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Efficient Protocol for *In vitro* Regeneration of *Ocimum sanctum* using Nodal Segments as Explants

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Abstract. *Ocimum sanctum* commonly called (holy basil) an herb containing medicinal, ornamental values, is often used in culinary applications. This research focuses on the improved and efficient protocol for the direct regeneration and acclimatisation of *Ocimum sanctum* using nodal segments. Organogenesis and multiplication from explants were observed to a maximum on Murashige and Skoog (MS) medium supplemented with 0.1 mg/L of 6-Benzyl amino purine (BAP) and 0.025 mg/L of Indole-3-acetic acid (IAA). Furthermore, same medium was found effective for the induction of roots, in the *in-vitro* grown plantlets. A series of experiments were conducted to optimise the acclimatisation of *in-vitro* grown rooted plantlets of *Ocimum sanctum.* For this study different types of potting mix in assorted ratios were used to obtain best supporting media for the acclimatisation, A7 media containing soil : farmyard manure (75:25) and A1 media containing (100%) sand were found best supporting medium for the acclimatisation and hardening of *Ocimum sanctum.*

Keywords: organogenesis, nodal segments, acclimatization, regeneration, *Ocimum sanctum*

Introduction

Aromatic plants have played a significant role in the combating diseases, since ancient times, including, *O. sanctum* is a great contender for new investigations due to vast array of activities (Triveni *et al.*, 2013). *Ocimum sanctum* L. (holy basil) is considered a very sacred plant and rich source of essential oil (Kumar *et al*., 2011). The chief component is eugenol and the oil contains other chemical compounds like l- methyl chavicol, cineole, citral, 1- 8-cineole, carvacrol, ∝-pinene, eugenol, eugenol methyl ether, methyl eugenol, *p*-cymene, linalool, bornyl acetate, and eugenol (Kothari *et al*., 2004). Essential oils and herbal extracts have great potential as natural flavours and enormous uses in traditional practices. Ursolic acid is the principal component of the *O. sanctum* (tulsi) leaves (Shanmugam *et al*., 2013; Fontanay *et al*., 2008). *O. sanctum* used in various purposes such as leaves, flowers, stem, root, seeds etc. are known to have potential pharmacological activity. It also plays a significant role in treatment of fevers, arthritis, convulsions, bronchitis etc. in traditional medical practices (Kumar *et al*., 2011).

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Therapeutically, *O. sanctum* is used for treatment of eye diseases and its oil also helps in eye sight improvement (Rajeswari, 1992), while its extract have been shown to possess antimalarial, insectical and larvicidal activity (Kumar *et al*., 2011). It also has anthelmintic (Sen, 1993), antidiabetic (Hannan *et al*., 2015), analgesic, antioxidant (Khanna and Bhatia, 2003; Rajeswari, 1992), immune-modulatory (Jeba *et al*., 2011), antiulcer (Dharmani *et al*., 2004), hepatoprotective (Chattopadhyay *et al*., 1992) and anti-inflammatory activity (Malick, 2014).

There are previous reports available on *O. sanctum in-vitro* propagation using other explants sources, but this is the first report of *in-vitro* multiplication using nodal segments as explants. The main rationale of this study was to develop a sterile germ-plasm source that can be used for other *in-vitro* studies like bio-transformation (Zafar *et al*., 2012) and protoplast manipulations.

Material and Methods

Plant material. Seeds were collected from the *O. sanctum* plants grown in the green house of International Center for Chemical and Biological Sciences (ICCBS), University of Karachi.

Sterilization. Seeds of *O. sanctum* were surface sterilized using 20% (v/v) commercial bleach in autoclaved distilled water for 20 min with continuous shaking. Two drops of Tween-20 were also added with the sterilant as a surfactant. The seeds were then sieved through a pre-sterilized tea strainer and rinsed thrice with autoclaved distilled water in a petri-dish (Khan *et al*., 2008). Finally, seeds were placed on a filter paper in a petri-dish to remove moisture. The entire experiment was performed in a laminar flow cabinet.

Seed germination. For germination, MS medium (Murashige and Skoog, 1962) was used with 25 g/L sugar and 6 g/L agar without plant growth regulators (PGRs). The pH of the media was adjusted to 5.75 before autoclaving. Media sterilization was performed by autoclaving at standard temperature and pressure (121°C, 15 p.s.i., and 15 min). The seeds were transferred to the germination media and the cultures were kept under 16:8 photoperiod provided by white fluorescent light (1000 Lux) at 25±1 °C for 4 weeks.

Shoot induction and multiplication. Nodal segments (1-2 cm) of *Ocimum sanctum* were taken from the seed germination medium under aseptic condition. 28 days old nodal segments were cultured on shooting and multiplication medium.

The media were corresponding with the formulation of MS medium containing BAP and Kinetin (KN) in various concentration having media code OS1 – OS8 as shown in Table 1. MS medium was considered as control without growth regulators. All the cultures were incubated at 25±1 °C with 16:8 photo periods. Data of the plant growth parameters *i.e.*, shoot regeneration; average number and length of shoot were recorded weekly for five consistent weeks.

Root induction. For *In vitro* root propagation, *Ocimum sanctum* shoots were taken on various roots induction media in order to propose an optimized medium for root induction shown in (Table 2) The media was formulated with MS media containing various kinds of auxins IAA, 1-naphthelene acetic acid (NAA) and indole-3-butyric acid (IBA) individually, and in combination with varied concentration (Table 2). In this experimental setup, Ten different combinations bearing media code OR1 to OR9 were used, while basal MS medium with no growth regulators was control.

Auxin-cytokinin combination media. To test the effect of auxin:cytokinin combination medium on the plant growth, *viz-a-viz* shooting and rooting, an experimental set up shown in Table 3 was applied under controlled environment for five weeks.

Table 1. Effect of cytokinins on shoot induction in *O. sanctum*

Code	Cytokinin (mg/L)		% Shoot regeneration/ explant	No. of shoots*	Average shoot length (cm)*
	BAP	KN			
MS	0.0	0.0	54	2.73±0.34	1.80±0.24
OS1	0.05	0.0	87	9.98±0.09	4.93±0.15
OS2	**0.1**	0.0	**100**	**11.06±0.22**	**5.53±0.13**
OS3	0.25	0.0	100	11.00±0.23	5.23±0.12
OS4	0.5	0.0	100	10.86±0.24	4.96±0.15
OS5	1	0.0	67	10.46±0.23	4.61±0.24
OS6	0.0	0.25	67	4.03±0.03	2.30±0.18
OS7	0.0	0.5	75	4.83±0.14	3.03±0.19
OS8	0.0	1	87	5.03±0.07	3.96±0.07

*Values are mean ± standard error (X ± SE)

Table 2. Effect of auxins on root induction

Code	Auxins (mg/L)			% Root induction/ explant	No. of roots*	Average root length (cm)*
	IAA	IBA	NAA			
MS	0.0	0.0	0.0	60	2.06±0.33	2.00±0.27
OR1	0.1	0.0	0.0	100	4.00±0.23	4.86±0.09
OR2	0.5	0.0	0.0	100	4.60±0.23	4.40±0.13
OR3	1	0.0	0.0	100	5.80±0.10	3.76±0.20
OR4	0.0	0.1	0.0	93.3	9.93±0.18	4.87±0.08
OR5	0.0	0.5	0.0	100	11.06±0.15	5.01±0.05
OR6	0.0	**1**	0.0	**100**	**12.93±0.22**	**5.96±0.03**
OR7	0.0	0.0	0.1	93.3	8.60±0.23	4.73±0.11
OR8	0.0	0.0	0.5	100	9.20±0.14	5.06±0.12
OR9	0.0	0.0	1	100	12.06±0.18	5.93±0.20

*Values are mean ± standard error (X ± SE)

Acclimatization. Acclimatization of *Ocimum sanctum* plantlets was done in which different supporting materials *i.e.* soil, charcoal, farm yard manure (FYM) were used in different ratios and combinations as shown in Table 4.

Statistical analysis. All the parameters of recorded data for each treatment were analyzed by analysis of variance (ANOVA) in MSTAT-C software (MSTATC, 1989). Fifteen replicates were used for each treatment applied.

Results and Discussion

In this study, a modified protocol of direct regeneration and acclimatisation of *Ocimum sanctum* plantlets was

Table 3. Effect of synergistic/single medium on direct regeneration in *O. sanctum*

Code	BAP	IAA	No. of shoots	Average shoot length (cm)*	No. of roots*	Average root length (cm)*
OC1	**0.1**	**0.025**	**12.40±0.22**	**5.94±0.05**	**15.00±0.23**	**6.05±0.06**
OC2	0.1	0.05	11.33±0.12	5.59±0.24	14.46±0.13	5.83±0.11
OC3	0.1	0.1	10.65±0.12	5.38±0.24	13.06±0.06	5.10±0.24
OC4	0.1	0.25	10.10±0.22	5.02±0.24	12.13±0.09	4.98±0.12
OC5	0.1	0.5	10.03±0.26	4.83±0.11	10.93±0.06	4.55±0.12

*Values are mean ± standard error (X ± SE)

Table 4. Acclimatization of *Ocimum sanctum*

Code	Formulation	Ratio	% survival/leaf colour and morphology
A1	**Soil**	**100**	**87%, green/normal**
A2	Charcoal	100	5%, green/normal
A3	FYM	100	50%, green/normal
A4	Soil: Charcoal	75:25	70%, green/normal
A5	Soil: Charcoal	50:50	60%, light green/stunted
A6	Soil: Charcoal	25:75	50%, light green/stunted
A7	**Soil: FYM**	**75:25**	**87%, green/normal**
A8	Soil: FYM	50:50	75%, green/normal
A9	Soil: FYM	25:75	60%, green/normal
A10	FYM: Charcoal	75:25	60%, light green/normal
A11	FYM: Charcoal	50:50	50%, light green/stunted
A12	FYM: Charcoal	25:75	35%, light green/stunted

optimized. The germination and development of seeds were recorded in MS medium after three weeks of culture. The auxiliary bud explants showed 100% regeneration after 21 days of culture when placed in media supplemented with auxins and cytokinins alone and in combination.

Shoot induction and multiplication. In this study, the cultured nodal segments on MS medium with cytokinins (BAP and KN) showed budding. Concentration and cytokinin type both exhibited their role in successful *in-vitro* growth. There are some reports of favouring KN over BAP previously (Kumar and Rao, 2007; Bhattacharya and Bhattacharya, 2001), however, Begum *et al*. (2000) and Gopi *et al*. (2006) reported direct multiple shoots differentiation of *O. sanctum* and *O. gratissimum* L. respectively in BAP supplemented medium. Preece (1995) stated that apical dominance and cell division due to cytokinin, influences the growth and shoot induction. The maximum induction of multiple shoots after four weeks of initiation (11.06±0.22) was attained on medium (OS_2) containing 0.1 mg/L BAP, having 5.53±0.13 cm average shoot length. As the concentration got higher, the corresponding number of shoot and shoot lengths were on gradual decrement, while application of KN did not yield any remarkable results (Table 1).

Root induction. After four weeks, the regenerated shoots attained the height (up to 1-1.5 cm). Root induction occurs when regenerated shoots were transferred on rooting media which contains MS media with different concentration of auxins (IBA, IAA, NAA) as shown in Table 2. The comparative study of different auxins shows that the root induction response is vigorous in IBA as compared to IAA and NAA. The optimum media type was thus determined. A relationship was found between the rooting percentage and number of roots *in-vitro* differentiated plant was found in Table 2. The results showed that, the 100% root formation with increase in number and length was observed in OR6 medium which contains IBA 1 mg/L i.e., 12.93+0.22 root induction and average root length is 5.96±0.03 cm as shown in Table 2. Concurrently, it is also observed that the effect of IAA and NAA during the induction of roots gives 100% root induction with undersized root number and length. Deklerk *et al.* (1997) suggested that high concentration inhibits, while lower auxin concentrations having growth stimulating effect on root induction.

Auxin. *Cytokinin medium effect on plant regeneration*. In third set of experiment, auxin in combination with optimized cytokinin i.e., BAP 0.1 mg/L (OC2) was evaluated to testify synergistic medium potential (Table 3). Emergence of shoot buds was observed on MS medium having combination of BAP and IAA illustrated the good response (Fig. 1a). It is observed that analysis of variance revealed highly significant differences in OS1 medium formulated with (IAA 0.025 mg/L and BAP 0.1 mg/L) respectively, which showed vigorous effect on shoot multiplication (12.40±0.22) and shoot length (5.94±0.05 cm) as observed (Fig. 1b and c). Auxin (IAA) oxidized and metabolized swiftly, supporting the formation of shoots and embryos hence low concentration of IAA encourage root growth and retain the apical dominance (George, 1993). Cytokinins contribute a vital role in plant regulation which provokes calli division in the presence of auxin, leading to bud or root formation directly on the explants or from calli (Taiz and Zeiger, 2004). The synergistic effect of auxins and cytokinins has also been reported in other *Ocimum* spp. (Phippen and Simon, 2000; Singh and Sehgal 1999;

Patnaik and Chand, 1996; Vasil and Thorpe, 1994; Evans *et al*., 1981). In the same medium, rooting was vigorous when the shoots were placed in medium supplemented with IAA and BAP in combination. The number of roots induced was 15.00±0.23 having average root length 6.05±0.06 cm (Table 3). New individual plantlets propagated well from axillary buds on further multiplication (Fig. 1d).

Acclimatization. Regenerated shoots having vigorous roots from rooting media were transferred to green house for acclimatization. Three different types of potting mix (soil, charcoal and farm yard manure) were used in different ratio as shown in Table 4. In order to study the phenotypic variation of *in-vitro* propagated plantlets in *ex-vitro* conditions. In this experimental design, it is observed that the A7 media containing Soil: FYM at the ratio of 75:25 showed 87% survival rate and plantlets were healthy and green with normal proliferation, whereas A1 media containing 100% sand also exhibited optimum growth for the acclimatization and hardening of *Ocimum sanctum,* albeit the foliage colour was on the lighter green shade. Previous reports showed that acclimated plants survival under *ex vitro* condition ranges from 70% (Begum *et al*., 2000) to 85% (Singh and Sehgal, 1999), but as the waxy cuticle layer of young plants develop, they become more resistant to environmental stresses (Khan *et al*., 2007).

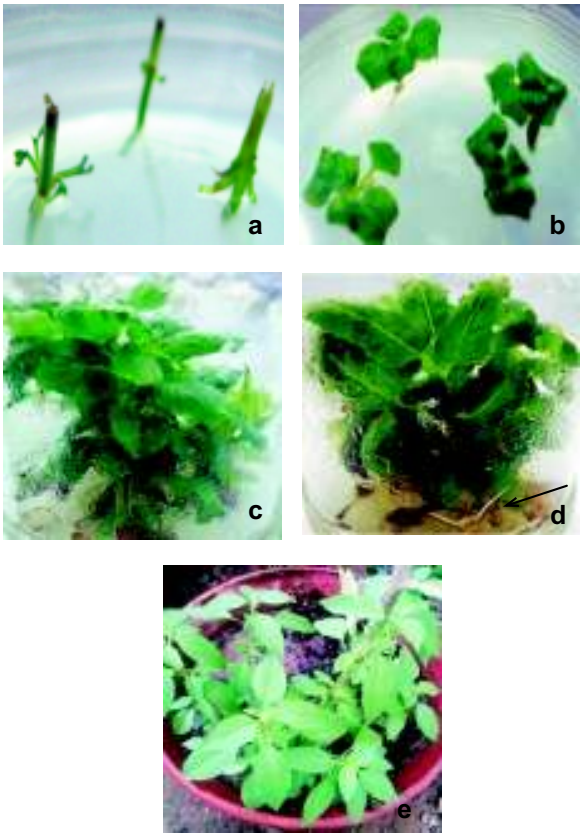

Fig. 1. Direct regeneration in *Ocimum sanctum* (a) budding of nodal segments after one week (b) plantlet formation after three weeks (c) shoot multiplication (d) rooting (arrow) and plantlet formation (e) acclimatized plant.

Conclusion

In this study, the identical plantlets of *O. sanctum* were produced in massive quantity from nodal segment which reveal that direct regeneration is more efficient than indirect (callus) regeneration method. Shoots were vigorously propagated from nodal segment under *in-vitro* conditions, which lead to root proliferation on optimized MS medium supplemented with Growth regulators (BAP and IAA) respectively. This study is concluded with the approach for producing the identical plantlets of *O. sanctum* in immense number from nodal segments.

Conflict of Interest. The authors declare no conflict of interest.

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