011). However, its success depends on stringent optimization of culture conditions (nutrient media, light, temperature) and sterilization protocols to mitigate contamination risks (Reed et al., 1995). Thus, micropropagation emerges as a vital tool for the conservation, improvement, and sustainable exploitation of medicinal plants (Hussain et al., 2012).

This study focuses on callus induction and micropropagation of two Algerian medicinal plants, addressing gaps in scalable cultivation and bioactive compound production. By leveraging tissue culture techniques, such as Murashige and Skoog (MS) media supplemented with growth regulators (e.g., 2,4-D, BA), we aim to establish efficient protocols for plant regeneration and callus biomass enhancement (Ahmadian et al., 2017; Murashige & Skoog, 1962; Pandey et al., 2016). Such methods not only preserve genetic resources but also facilitate standardized phytochemical extraction for drug development.

II) Material and methods:

1. Section One: Callus induction and micropropagation:

1.1 Preparation of *Pistacia atlantica* Galls and *Cinnamomum* Aqueous Extracts:

Shade dried galls of *Pistacia atlantica* and *cinnamon* (*Cinnamomum* spp.) sticks were ground separately using an electric mill and sieved (250 µm mesh) to obtain a fine powder, which was stored in airtight glass jars at room temperature until use. For extraction, 20 g of each powdered material was macerated in 200 mL of distilled water for 24 h at room temperature (25 ± 2 °C). The resulting mixtures were filtered through Whatman No. 1 filter paper, and the filtrates were concentrated in an oven at 40 °C until complete solvent evaporation. The dried extracts were scraped, weighed, and stored in sterile amber vials at 4 °C for further phytochemical and biological analyses.

1.2 Plant Material and Surface Sterilization:

The study utilized three aromatic and medicinal plant species *Origanum* sp, *Thymus sp*, and *Mentha sp*, selected for their significant economic, culinary, and therapeutic value. These species are widely recognized for their essential oils, which possess antimicrobial, antioxidant, and anti-inflammatory properties, making them ideal candidates for in vitro propagation studies. Additionally, their high demand in the pharmaceutical, food, and cosmetic industries underscores the importance of developing efficient micropropagation protocols to ensure consistent and disease-free plant material.

This meticulous sterilization process was crucial to establishing contamination-free cultures, thereby enhancing the success rate of micropropagation for these valuable medicinal species.

1.3 Culture Media Preparation and Supplementation:

The Murashige and Skoog (MS) basal culture medium was prepared in advance using fresh prepared stock solutions containing 3.0% (w/v) sucrose, 0.7% (w/v) agar, the pH was adjusted to 5.7 with NaOH (1M) and HCl (1 M). The medium was sterilized by autoclaving at 121 °C for 35 minutes after the addition of plant growth regulators (PGR) and plant extracts.

All subsequent manipulations were carried out under aseptic conditions in a horizontal laminar airflow hood (Figure 01).

1.4 Preparation and Inoculation of Explants:

Following surface sterilization, the explants were subjected to a final disinfection procedure. The explants were immersed in 70% ethanol for 30 seconds, followed by immersion in 1% sodium hypochlorite solution for 10 minutes. They were then rinsed thoroughly with sterile distilled water for 15 minutes to eliminate any remaining traces of disinfectant.

Figure 1 :Horizontal laminar airflow hood

After rinsing, the explants were placed on sterile filter paper to dry under aseptic conditions. Once dried, the required explant parts (leaves stems or nodal segments) were carefully excised using a sterile scalpel.

In the meantime, the previously prepared and sterilized Murashige and Skoog (MS) medium was poured into sterile culture vessels and allowed to solidify. Once the medium had solidified, the explants were properly inserted into the medium to ensure proper contact and orientation for optimal growth. Each culture vessel was tightly sealed with parafilm to maintain sterility and prevent contamination.

2. Experimental design :

2.1 **Part one: Micropropagation**:

In this section nodal explants (1 to 2 cm) of *Origanum* sp and *Mentha* sp (Figure 02), were used for micropropagation assay and aseptically excised from doner plant and inoculated onto 100 ml sterile glass culture bottles containing autoclaved 20 mL of MS medium. The PGR and plant extracts were added to the culture media before pouring the MS media according to the experimental design outlined in the table below.

All the cultures were maintained in the plant growth chamber at 24 °C under a 16/8 h photoperiod irradiance provided by fluorescent lights.

Table 01: the Experimental Treatments Applied for micropropagation

Treatment Code	PGR	Concentration
T^0	Control (no addition)	–
T^1	BAP	0.5 mg/L
T^2		1.0 mg/L
T^3	Kinetin	0.5 mg/L
T^4		1.0 mg/L
T^5	Galls extract	50 mg/L
T^6		100 mg/L
T^7	Cinnamon extract	50 mg/L
T^8		100 mg/L

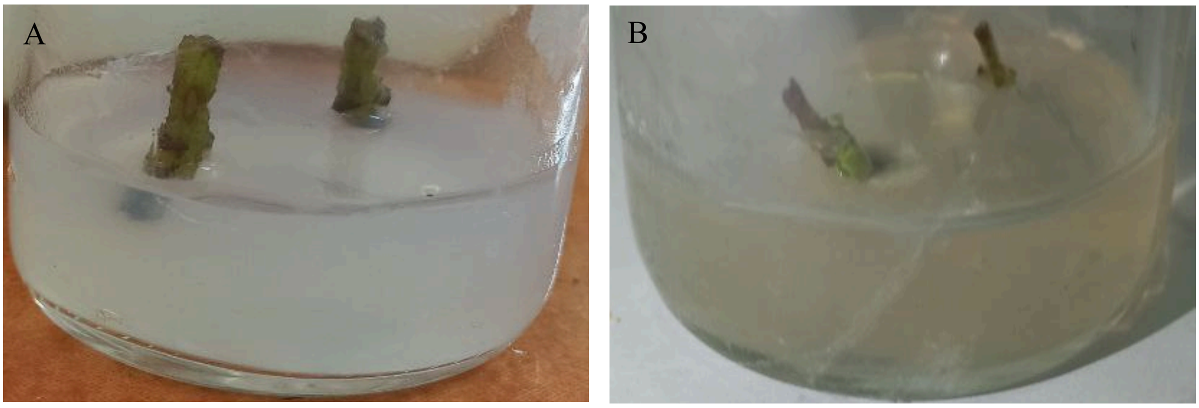

Figure 2:Nodal explants of mentha sp(A)and origanum sp (B)

2.2 PartTwo:CallusInduction and Culture Conditions:

The callus induction assays were conducted in two separate experiments using different combinations of PGR and species. It is of prime importance to select a suitable explant from stock plant for successful tissue culture process.

In the first experiment, Leaves and stems explants of *thymus* sp and *mentha* sp were selected and aseptically excised from donner plant (described above). Segments of about 0.5 cm were inoculated onto petri dishes (90 × 15 mm) containing autoclaved 20 mL of MS medium supplemented with different concentration of BAP (0.01, 0.5, 1 and 2 mg/l) and NAA (0.1, 0.5, 1 and 2 mg/l) to select the optimal medium for callus induction.

In the second experiment, Leaves, stems explants of micropropagated *organum* sp were used for callus induction. The explants were inoculated onto petri dishes (90 × 15 mm) containing 20 mL of MS medium supplemented with different concentration of 2, 4D (0.5 and 1 mg/l) and Kinetin (0.5 and 1 mg/l).

The petri dishes were sealed with two layers of parafilm and incubated in complete darkness for 4 days, after they were transferred to a growth chamber at 24 °C under a 16/8 h photoperiod irradiance provided by fluorescent lights.

III) Results and Discussion:

1.1 Section One: Callus induction and micropropagation :

1.2 **Part one: Micropropagation** :

This study aimed to develop an efficient micropropagation protocol for *Origanum sp* and *mentha sp*. by evaluating the individual effects of various synthetic and natural growth-promoting substances. The tested substances are 6-Benzylaminopurine (BAP), Kinetin, *Cinnamomum* and galls extract.

Each treatment consisted of a separate culture medium containing a specific concentration of only one of the tested substances, without any combination between them. This experimental design allowed for the assessment of the isolated effect of each compound on different stages of in vitro development, including shoot induction and multiplication.

The micropropagation of *Origanum* and *Mentha* species showed promising results, with some explants exhibiting healthy growth and shoot proliferation under specific treatments. However, the study faced significant challenges due to intense microbial contamination in the majority of replicates of *origanum sp* and the totality for *mentha sp*, which hindered our ability to conduct statistical analyses or make robust comparisons between treatments. As a result, all comparative assessments were limited to visual observations of the uncontaminated explants. Despite these obstacles, the surviving cultures demonstrated encouraging morphological responses, suggesting that, under optimized sterilization protocols and culture conditions, may enhance regeneration efficiency. While the lack of quantitative data prevents definitive conclusions, these preliminary findings provide a valuable foundation for optimizing future micropropagation protocols in these aromatic species, with an emphasis on improving contamination control measures

During the first week of culture (Figure 03), the initial signs of shoot initiation were observed in *Origanum* explants across all tested treatments. This early response occurred under the influence of both synthetic plant growth regulators (BAP and Kinetin) and natural extracts (galls and *Cinnamomum* extract) at both concentrations used (50 mg/L and 100 mg/L). The appearance of shoot primordia at this early stage indicates the responsiveness of *Origanum* tissues to a range of biostimulants, regardless of their origin or nature. These findings suggest that the applied substances were effective in triggering cellular reactivation and meristematic activity shortly after culture initiation.

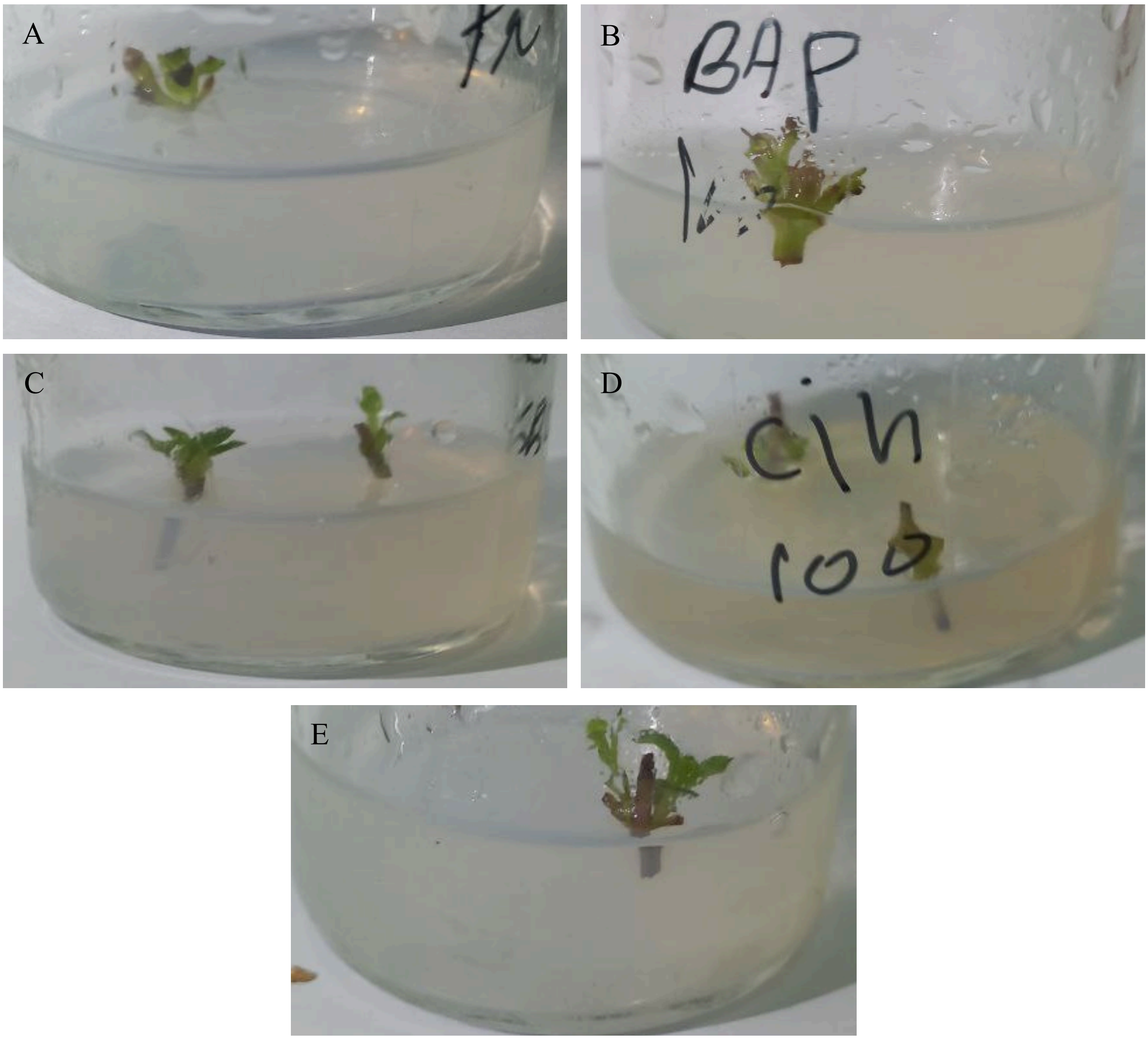

Figure 03 ; Effect PGR and plants extracts on shoot regeneration. A: Control, B: BAP (1mg/l); C: Galls extract (50 mg/l); D: Cinnamomum extract (100 mg/l) and E: Kinetin (1mg/l).

observed across most treatments and concentrations. However, notable exceptions occurred with the *Cinnamomum* extract at 50 mg/L and kinetin at 1 mg/L, where shoots exhibited sustained growth. Moderate development was also observed in treatments containing kinetin at 50 mg/L and BAP at 100 mg/L. Among all tested conditions, kinetin at 1 mg/L yielded the most promising results, producing the highest shoot elongation and overall vigor. These findings highlight the critical influence of both growth regulator type and concentration in maintaining shoot development beyond the initial induction phase.

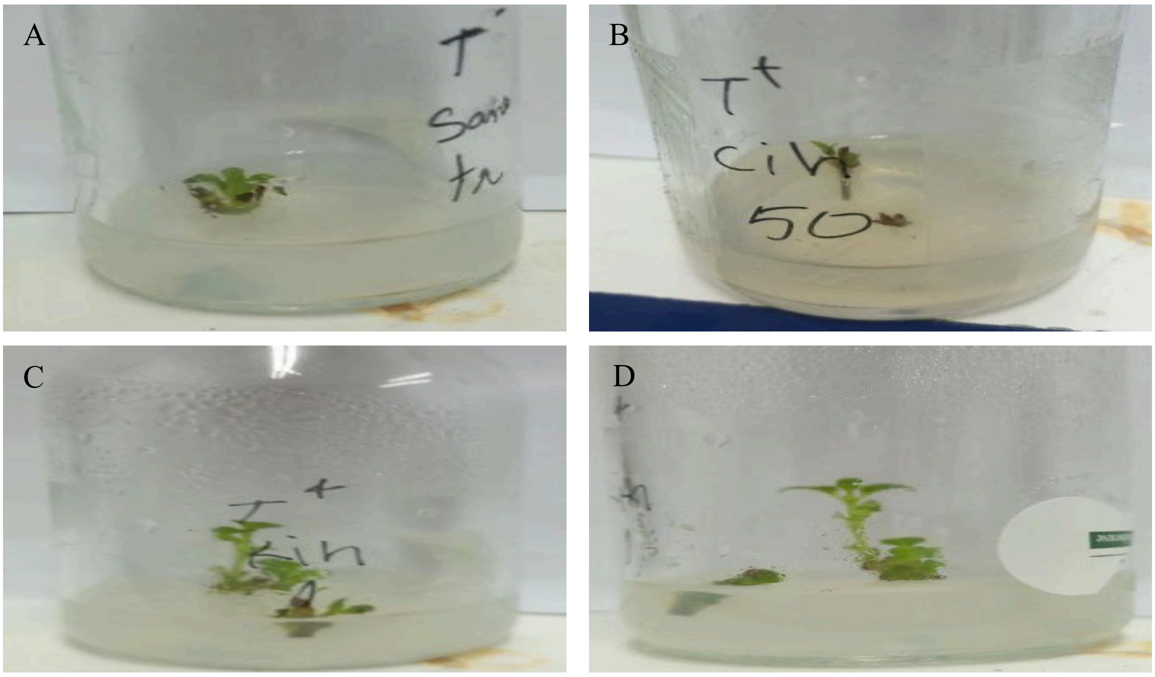

Figure 04: Shoot regeneration at the second week. A: Control, B: Cinnamomum extract (50 C: Kinetin (1mg/l). Kinetin (0.5 mg/l).

By the third week of culture (figure 05), significant improvements in plantlet development were observed in specific treatments. Explants exhibited robust shoot elongation and leaf expansion, indicating active morphogenesis. The most pronounced growth occurred in media supplemented with kinetin (1 mg/L and 0.5 mg/L) and galls extract (50 mg/L), which outperformed the control group that showed only slow, limited development. These treatments promoted vigorous stem elongation and the formation of well-developed green leaves, suggesting that optimized kinetin concentrations and natural biostimulants can effectively enhance organogenic activity in *Origanum* explants.

Figure 05: Shoot regeneration at the third week. A: Galls extract (50mg/l); B: Kinetin (1mg/l); C: Kinetin (0.5 mg/l) and D: Control.

By the fourth week of culture (figure 06), the trends observed in the previous week were further reinforced, with even greater growth recorded in the same effective treatments. Explants cultured on media containing Kinetin at 1 mg/L and 0,5 mg/L and galls extract at 50 mg/L, the control treatment, continued to exhibit vigorous development. An increase in both shoot length and leaf number was clearly noticeable compared to the third week. The plantlets appeared healthier and more robust, indicating that these treatments provided sustained support for shoot proliferation and foliar development over time.

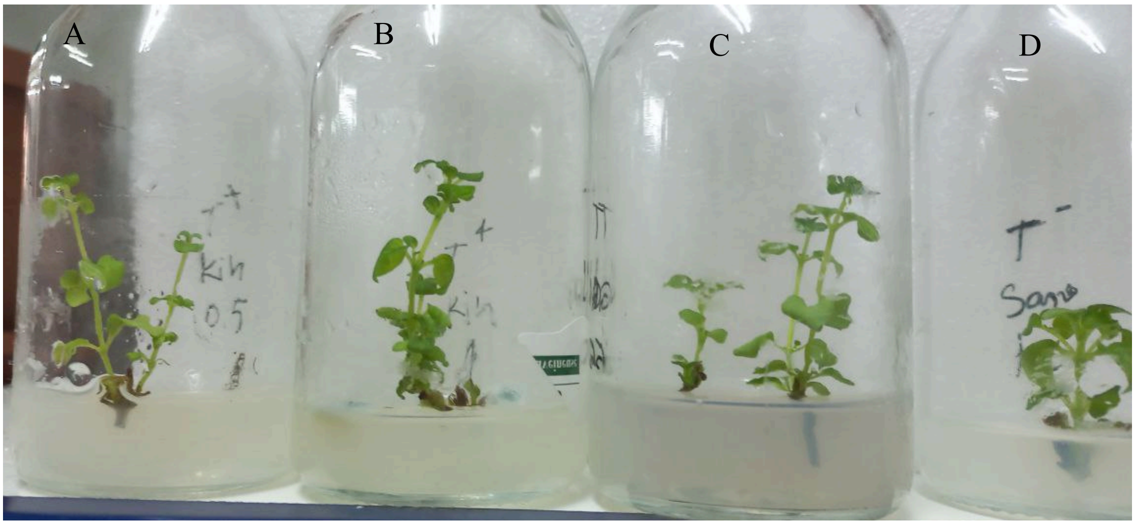

Figure 06: Shoot regeneration at the fourth week. A: Kinetin (0.5 mg/l); B: Kinetin (1mg/l); C: Galls extract (50mg/l) and D: Control.

The micropropagation of Origanum species is significantly influenced by multiple factors including plant growth regulator (PGR) types and concentrations, culture media formulations, and explant selection. Our findings demonstrate that explant responses vary considerably based on treatment conditions, highlighting the necessity for optimized protocols to achieve efficient shoot induction, proliferation, and rooting. These results align with previous research on Origanum species micropropagation. For example, Özkum (2007) reported that Murashige and Skoog (MS) medium containing 2.0 mg·L^{-1} BAP and 0.1 mg·L^{-1} NAA was most effective for shoot regeneration in *Origanum minutiflorum* when using single nodal segments as explants. Similarly, Benkaddour et al. (2022) found that Margara medium supplemented with 0.5 mg·L^{-1} BAP optimized shoot development in *Origanum compactum*, with improved rhizogenesis when combined with 1.1 mg·L^{-1} IAA.

In our study, kinetin concentrations of 0.5 and 1.0 mg·L^{-1} demonstrated superior performance in promoting shoot proliferation and elongation. These results are consistent with findings by

Benkaddour et al. (2022), who observed optimal regeneration in *Origanum vulgare* using 0.67 mg·L⁻¹ kinetin combined with 1.2 mg·L⁻¹ IBA and 0.1 mg·L⁻¹ putrescine. The effectiveness of lower kinetin concentrations (0.5-1.0 mg·L⁻¹) in our study suggests that these levels may provide an optimal balance between promoting growth and minimizing potential negative effects such as hyperhydricity, a common physiological disorder in tissue culture systems (Zayova et al., 2019).

Comparative analysis reveals interesting variations in kinetin response among different Origanum species. While our results show excellent performance at 0.5-1.0 mg·L⁻¹, Atar and Çölgeçen (2019) reported successful shoot formation in Origanum onites using slightly higher kinetin concentrations (1.5 mg·L⁻¹). These differences likely reflect species-specific requirements and suggest that while kinetin is generally effective for Origanum micropropagation, optimal concentrations may need to be determined for each particular species (Sarropoulou et al., 2023).

Among synthetic cytokinins, BAP at 1 mg·L⁻¹ showed particularly strong effects on shoot proliferation in our Origanum cultures. This aligns with previous reports where 0.8 mg/L BAP produced 10.28 shoots per explant in O. vulgare (Kizil & Khawar, 2017). However, our results suggest that kinetin at 0.5-1.0 mg·L⁻¹ may offer advantages over BAP in certain applications, particularly in reducing the risk of callus formation and vitrification sometimes associated with BAP use (Sarropoulou et al., 2023).

The effectiveness of natural extracts in micropropagation was also demonstrated in our study. Cinnamon extract at 50 mg/L promoted early bud initiation and stable growth, likely due to its rich content of cinnamaldehyde and phenolic compounds known to enhance cell division and reduce oxidative stress (Ranasinghe et al., 2013; Srinivasan, 2014). These findings are supported by similar observations in *Gardenia jasminoides*, where cinnamon extract improved root formation independent of synthetic PGRs. Further evidence highlights the broad applicability of plant extracts in tissue culture systems. For instance, coconut water rich in cytokinins and sugars enhanced shoot proliferation in *Lamprocapnos spectabilis* 'Gold Heart' (Kulus and Miller, 2021) and significantly improved shoot multiplication, biomass, and chlorophyll content in *Rosa hybrida* (Chauhan et al., 2018). Similarly, in *Adenophora liliifolia*, coconut water at 50 mL L⁻¹ promoted root formation, though higher concentrations inhibited it while stimulating shoot growth (Kovács et al., 2024). However, the efficacy of extracts can vary by species and cultivar; sesame extract suppressed explant development in *L. spectabilis* due to polyphenol toxicity (Kulus and Miller, 2021), whereas thyme essential oil and microalgae consortia enhanced rooting and antioxidant activity in *Fragaria* × *ananassa* (Chaouch et al., 2023). Natural extracts like vermicompost-coconut water mixtures (Vermi-Co) also outperformed synthetic PGRs in shoot regeneration of *Lindernia minima* (David Raja et al., 2025), underscoring their potential as cost-effective alternatives. Additionally, seaweed extracts such as *Ascophyllum* Marine Plant Extract Powder (AMPEP) mitigated abiotic stresses in *Kappaphycus alvarezii* micropropagation (Hurtado and Critchley, 2018), while brassinosteroids and abscisic acid improved acclimatization efficiency in *Echinacea purpurea* (Gerzelak et al., 2024). Collectively, these studies affirm that plant extracts can enhance micropropagation outcomes through species-specific mechanisms, though their composition, concentration, and synergistic effects with conventional PGRs require careful optimization.

On the other hand, galls possess distinct biochemical profiles compared to normal plant tissues, primarily due to metabolic alterations induced by gall-forming insects. For instance, aphid phylloxera modifies grape leaves by inducing abnormal stomata formation on the upper epidermis, enhancing nutrient acquisition for the insect (Nabity et al., 2012). These changes extend to transcriptional pathways, disrupting sucrose mobilization and glycolysis (Nabity et al., 2012). Phytochemical analyses reveal that galls are rich in bioactive compounds, including triterpenes, gallic acid, ethyl gallate, catechin, epicatechin, and tannic acid, which accumulate in response to heightened oxidative stress (Huang et al., 2015). The reddish pigmentation observed in some galls is attributed to anthocyanins, indicating a shift from chlorophyll synthesis to secondary metabolite production as a defense mechanism against stressors such as cold, nutrient deficiency, and herbivory (Karageorgou and manitas, 2006; Steinbauer et al., 2014,). Similarly, galls induced by *Slavum wertheimae* on wild pistachio trees contain high terpene levels, likely as an insect-derived strategy to deter predators (Rostás et al., 2013). Additionally, gall-forming insects like *Bruggmanniella* sp. redirect photosynthetic activity in host plants (*Litsea acuminata* and *Machilus thunbergii*), reducing carotenoids and chlorophyll while increasing soluble sugars and free amino acids (Huang et al., 2015). Notably, purpurogallin, a potent antioxidative phenol found in certain galls, exhibits antibacterial activity against methicillin-resistant *Staphylococcus aureus* (MRSA) (Inamori et al., 2014), inhibits polo-like kinase 1 (Plk1) (Watanabe et al;, 2009), and suppresses proinflammatory cytokines (IL-6, TNF-α). Despite these documented therapeutic properties, no studies have explored gall extracts as biostimulants in plant micropropagation until now.

In our study on *Origanum* sp. micropropagation, gall extract applied at concentrations of 50–100 mg/L significantly enhanced shoot development, demonstrating its potential as a novel biostimulant. This aligns with the extract's high phenolic and growth-modulating content, suggesting that gall-derived metabolites could optimize in vitro plant regeneration protocols. Further research is needed to elucidate the mechanisms behind this stimulatory effect and to expand applications to other economically important species.

2. Part two: Callus induction:

The present study evaluated the callus induction potential of leaf and stem explants from *Thymus* sp., *Mentha* sp., and *Origanum* sp. cultured on MS medium supplemented with varying concentrations of plant growth regulators (PGRs). The explant responses to different hormonal combinations were assessed based on callus initiation frequency, morphological characteristics of the calli, and the time required for callus formation. The results from both experimental sets are presented below and discussed in comparison with previous literature findings.

2.1 The first set of experiments using Thymus sp. and Mentha sp:

In this experiment where explants are collected directly from wild field growing donor plants, encountered a high level of contamination across all treatments (Figure 07). Despite following aseptic techniques, microbial contamination appeared within the first few days of incubation, preventing the proper evaluation of callus induction. This outcome highlights the challenges of working with field-grown plant material, which may harbor endophytic microorganisms difficult to eliminate completely through surface sterilization alone.

The high contamination rates observed in the first set of experiments, which used explants from field-grown *Thymus* sp. and *Mentha* sp., are consistent with well-documented challenges in plant tissue culture. Despite rigorous surface sterilization procedures, microbial contamination emerged rapidly, indicating the presence of endophytic or systemic microorganisms embedded within plant tissues. Field-grown plants are frequently exposed to diverse microbial communities, including bacteria, fungi, and actinomycetes, which colonize both external surfaces and internal tissues (Cassells, 2012; Leifert & Cassells, 2001).

Contaminants such as *Bacillus*, *Pseudomonas*, *Aspergillus*, and *Penicillium* are commonly reported in explants derived from natural environments (Odutayo et al., 2012). These microorganisms may persist in vascular tissues and are not easily eliminated by surface sterilants like sodium hypochlorite or ethanol (George et al., 2008). Moreover, excessive sterilization often leads to tissue necrosis, reducing explant viability without fully preventing contamination (Cassells & Curry, 2001).

In this study, contamination appeared within the first 3–5 days of incubation, suggesting that microorganisms were endogenous rather than superficial. Such contamination can severely compromise tissue culture outcomes by competing with plant cells for nutrients, releasing phytotoxic metabolites, and altering pH and osmotic conditions in the medium (Ali et al., 2018).

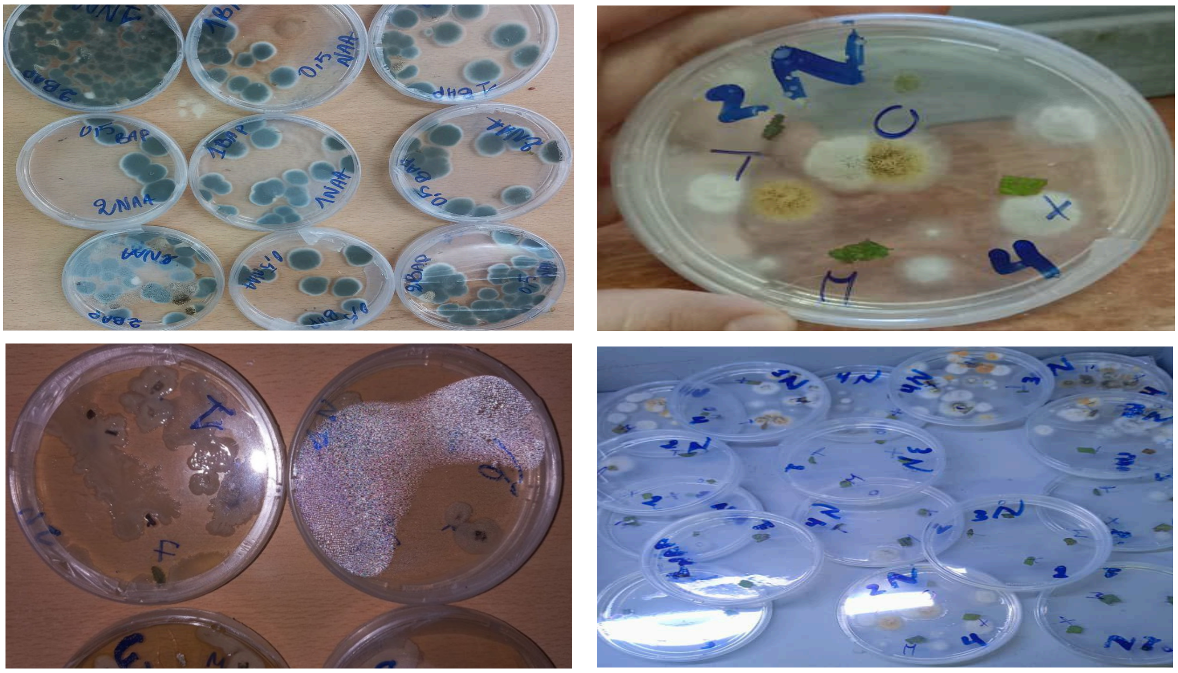

Figure 07: The impact of different contaminants in various assays

Previous research has proposed various strategies to mitigate this issue, including the use of antibiotics, antifungal agents (Cheng et al., 2004), or even silver nanoparticles (Parzymies, 2021). However, these treatments may also have phytotoxic effects or lead to microbial resistance over time. A more sustainable and reliable solution is the use of *in vitro*-propagated donor material, which offers a cleaner and more uniform source for explants.

The results of the present study reinforce the conclusion that explants derived from field-grown plants particularly aromatic and medicinal species such as *Mentha* and *Thymus* are highly susceptible to microbial contamination. Consequently, careful selection of source material, optimization of sterilization protocols, and possibly pre-culture of donor plants under controlled greenhouse conditions should be considered in future experiments.

2.2 The second experiment using micropropagated origanum as explants:

In contrast, the second experiment, which utilized *Origanum* sp. explants derived from micropropagated plantlets, showed no signs of contamination. Under these conditions, successful callus formation was observed in response to different concentrations of 2,4-D and kinetin. After 7 days of culture (Figure 08), callus initiation became evident across nearly all hormone combinations, particularly in treatments where the concentration of 2,4-D exceeded that of kinetin. These results confirm that the use of micropropagated material significantly improves the reliability and sterility of *in vitro* cultures, thereby enhancing the chances of successful callus induction. The early onset of callus proliferation in high-2,4-D treatments suggests a stronger auxin-driven response, likely due to the hormone's role in promoting cell dedifferentiation and division

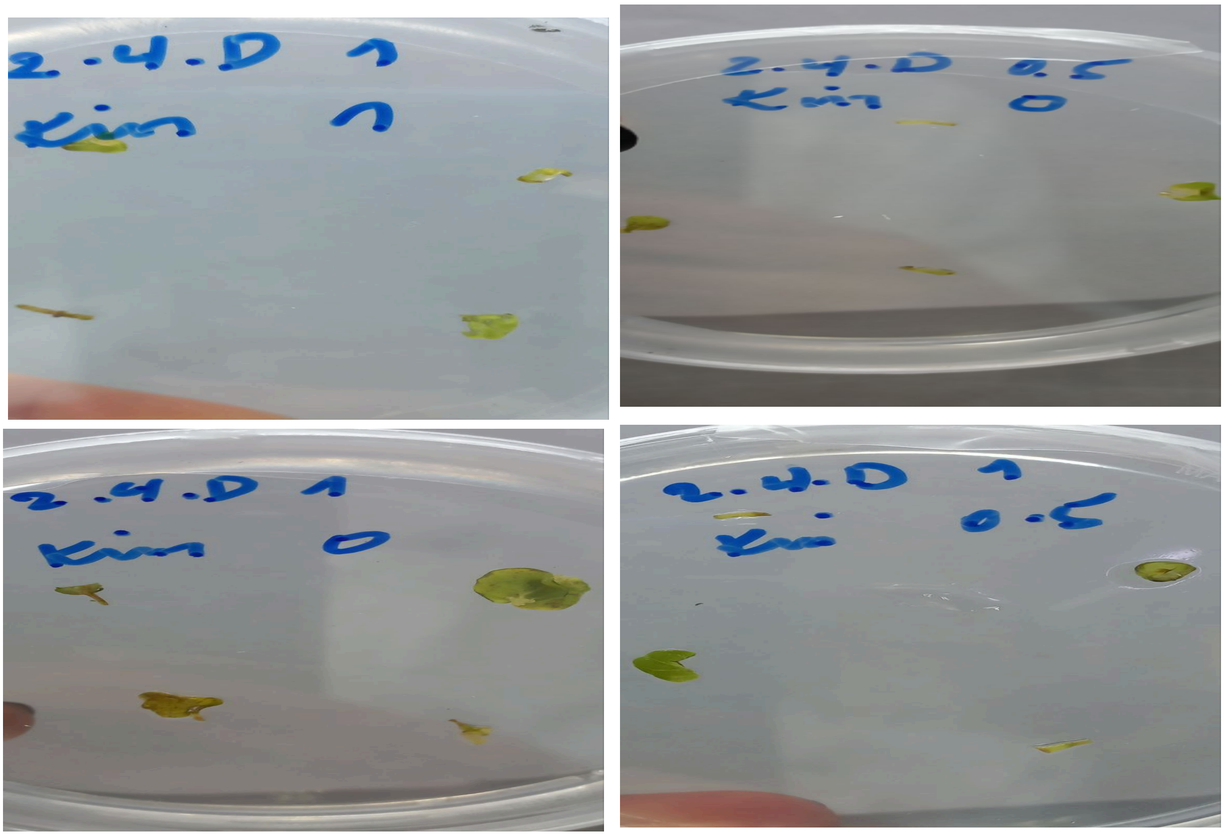

Figure 08: callus induction of both explants used (leaf, stem) in most combinations

The use of micropropagated plants as explant source in tissue culture significantly reduces contamination levels compared to explants derived directly from field-grown plants. This finding aligns with previous research that highlights the advantages of in vitro-propagated donor material, which is considered cleaner and more uniform, thereby minimizing the introduction of endogenous and surface contaminants (George et al., 2008; Thorpe, 2007).

Micropropagated explants benefit from the controlled aseptic environment during multiplication, which limits exposure to microbial contaminants such as bacteria and fungi commonly found on field plants (Cassells, 2001). Several studies have demonstrated that explants sourced from micropropagated plants show higher callus induction rates and better growth performance due to reduced microbial interference (Ali et al., 2018).

Moreover, the lower contamination rates contribute to better nutrient availability and optimal medium conditions, which are critical for successful callus formation and subsequent regeneration. The absence of competing microorganisms prevents the production of phytotoxic metabolites and maintains stable pH and osmotic conditions, both essential for healthy tissue culture growth (Cassells, 2001).

In conclusion, sourcing explants from micropropagated plants provides a reliable and sustainable approach to enhance callus induction while minimizing contamination, improving overall tissue culture outcomes. This strategy should be prioritized in future protocols for the propagation and genetic improvement of medicinal and aromatic plants

The successful induction of callus largely depends on the appropriate selection and concentration of plant growth regulators (PGRs), primarily auxins and cytokinins. Auxins such as 2,4-dichlorophenoxyacetic acid (2,4-D) and naphthalene acetic acid (NAA) are widely recognized for their critical role in promoting cell division and dedifferentiation, which are essential for callus formation (Gaspar et al., n.d.; Thorpe, 2007).

Auxins, particularly 2,4-D, are among the most commonly used PGRs in callus induction during plant tissue culture. Multiple studies have demonstrated that 2,4-D possesses strong auxin activity, promoting rapid cell division and the formation of undifferentiated cell masses. For instance, in Salvia moorcroftiana, the highest fresh weight of callus was observed with 1 mg/L of 2,4-D, while the greatest dry weight was obtained with 2 mg/L, highlighting the auxin's efficacy in stimulating callus growth (Bano et al., 2022).

In addition to auxins, cytokinins such as benzylaminopurine (BAP) and kinetin play a crucial role in stimulating cell proliferation and morphogenesis. The balance between auxins and cytokinins significantly influences callus behavior and developmental direction. A higher auxin-to-cytokinin ratio generally favors callus formation and root induction, whereas a higher cytokinin-to-auxin ratio tends to promote shoot regeneration. These findings emphasize the importance of selecting the appropriate type and concentration of PGRs to achieve successful callogenesis and organogenesis in vitro (Long et al., 2022).

2.3 Callus browning after 4 weeks:

After four weeks of culture, oregano (*Origanum*) calli exhibited varying degrees of browning across different treatments, influenced by growth regulators and culture conditions (Figure 09).

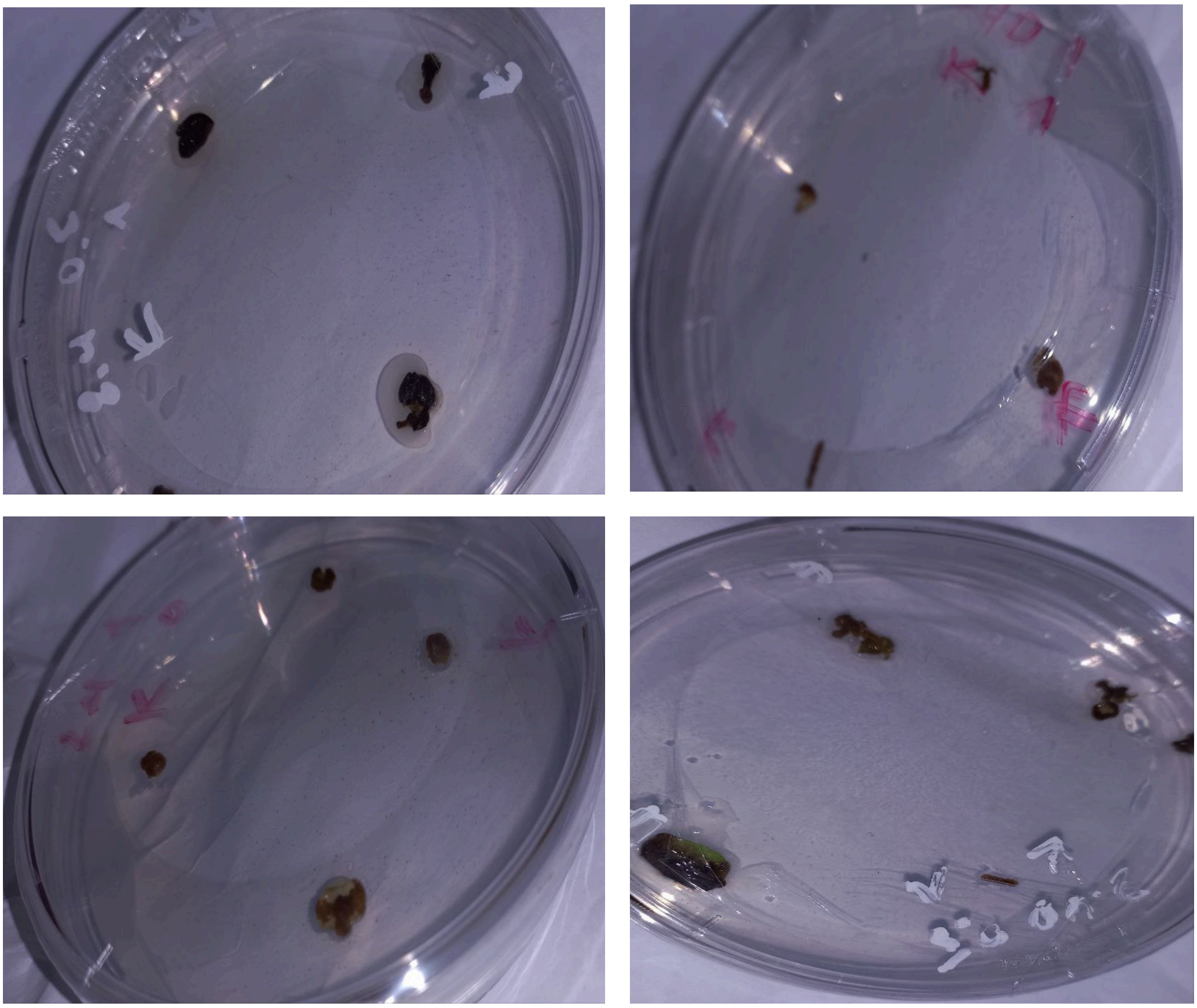

Figure 09: callus browning of explants used (leaf, stem) in most PGR combinations

The browning of oregano (*Origanum*) callus cultures is a critical challenge in plant tissue culture, significantly impacting callus viability, secondary metabolite production, and regeneration potential. This phenomenon is influenced by multiple factors, including oxidative stress, phenolic metabolism, hormonal imbalances, and suboptimal culture conditions. Below, we provide an expanded discussion integrating findings from recent studies to elucidate the mechanisms behind callus browning and propose mitigation strategies.

One of the primary causes of callus browning is the oxidation of phenolic compounds, which occurs when polyphenol oxidases (PPOs) interact with oxygen, leading to the formation of quinones and subsequent melanin-like pigments (Kaviani, 2023; Yang & Shetty, 1998). In oregano callus cultures, the rapid proliferation of cells in Murashige and Skoog (MS) medium supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) and kinetin (Kin) can trigger oxidative stress, particularly when explants are transferred to fresh media after four weeks (Kaviani, 2023). The study by Hoenemann et al. (2010) on *Camellia hainanica* callus demonstrated that browning is linked to the upregulation of phenylpropanoid and flavonoid biosynthesis pathways, including key enzymes such as phenylalanine ammonia-lyase (PAL), chalcone synthase (CHS), and flavonoid 3-hydroxylase (F3H). Similarly, oregano callus may experience enzymatic browning due to the disruption of cellular compartmentalization, allowing phenolics and PPOs to interact (Kaviani, 2023; Yang & Shetty, 1998).

The accumulation of reactive oxygen species (ROS) exacerbates oxidative stress, leading to cellular damage and necrosis (Shetty, 2004). Studies on *Zataria multiflora* callus cultures have shown that excessive phenolic secretion can be mitigated by optimizing carbon sources, with maltose proving particularly effective in reducing oxidative browning (Bernard et al., 2006). This suggests that modifying the carbohydrate composition of the culture medium could help control phenolic oxidation in oregano callus cultures.

The choice and concentration of plant growth regulators (PGRs) significantly influence callus browning. In oregano, callus induction is faster with 2,4-D than kinetin, likely due to 2,4-D's stronger auxin-like activity, which promotes rapid cell division but may also induce oxidative stress (Kaviani, 2023). However, prolonged exposure to high auxin levels can lead to cellular damage, as seen in wheat anther cultures where excessive 2,4-D concentrations reduced regeneration capacity (Boase et al., 2002).

The transition to fresh media after four weeks may further stress cells, as they must readjust to new hormonal and nutrient conditions, exacerbating browning (Kaviani, 2023). Light conditions also play a role; some studies suggest that dark incubation reduces browning by minimizing photo-oxidation, whereas light exposure can accelerate phenolic oxidation (Yang

& Shetty, 1998). Additionally, the presence of antioxidants such as ascorbic acid has been shown to enhance callus induction while reducing oxidative browning (Kaviani, 2023)

Future Directions and Conclusions:

This study successfully investigated **callus induction** and **micropropagation** of two medicinal plants, *Origanum* sp. and *Mentha* sp., leveraging both synthetic plant growth regulators (PGRs) and natural extracts. The research achieved significant findings in both callus development and shoot propagation, while also identifying key challenges that require further optimization.

Key Achievements in Callus Induction

The study demonstrated that:

- **Optimal hormonal combinations** using **2,4-D (0.5–1 mg/L) with kinetin (0.5–1 mg/L)** effectively induced callus formation in *Origanum* sp., confirming the importance of auxin-cytokinin balance in cellular dedifferentiation.
- **Micropropagated explants** proved superior to field-collected material, as seen in the successful callus initiation from *Origanum* sp. cultures versus the high contamination rates encountered with field-derived *Thymus* sp. and *Mentha* sp. explants. This highlights the critical need for sterile starting material in tissue culture protocols.
- **Callus browning**, observed after four weeks of culture, emerged as a major challenge, likely due to phenolic oxidation and oxidative stress. This finding underscores the necessity for incorporating antioxidants (e.g., ascorbic acid) or adjusting culture conditions (e.g., dark incubation periods) in future studies.

Advances in Micropropagation

The micropropagation experiments revealed that:

- **Kinetin (0.5–1.0 mg/L)** and ***Pistacia atlantica* galls extract (50 mg/L)** significantly enhanced shoot proliferation and elongation in *Origanum* sp., demonstrating their potential as effective growth promoters.
- **Natural extracts**, particularly *Cinnamomum* extract (50 mg/L), showed promising results in stimulating early bud initiation, suggesting their viability as cost-effective, natural alternatives to synthetic PGRs.

- **Contamination issues**, especially in *Mentha* sp. cultures, emphasized the limitations of current sterilization techniques and the importance of developing more robust protocols for field-derived explants.

Future Research Directions

To build on these findings, subsequent studies should focus on:

1. **Refining callus induction protocols** by testing alternative auxins (e.g., NAA, IAA) and incorporating antioxidant supplements to mitigate browning.
2. **Scaling up production** through bioreactor-based callus culture systems to enhance secondary metabolite yields for pharmaceutical applications.
3. **Assessing genetic stability** in regenerated plants to ensure clonal fidelity and suitability for commercial cultivation.
4. **Expanding species applications** to include other economically valuable medicinal plants, particularly those threatened by overharvesting or habitat loss.

Overall Implications

This research bridges traditional ethnobotanical knowledge with modern biotechnological techniques, providing a foundation for the sustainable conservation and commercial utilization of Algeria's medicinal flora. By optimizing callus induction and micropropagation protocols, the study contributes to global efforts in biodiversity preservation and phytopharmaceutical development**.** The integration of natural extracts as biostimulants offers an innovative, eco-friendly approach to plant tissue culture, aligning with the growing demand for sustainable agricultural practices.

Based on our findings and existing literature, several key areas warrant further investigation:

1. Species-specific protocol optimization to determine ideal PGR concentrations for different Origanum varieties
2. Exploration of synergistic effects between PGR and natural extracts to potentially enhance regeneration efficiency
3. Long-term studies assessing genetic stability in plants propagated using these optimized protocols

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