APPLICATIONS OF PHYTOHORMONES FOR CANNABIS SATIVA CALLUS, SHOOT, AND ROOT INDUCTION UNDER TISSUE CULTURE TECHNIQUE

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Abstract

Cannabis sativa in vitro cultivation interest has been invigorated/renewed due to health and economic benefits, unconventional strategy, quality plants, and industrial fiber. The in vitro regeneration of cannabis encountered establishment and disinfection aspects globally. the research on plant growth regulator composition, combination, concentrations, decontamination, significant influence on, callus, shoot, and root induction, and regeneration is needed to shed light on desirable, healthy plants for the cannabis industry. The study outcomes indicated some disinfectant duration and concentrations exposure to ex-plant have a toxic impact and reduced growth while 5 % sodium hypochlorite and mercury chloride 0.1-1% are efficient sterile and gave good seed germination. Sufficient increase in callus proliferation was noticed on combination and concentrations of MS (Murashige and Skoog) media treated with 450 µl kinetin +450 ml BAP+450 µl 2,4-D from cannabis sativa leaves. Substantial influence on shoot survival was recorded on MS media supplemented with increasing 2-3 ml BAP concentration. Maximum rhizogenesis observed on PGR (plant growth regulator) 5ml (indole-3-acetic acid) IAA and 8ml (indole-3-butyric acid) IBA combinations. The study output suggests that in vitro cultivation using phytohormones protocols is applicable and appropriate for cannabis survival and productivity and can be valuable for medical and industrial purposes.

Keywords: Cannabis sativa, Murashige and Skoog, indole-3-acetic acid, Kinetin 2,4D, BAP, Kinetin, IAA, IBA.

Introduction

Cannabis sativa, an annual plant with herbal flora, belongs to the family of *cannabaceae*. The common name is hemp, natively to Eastern Asia but currently cultivated worldwide. In 1753, Carl Linnaeus first classified the sativa and believed to be as a cross-pollinator plant. Various environmental factors contribute to cannabinoid growth and development [1]. *Cannabis* is an adaptable herb with noticeable pharmacologic characteristics, which produces several medicines depending on the composition of cannabinoids and phytochemicals. Tetrahydrocannabinol (THC) is a well-known plant cannabinoid that is an essential agent for antinausea and muscle relaxation. Currently, cannabidiol (CBD) is an

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available product in the market. The ratios of tetrahydrocannabinol/Cannabidiol (THC/CBD) were genetically established. The female plants produce enough THC for drug production.

Cannabis sativa has a vital role in the field of medicine, such as enhancing lung capacity, relief of chronic pain, fighting cancer, regulating and preventing diabetes, helping to cure depression, and alleviating anxiety. Clinically, cannabis is involved in the prevention of cancer. Cannabis is used for the medication of nausea/diarrhea, to improve appetite in AIDS/HIV patients, and to relieve severe body pain and spasms. District of Columbia and thirty-three states legally allow *cannabis* for their utilization. In 1996, California first legalized cannabis for medical use [2]; [3]. In China, *Cannabis sativa* has been cultivated as a fiber, food, and medicine. China is the oldest country for cannabis cultivation, bringing different germplasm with hereditarily unmistakable local assortments of fiber-rich cannabis. Cultivation of plant cells and tissues mediates aseptic controlled nutrient culture media to expand plant cells, tissues, and organs [4]. *In vitro* culture methods are vital for creating infection-free plants, speedy growth for uncommon plant genotypes, plant genome alteration, and creation of plant-inferred metabolites of significant business value. There is a minimal amount of tissue culture studies on *cannabis sativa*. According to the increased demand for cannabis in the field of medicine, the rate of cannabis through micropropagation is the most efficient and reliable. Different factors such as medium, stock solution concentration, light, pH, temperature, concentration of sucrose, and type of hormones can affect seed growth under in vitro conditions (Shi et al. 2024). The concentration of phytohormones influences the induction of shoots, callus, meristem, root, and multiplication during *in vitro* regeneration of plants (Page et al. 2020; Sharma et al. 2013). Some of the mainly utilized phytohormones include (BAP) 6-benzylaminopurine, cytokinins, (TDZ) thidiazuron, (ZT) zeatin, (KN) kinetin, auxins, and (mT) meta-Topolin, (NAA) naphthalene acetic acid, (IBA) indole butyric acid, indole acetic acid (IAA) and (2,4-D) 2,4-dichlorophenoxy acetic acid.

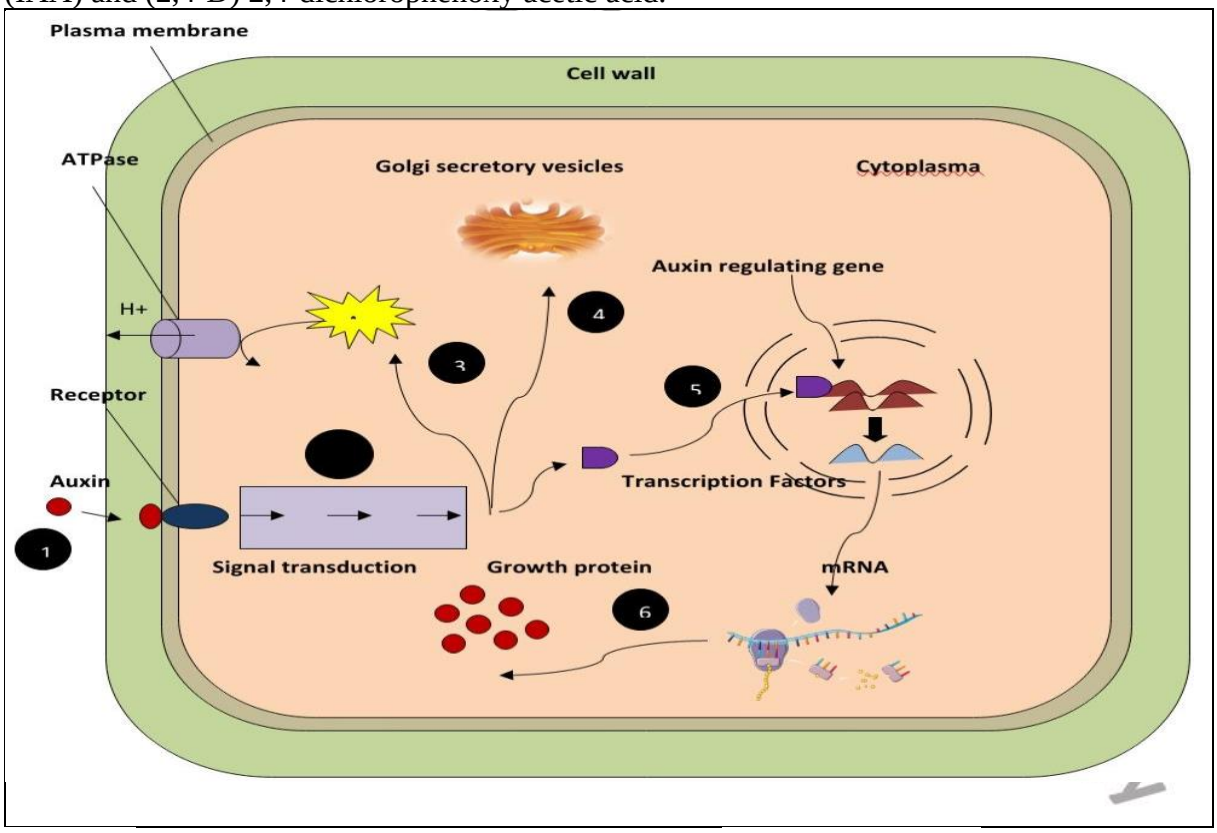

Figure 1: Signalling pathway of auxin in the plant cell (Bhatla & Lal. 2023)

The study of Miller *et al.* (1955); Truta *et al.*(2011) proposed that kinetin from autoclaved herring sperm, where cell division is stimulated by plant growth regulator (2,4-D) dichlorophenoxyacetic acid, and (auxin) in low-concentration and an efficient regulator of large-leaf plant development. In (Miller *et al.* 1955) study, different phytohormones such as (BAP) 6-benzylaminopurine, Kinetin, (2,4D) (2,4-Dichlorophenoxy acetic acid, (IAA) Indole-3-acetic acid were used for the plant induction and proliferation, for the induction of callus (myoinositol, sugar, stock solution, BAP, kinetin, 2, 4 D, and celery gel) is used as a media. For callus media, the concentration of BAP is used in microliters. BAP is essential for plant growth and development responses, cell division, shoot elongation, flowering, and plant fruiting. For root and shoot induction, 3ml BAP was added in 1L for fast growth [5].

Materials and methods

Sample collection

In the current study, the seeds of disease-free cannabis were taken from two cities in Pakistan, D.G Khan and Lahore, and the collected seeds were placed within controlled conditions in the culture room of the tissue culture lab.

Washing and sterilization of seeds

Seeds were washed in a series of disinfectants to eliminate all dust particles. The seeds were dipped in tap water for 30 minutes, 70% ethanol manufactured byUnicol limited for 2 minutes. The ethanol residues were removed and washed with deionized distilled autoclaved water. After washing detergent for 4 to 5 minutes used and seeds were rewashed with autoclaved double-distilled water to remove detergent residues. Seeds were further treated with Unichem chemicals supplier's mercury chloride and 5% sodium hypochlorite manufactured by Ittehad Chemicals Limited. Finally, three times seeds were washed with autoclave deionized distilled water and sterilized seeds were ready for further experiment.

Seed inoculation media and conditions

Sterilized seeds of both varieties (Lahore and DG Khan) were inoculated on (Murashige & Skoog 1962) MS media petri plates under a BIOBASE laminar flow cabinet. Polypropylene sheets tightly covered petri plates and placed in the tissue culture room (temperature 22-28 ° C, humidity 30-40%, light 5000 lux).

Callus induction preparations

For callus induction three weeks old 0.6 cm cannabis leaves grown in tissue culture lab, were cut into small pieces with the help of a scalpel blade, and sterilized with (Ioannidis et al. 2022) method under laminar flow cabinet. The sterile leaves were inoculated in MS media incorpated PGR, BAP manufactured by EMCO Industries Ltd, Bayer 2,4 D, and AdooQ® Bioscience kinetin with various combination and concentrations include 50µl BAP,kinetin 25µl, 2, 4D 25µl, combine treatement of 50µl BAP + 25µl 2, 4D, 50µl kinetin + 50µl BAP +50µl 2, 4D, BAP 250µl +250µl kinetin, 50µl kinetin + 50µl BAP, 450µl + 450µl 2, 4D and kinetin 450µl+ BAP 450ml+ 2, 4D 450µl was added in series of flask and pH was set on 5.7 by using ChromTech pH meter. After addation of growth regulators combinations put solidifying agent 3.5g celery gel made of EMCO Industries Ltd. The sterile leaf pieces were inoculated in media under a laminar flow cabinet with the help of sterilized forceps, and flasks were covered tightly with polypropylene sheets and rubber bands and placed in a tissue culture room (temperature 22-28 ° C, humidity 30-40%, light 5000 lux). after 20 days of culturing callus efficiency (%), length (cm), growth rate, and color indication were evaluated.

Shoot induction media and conditions

Leaves derived from callus were cut into small pieces by a scalpel blade under sterile conditions. adjusted 5.7 pH of media and autoclaved in BIOBASE manufacture autoclave for 1 hour at 121°C. Callus small pieces were inoculated on cotton dipped in semi-solid media with three different BAP (2ml BAP, 2.5ml BAP, and 3ml BAP) concentrations. Flasks were put in

a tissue culture lab under a controlled environment (temperature 22-28 ° C, humidity 30-40%, light 5000 lux). the results were described by evaluating shoot length, shoot efficiency (%), color, and growth rate.

Root induction

Shoots of cannabis 0.5 cm were cut into pieces with the help of a scalpel blade in laminar flow under sterile conditions for the induction of root. Shoots were inoculated on MS media treated with combination and concentrations of root growth regulators 5ml IAA, 8ml IBA, and combination IAA + IBA. After inoculation, flasks were placed under a controlled environment (temperature 22-28 ° C, humidity 30-40%, light 5000 lux) in a tissue culture room. Root efficiency (%), length (cm), and growth rate were evaluated.

Statistical analysis

The phytohormone concentration and combination were applied in three replicates and analyzed by using Microsoft Excel software and Tukey's significant differences for variance ANOVA and LSD to compare mean values.

Results

Seed germination of *Cannabis sativa* from two different areas

Both seed varieties were sterilized with 0.1-1% mercury chloride and 5% sodium hypochlorite for 5 minutes and inoculated on MS media. The Lahore seeds did not show germination due to immaturity after one week and D.G Khan's seed growth in the medium was three leaves and 4cm tall.

Figure 2: The image (a,b,d) showed cannabis seed sterilization and (c,e,f,g) germination of seeds under in vitro conditions

Sterilization chemicals used in *cannabis* tissue culture preparation. Disinfectants for cannabis tissue culture germination To culture the apical meristem and seeds of cannabis it's sterilized with different detergents to remove all the possible contaminants sterilized 2-5 times with tap water, and distilled water, 10-20 minutes with one drop of tween 20 into 100 ml of water and washing detergent 2-4 drops was used for 5-15 minutes in the second step but growth did not occur. After the failure of attempts, 5% sodium hypochlorite for 5-30 minutes and

germination was observed. Mercury chloride 0.1-1% for 2-10 minutes and good seed germination was noticed as compared to sodium hypochlorite treatment. .

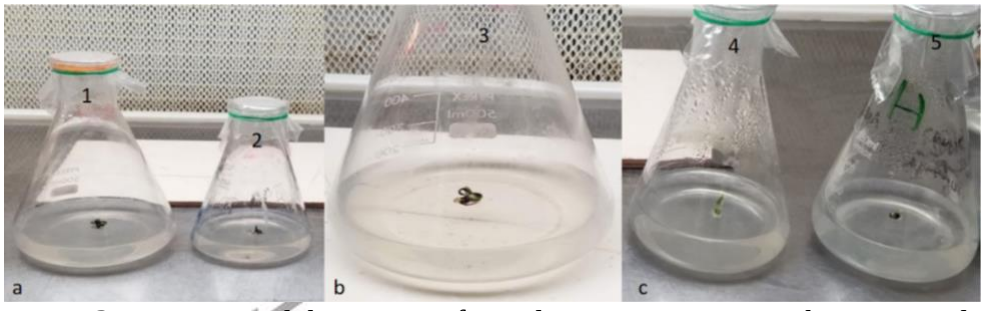

Figure 3: Represented the impact of sterilizing agent in cannabis tissue culture. In image (a,b) flask 1,3 was treated with washing detergent, flask 2 with tween twenty, in (c) image flask 4,5 supplemented with sodium hypochlorite and mercury chloride.

Growth regulators combination and concentrations for callus induction

In Table 1 , various combinations and concentrations of growth regulators were supplemented to the callus media to check the callus growth rate and suitable concentrations and combinations of callus. 50µl BAP, kinetin 25µl and 2, 4D 25µl treatments showed no callus growth. The combination of 50µl BAP + 25µl 2, 4D represented asmall amount of blackish colour callus growth after 20 days with low growth rate , combine treatment of 50µl kinetin + 50µl BAP +50µl 2, 4D indicated a greenish colour callus start grow within 20 days with good growth rate. BAP 250 µl +250 µl kinetinshowed low callus growth rate , the 50 µl kinetin + 50 µl BAP represented low growth rate of callus, kinetin 450 µl + 450 µl 2, 4D showed a very small amount of dark red colour callus growth within 20 days and kinetin 450 µl+ BAP 450 ml+ 2, 4D 450 µl showed very good amount of dark green colour callus growth on the callus media within 20 days.

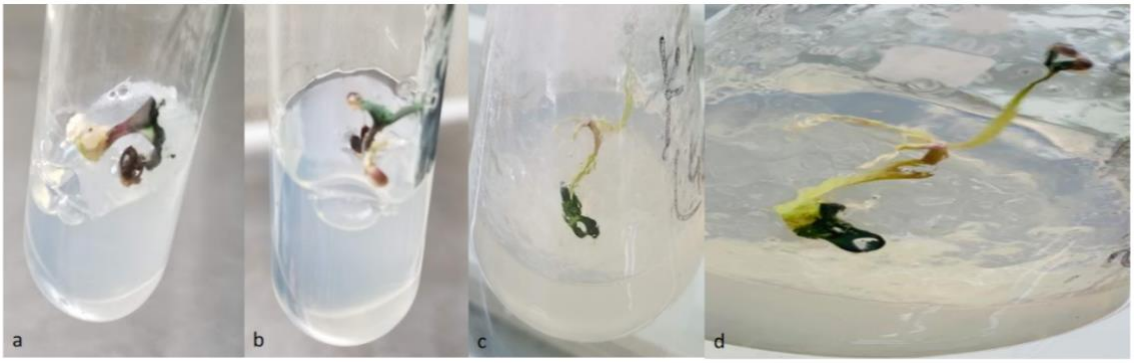

Figure 4: The image (a,b,c,d) indicated callus growth with concentration of hormones combinations

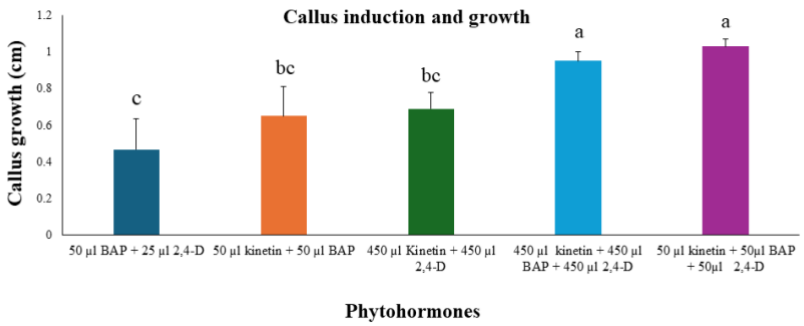

Figure 5: Cannabis sativa callus induction and growth by different phytohormone concentrations. The figure showed various lowercase letters with significant differences (P<0.05) among treatments and means ± standard error.

Impact of BAP for shoot induction BAP combination and concentration alone and combined behave differently on cannabis shoots for 25 days. The results indicated increasing the concentration of BAP improves shoot efficiency. At 2 ml BAP shoots were grown with a small growth rate, 2.5ml BAP showed shoots were grown with good growth rate, and 3 ml BAP concentration represented highest shoots growth on shoot media.

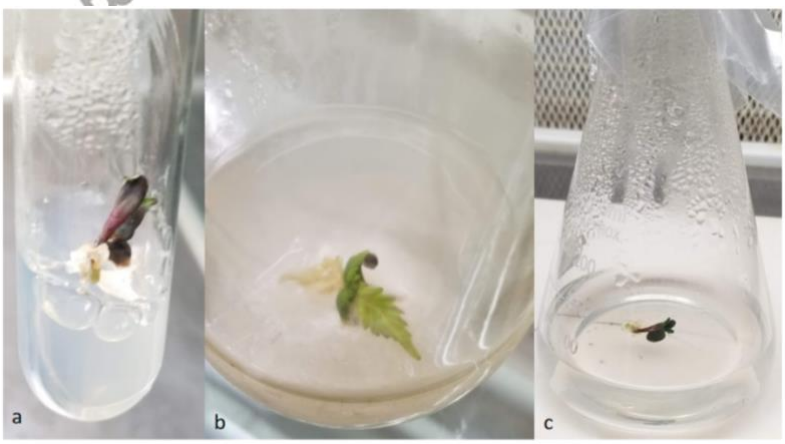

Figure 6: Effect of various BAP concentrations on Cannabis sativa shoot growth. Image (a) represented shoot regeneration supplemented with shoot media 2.5ml BAP, (b) 3ml BAP, and (c) 2ml BAP.

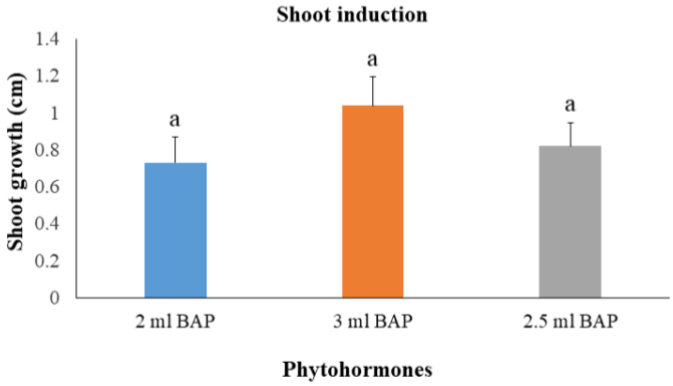

Figure 7: Shoot induction and regeneration with different BAP concentrations and lowercase letters showed significant differences (P<0.05) among treatments and means ± standard error.

Growth regulators combination for root induction

Roots were inoculated on MS media incorporated root growth regulators 5ml IAA resulted in minimal root induction, 8ml IBA addition illustrated moderated root induction after 25 days. combinations of both IAA+IBA were added for root induction and maximum root efficiency was calculated after 25 days.

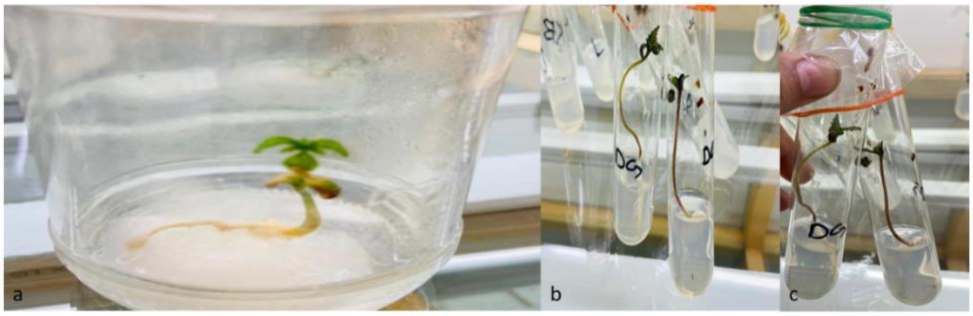

Figure 8: The image (a) illustrates cannabis roots regeneration with 5ml IAA and (b,c) 5ml IAA+ 8ml IBA treatment impacts.

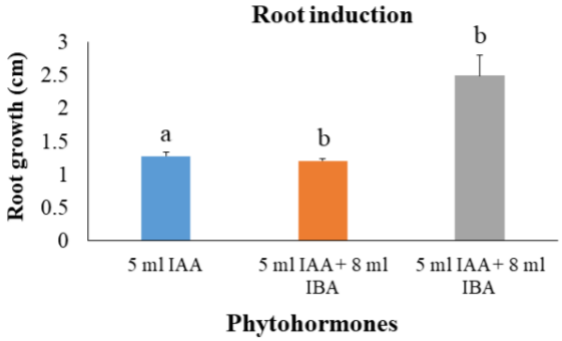

Figure 9: Root induction and growth with different IAA and IBA concentrations and lowercase letters showed significant differences ($P<0.05$) among treatments and means ± standard error.

Table 1: Growth regulators combination and concentrations for callus induction indicated various combinations and concentrations of growth regulators were supplemented to the MS media to check callus, shoot and root induction, growth rate, suitable concentrations, and combinations. The (+ and – are the indications of callus growth (- shows no growth of callus, + shows growth in a tiny amount, ++ good growth, and +++ indicates growth was excellent).

Callus growth regulators	Concentrations	Growth rate	Days
BAP	50 µl	-	20
Kinetin	25 µl	-	20
2,4-D	25 µl	-	20
BAP + 2,4-D	50 µl + 25 µl	+	20
Kinetin + BAP	50 µl + 50 µl	+	20
Kinetin+2,4-D	450 µl + 450 µl	+	20
kinetin + BAP +2,4-D	450 µl + 450 µl+ 450 µl	++	20
kinetin + BAP +2,4-D	50 µl + 50 µl+ 50 µl	+++	20
Shoot growth regulators			
BAP	2 ml	+	25
BAP	2.5 ml	++	25
BAP	3 ml	+++	25
Root growth regulators			
IAA	5 ml	+	25
IBA	8 ml	+	25
IAA + IBA	5 ml+8 ml	+++	25

Discussions

Recent *Cannabis sativa* plants in vitro production due to their medicinal purposes, and economical benefits, to save time and labour force a biotechnological tool success is needed that encountered establishment and disinfection aspects under in vitro regeneration. In this respect, our findings indicated the plant growth regulator media composition, combination, and concentrations, origin of explant, decontamination, had a significant influence on, callus, shoot, and root induction and regeneration which is in agreement with (Thacker et al. 2018; Feeney & Punja, 2003; Lata et al. 2010; Monthony et al. 2021; Wielgus et al. 2008)
presented a resemblance with (Mishra et al. 2006; Manreet et al. 2000). The study outcomes illustrated before tissue culture initiation the contamination recognition and address is a crucial factor that devastated the culture after dissemination and showed a resemblance with (Polivanova & Bedarev, 2022). the disinfectant duration and concentrations are utmost factors for ex-plant minimal toxicity and survival. In our study, Some sterilizer exposure to ex-plant had a toxic impact and reduced growth while sodium hypochlorite and mercury chloride 0.1-1% exposed plants showed good germination rate and also exhibited similarity with (Sorokin et al. 2022; Burbulis et al. 2015). The present study result callus induction on MS media supplemented with 2,4-D in combination with kinetin proved optimal growth regulator mixtures treatment and produced the greatest callus showed similarity with the findings of (Mandolino & Ranalli, 1999; Slusarkiewicz-Jarzina et al. 2005). The 2,4-D several-level treatments enhance callus proliferation supported by (Page et al. 2020) study. Callus-regenerated plants derived from stem ex-plants showed a sufficient increase in MS media treated with various BAP, kinetin, and 2,4-D combinations and concentrations. Such outcomes are by (Yasemin & Beruto, 2024).
Our findings indicated that the greater rise observed in shoot growth with increasing concentration of BAP in MS media and comparable with findings of the (Rusea et al. 2018).
The present study discovered a combination of IAA and IBA growth regulators efficiently induced the roots from the shoot of Cannabis. (Yu et al. 2023; Jdaidi et al. 2024) documented that medicinal plants in vitro rooting was significantly enhanced on MS media treated with IBA. Others (Marimuthu & Muthuchelian 2024) are also in favor of the combination of IBA and IAA-mediated root formation.

Conclusions

Cannabis sativa *in vitro* cultivation by following various disinfection and phytohormones combination and concentration protocols conclude that Some sterilizer exposure to ex-plant has a toxic impact and reduced growth while sodium hypochlorite and mercury chloride duration and concentrations exposure have efficient impact on seed germination. In addition, a combination and concentrations of MS media treated with 450 µl kinetin +450 ml BAP+450 µl 2,4-D from cannabis sativa leaves noticed a sufficient increase in callus proliferation. Maximum shoot induction was achieved on MS media containing increasing concentrations of 2-3 ml BAP. The MS media supplemented PGR 5ml IAA and 8ml IBA combination were considered effective for the highest rhizogenesis. As phytohormones combination serves as a promising base for in vitro better cultivation more possibilities are required in protocoals formulation of hemp propagation. This fact is interesting for further studies in the regeneration of cannabis plants from callus and metabolic engineering studies. Ongoing working endeavors are crucial in the efficacy enhancement of micropropagation, clean plant productivity, and germplasm preservation.

Declarations

Consent for publication: Not applicable

Availability of data and materials: on request by corresponding author

Competing interests: the authors have no competing interest

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Authors' contributions: all authors contribute equally

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Ethical statement: Ethical approval not required for this study

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