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In vitro Propagation of *Ocimum Sanctum* Linn by Using Growth Hormone Shoot Induction

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Author's contribution

The sole author designed, analyzed, interpreted and prepared the manuscript.

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ABSTRACT

The majority of Indian homes include tulsi, often known as holy basil (Ocimum sanctum L). Although it is regarded as a spiritual plant, physiologically speaking, it is one of the most readily available antibiotics. Tulsi is the most significant herb in Ayurveda, and current research is confirming its health advantages. It is also one of the plants that are utilised widely in Ayurvedic treatments.It relieves physical, physiological, metabolic, and psychological stress due to its special mix of pharmacological activity. A substrate for the quick development and multiplication of commercially significant plants is provided by plant tissue culture. Determining the optimal explants type and medium conditions for large-scale in vitro Tulsi shoot induction is the aim of the current effort. In the current investigation, the nodal segment and shoot tips were employed as explants. The nodal segment reacted well with a frequency rate of about 90% on all MS media utilized in the current investigation, including media with BAP and media with different combinations of BAP and IAA. It was seen that one or more shoots were emerging from the explants' nodal area after 10 days of culture. In this work, we examined the effects of various PGR combinations and dosages on the in vitro micro propagation of Tulsi, a fragrant and therapeutic plant (Ocmium sanctum L.). Three distinct PGRs were utilized, namely 6-benzylaminopurine (BAP), naphthalene acetic acid (NAA), and indole-3-acetic acid (IAA). The optimal medium for inducing and multiplying shoots was found to be Murashige and Skoog (MS) medium supplemented with 0.25 mg/l BAP and 0.1 mg/l NAA. The MS medium exhibits average shoot formation with 0.025 mg/l IAA and 0.1 mg/l BAP. Our results demonstrate that Tulsi may be successfully micro-propagated in vitro with the appropriate PGR.

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1. INTRODUCTION

Ocmium sanctum L., a revered indigenous Ayurvedic herb from India known as "Holy Basil" or "Tulsi," is a member of the mint family Lamiaceae [1-3]. It grows in the sub continental Himalayan region, rising up to 1,800 m, as well as on the Andaman and Nicobar Islands. It is typically found in two varieties: Shri Tulsi and Krishna Tulsi [4-6]. Numerous Ocmium species, including O. sanctum L. (Tulsi), O. bascilicum (Ban Tulsi), O. gratissimum (Ram Tulsi), O. americanum, O. canum (Dulal Tulsi), O. kilimandschricum, O. micranthum, and O. camphora, are found all over the world (Sharifi-Rad et al., 2017). O. sanctum is a sub-shrub that is upright, branched and tall (about 30-60cm). Its leaves are simple opposing elongate racemes in compact whorls that are green or purple in color, intensely scented, and have hairy stems. They are 2.5–5cm long and 1.6–3.2cm wide. It may flourish in a range of environments and soil types. It favors a temperate, sunny climate with regular rainfall and can withstand temperatures between 10°C and 35°C, but if the temperature rises above that, it may experience heat stress or frost damage (Naidu et al., 2005). Additionally, it needs at least 6 hours of direct sunlight to thrive in a variety of soil conditions, including loamy, clayey, or rocky. However, it favors a pH range of 6 to 7.5 in a well-drained, fertile, and slightly acidic soil. The present study is done to optimize in vitro micro propagation technique for O. sanctum. Tulsi (*Ocimum sanctum*), is a perennial plant native to the Indian subcontinent that belongs to the Lamiaceae family with immense cultural and therapeutic significance. It emits pleasant aroma due to green leaves and purple pink flower (Pant *et al*., 2020). Tulsi plants are not only a valuable medicinal and spiritual resource, but also an ecological and social one. It can purify the air, water, and soil from pollutants and pathogens, and can also be used as a natural pesticide, sanitizer, preservative, and cosmetic [8-10]. The plant can also provide food security, rural development, poverty alleviation, and climate change mitigation by promoting organic farming and biodiversity conservation [11-13]. Being a unique gift of nature, offers solutions to many of the modern challenges faced by humanity Agarwal, *et al.,* [14]. Tulsi leaves are also used in traditional Ayurvedic formulations and natural remedies for various ailments (Pattananayak, et al., 2010). The main methods of in vitro propagation of Tulsi are direct organogenesis (regeneration of organs from explants) and somatic embryogenesis (regeneration of embryos from somatic cells) Cardoso *et al.,* [15].

2. MATERIALS AND METHODS

The present study titled "Effect of different concentrations of BAP, IAA and NAA on in vitro shoot induction of Ocimum sanctum L." was undertaken in the Department of Biotechnology, Chaudhary Devi Lal University, Sirsa (Haryana).

2.1 Source of Explants

The explants of Ocmium sanctum was obtained from the, herbal park of Chaudhary Devi Lal University, Sirsa (Haryana). The in vitro research work was performed in the laboratory of plant tissue culture of the Department of Biotechnology, Chaudhary Devi Lal University, Sirsa (Haryana).

2.2 Instruments used for Tissue Culture

A pH meter (Control Dynamics Systems Pvt. Ltd., India), weighing balance (Metller-Toledo International Inc., USA), microwave oven (IFB Industries Ltd., India), water purification unit (Millipore Corporation, France), horizontal laminar airflow (Micro Flit, India) and autoclave were used in various steps. LAF was used for pretreatment of explants using $HgCl_2$, media pouring, explants inoculation, sub- culturing, etc.

2.3 Washing and Sterilization of Glassware

All the glassware such as beakers, flasks, measuring cylinders, petridishes, test tubes, pipettes, jam bottles, etc. were cleaned thoroughly with a liquid detergent (Teepol) and then rinsed under tap water to remove any traces of detergent before each use. Finally, they were washed with distilled water. The culture bottles used in the study were dried in an oven at 60-80°C for 3-4 hours before each use. The forceps, scalpel, petridishes, and other glassware that could be autoclaved were sterilized for 20 min. at 121°C and 15lbs /inch^2 pressure before every use for sterile handling and transfer of cultures. The forceps, scalpels, and scissors were further sterilized in the LAF by flaming till red hot prior the beginning of the working and also during the work at regular intervals and were kept in spirit inside the chamber of laminar airflow for further usage.

Workload during the experiment. The stocks of MS medium salts were prepared at 10 or 100X concentrations in double-distilled water. The preparation of separate stocks for different phytohormones was done by dissolving them in solvents like NaOH or HCI and then the final volume was made using distilled water. The stock solutions were then stored in the refrigerator at 4°C for future use and bought to room temperature prior to each use. The stock solution of EDTA and $FeSO_4$ was kept in the brown- colored bottles to protect from photo-oxidation.

2.4 Inoculation of Explants with the Essential Culture Conditions

The inoculation of explants is carried out inside the cabinet of laminar airflow. The UV light inside the cabinet was switched on before each usage for 15-20 mins. Succeeded by disinfection of the floor of the cabinet with spirit or 70% ethanol dipped cotton. All the equipment (forceps, scalpel, and scissor) used during inoculation were flame sterilized for decontamination with the use of spirit and they were kept immersed in spirit throughout the working in the laminar with a lightened burner with occasional sterilization of equipment till red hot. The cabinet floor was wiped properly with ethyl alcohol (70%) repeatedly during the working. The cut ends of surface sterilized explants were excised and then were carefully inoculated on the prepared nutrient medium in the culture vessels with the help of sterilized equipment like forceps, scalpel, scissor etc. in the aseptic conditions of laminar air flow cabinet.

2.5 Culture Establishment

MS basal medium supplemented with varied concentrations of growth regulators either alone or in various combinations for micro propagation study of Ocmium sanctum. The surface sterilized explants were then inoculated on MS basal medium augmented with varied concentrations of growth regulators like 6- Benzylaminopurine (BAP), Insole acetic acid (IAA) and Naphthalene acetic acid (NAA) for establishment and shoot induction in aseptic conditions inside laminar air flow cabinet.

A rapid in vitro regeneration protocol was developed for Ocmium sanctum L. by using shoot tips and nodal segments as explants. The explants were sterilized with 0.1% $HgCl_2$ for 8 mins and cultured them on Murashige and Skoog Basal Medium (MS) with different concentrations of different plant growth regulators. It found that MS medium with o.25 mg/l BAP and 0.1 mg/l NAA induced the maximum number of shoots per culture. Nodal segments were more responsive than shoot tips for micro propagation for Ocmium sanctum. The micro propagated plants were transferred in pots and under normal environmental conditions we successfully acclimatized and maintained. Our protocol can be used for large-scale production and conservation of O. sanctum germplasm, and for exploring its potential in modern medical health care system (Jamal *et al.,* 2016). The first step in micro propagation is shoot proliferation, which depends on several factors:

1. Genotype of the source plant
2. Type of explants
3. Pre-treatment of the explants
4. Composition of the media
5. Physical factors such as pH, temperature, light and humidity

To control microbial contamination, 6 surface sterilizing treatments were designed having different concentrations and combination of various sterilizing agents (Table 2). From these 6 surface sterilizing treatments the best one is selected in which there was least contamination and the explants were green i.e., very less damage to explants.

3. RESULTS AND DISCUSSION

3.1 Effect of Different Sterilization Treatment on *In vitro* Establishment of Explantsof Ocmium Sanctum

The effect of different sterilization treatment on in-vitro establishment of nodal segments and shoot tips on the survival percentage of Ocmium sanctum are presented. It indicates that the highest survival rate (80%) was found when explants were treated with $HgCl_2$ (0.1%) for 8 mins. There was no survival when the time duration of $HgCl_2$ (0.1%) was reduced to less than 6 mins. It was concluded that 8 mins treatment of $HgCl_2$ along with 70% ethanol treatment for 30secs was most effective amongst the different time periods used in almost all media composition.

Table 1. Stock solution preparation of MS (Murashige and Skoog, 1962) media

Stock	Component	Amount per liter of original media (g/L)	Conc. Of stock solution	Amountof stocksolution prepare (ml)	Amount of stock for 1L ofmedia (mg/L)
Stock 1	$MgSO_4.4H_2O$	7.4	10x	500ml	50
	KNO_3	38			
	NH_4NO_3	33			
	KH_2PO_4	3.4			
Stock 2	$Cacl_2.6H_2O$	0.005	10x	500ml	5
	$ZnSo_4.4H_2O$	1.720			
	$MnSO_4.4H_2O$	4.460			
	$NaMoO_4.2H_2O$	0.050			
	$CuSO_4.5H_2O$	0.050			
	KI	0.166			
Stock 3	$FeSO_4.7H_2O$	5.560	10x	500ml	5
	EDTA(Na)	7.460			
Stock 4	Thiamine-Hcl	0.020	10x	500ml	5
	Nicotinic acid	0.100			
	Pyridoxine-HCl	0.100			
	Glycine	0.400			
Components					
Myoinositol		100mg/l			
Glycin		2mg/l			
Pyridoxin		1mg/l			
Thiamine		1mg/l			
Sucrose		30g/l			
Agar agar		8g/l			

Chart 1. Steps involved in micro propagation of Ocmium sanctum

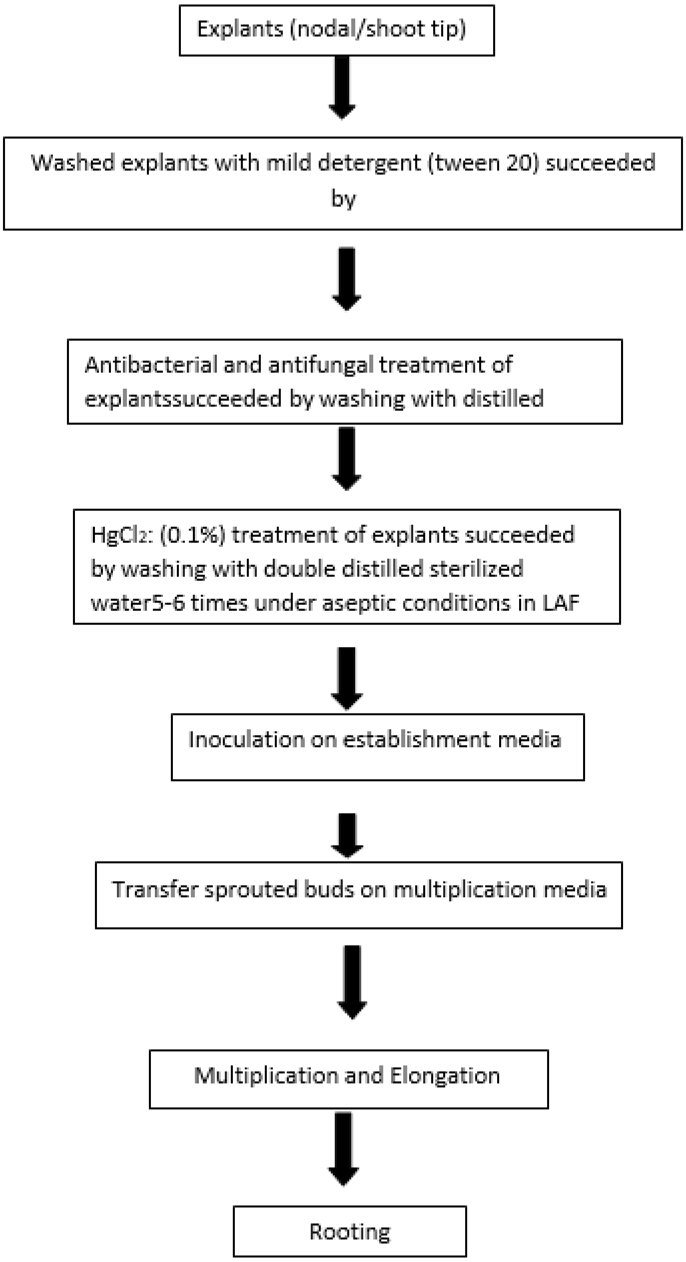

Table 2. Different surface sterilizing treatments (STs) given to explants tocontrol microbial contamination

Treatment No.	Tween-20	HgCl2	Alcohol
ST-1	1% (10min.)	0.1% (8 min.)	70% (30 sec.)
ST-2	1% (15 min.)	0.1% (8 min.)	70% (30 sec.)
ST-3	2% (10 min.)	0.1% (8 min.)	70% (30sec.)
ST-4	2% (10 min.)	0.1% (6min.)	70% (30 sec.)
ST-5	2% (10 min.)	0.1% (10 min.)	70% (30 sec.)
ST-6	2% (10 min.)	0.1%(8min.)	50% (30 sec.)

Table 3. Effect of different surface sterilizing agents on in vitro establishmentof O. sanctum. Optimization of conditions for in vitro shoot induction for different explants(shoot tips and nodal segments)

Sr. No.	Treatment No.	Total No. of explants inoculated	No.of contaminated cultures	No.of surviving cultures	Percentage survival (%)	Remarks
1.	ST-1	10	4	6	60	Low contamination Average Regeneration
2.	ST-2	10	2	8	80	Maximum regeneration Low contamination
3.	ST-3	10	2	8	80	High regeneration Low contamination
4.	ST-4	10	4	6	60	Low contamination with browning ofexplants
5.	ST-5	10	9	1	10	Minimum contamination but Explants Dead
6.	ST-6	10	5	5	50	Low contamination with browning ofexplants

Table 4. Different concentrations of growth regulators used for in vitro establishment of Ocmium sanctum

Sr. No.	BAP	IAA	NAA
1	0.10	-	-
2	**0.25**	-	-
3	0.50	-	-
4.	1.00	-	-
5	**0.10**	**0.02**	-
6	0.25	0.05	-
7	0.50	0.10	-
8	1.00	0.25	-
9	**0.25**	**-**	**0.10**
10	0.50	-	0.20
11	1.00	-	0.50
12	2.00	-	1.00

3.2 *In vitro* Shoot Induction from the Explants of O. Sanctum

The use of plant growth regulators in plant tissue culture is of fundamental importance. For shoot induction nodal segments and shoot tips are used as explants and were cultured on MS media supplemented with growth regulators BAP, NAA and IAA. Four concentrations of (0.1, 0.25, 0.5 and 1.0 mg/l) BAP singly, four of BAP and NAA combined and four concentrations of BAP and IAA combined were used. Data were recorded on percentage of shoot formation or induction after different periods of intervals of culture and their results are presented in below figures and graphs.

From these figures and graphs it is evident that BAP is the most efficient in shoot induction of O. sanctum and the highest percentage was recorded in 0.25mg/l BAP where nearly 90% response was obtained in the culture. It is also noticed that with the increase of concentration of BAP after certain level (0.25mg/l), the performance efficiency was found to be decreased as shown in figure 4.8. Out of all combinations of BAP and IAA the highest percentage of shoot formation was obtained with 0.10mg/l BAP and 0.02mg/l IAA which is 70%. The combinations of BAP and NAA shows relatively lower percentage of shoot formation as compared to other combinations of BAP and IAA. The maximum percentage of shoot formation in BAP and NAA combination was 60% which is obtained with 0.25mg/l BAP and 0.1mg/l NAA.

For optimization of conditions for in vitro shoot induction and multiplication of O. sanctum different combinations of three different plant growth regulators (BAP, IAA and NAA) were designed as shown in Table no. 2.

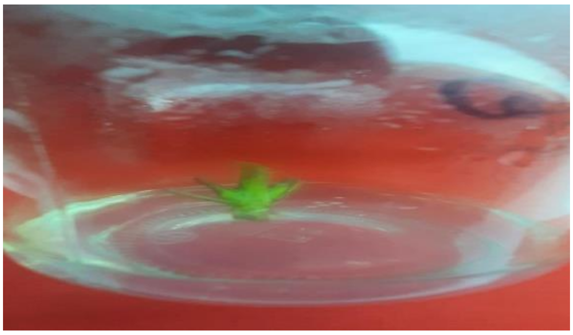

Fig. 1. Nodal segment at zeroday of culture

Fig. 2. Shoot induction after 7days of culturing on MS medium containing 0.25mg/l BAP

Fig. 3. Single shoot proliferating from nodalsegment after 10 days of culture

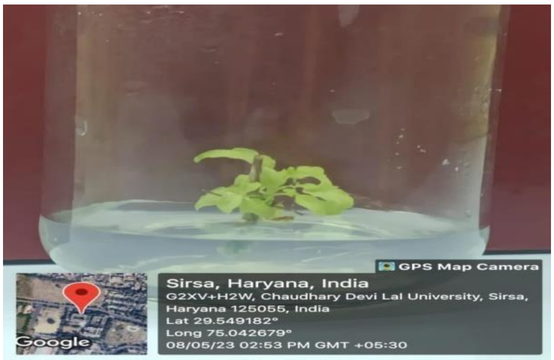

Fig. 4. Multiple shoots proliferating from nodalsegment after 10 days of culture.

Fig. 5. Shoot proliferating from shoot tip on MS medium containing 0.25mg/l BAP after 4 weeks of cultur

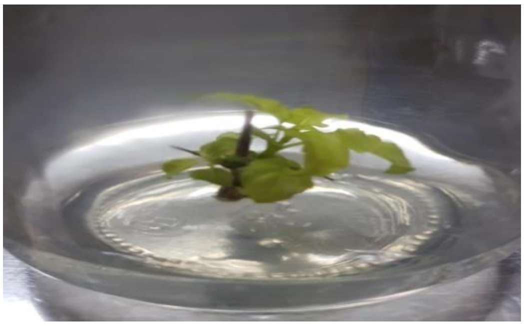

Fig. 6. Shoot proliferating from shoot tip and on MS medium containing 0.1mg/l BA 0.02mg/l IAA after 4 weeks of culture

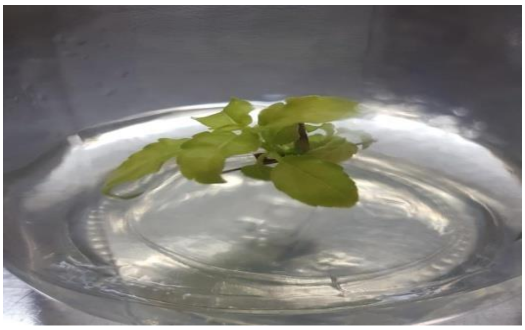

Fig. 7. Shoot proliferating from nodal segment on MS medium supplemented with 0.25mg/l BAP And 0.1mg/l NAA after 28 days of culture

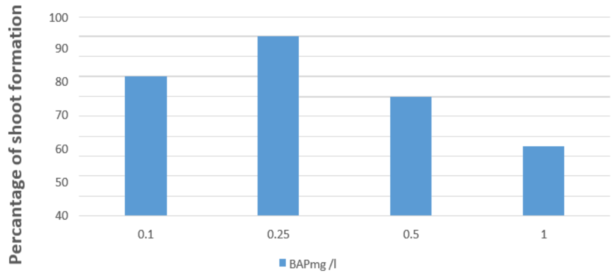

Fig. 8. Percentage of shoot formation on different concentration of BAP

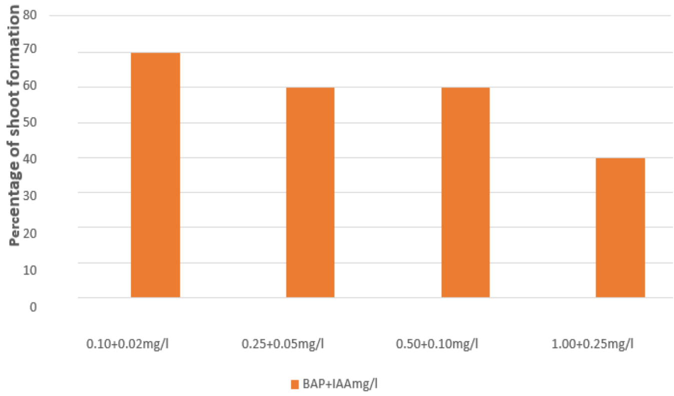

Fig. 9. Percentage of shoot formation on different concentration of BAP + IAA

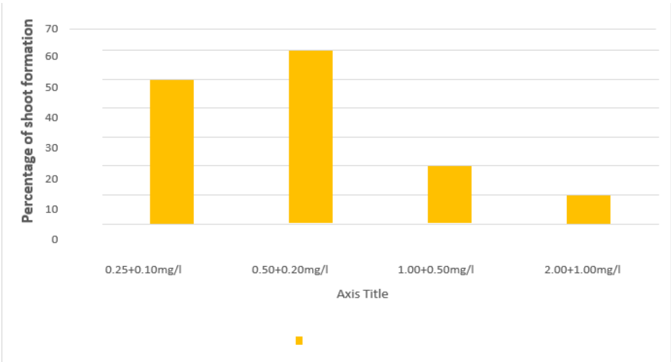

Fig. 10. Percentage of shoot formation on different concentration of BAP + NAA

4. CONCLUSION

The goal of the current work is to establish the ideal explants type and media requirements for Tulsi shoot induction on a large scale in vitro. The nodal segment and shoot tips were used as the explants in the current course of study. On all MS media used in the current study, including media with BAP and media with various combinations of BAP and IAA, nodal segment responded favorably with a nearly 90%% frequency rate. After ten days of culture, it was observed that single or multiple shoots were spreading from the nodal region of the explants. In this study, we looked at how different PGR's doses and combinations affected the in vitro micro propagation of the aromatic and medicinal plant Tulsi (Ocmium sanctum L.). We employed three different PGRs: indole-3-acetic acid (IAA), naphthalene acetic acid (NAA), and 6-benzylaminopurine (BAP). We discovered that Murashige and Skoog (MS) medium supplemented with 0.25mg/l BAP and 0.1mg/l NAA was the best medium for promoting shoot induction and multiplication. The MS medium with 0.025mg/l IAA and 0.1mg/l BAP shows average shoots formation. Our findings show that employing the right PGR, Tulsi can be successfully micro propagated in vitro.

COMPETING INTERESTS

Author has declared that no competing interests exist.

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