Invitro Propagation of Ocimum tenuiflorum

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Original Research Article

In vitro Propagation of *Ocimum tenuiflorum* var. CIM-AYU from nodal explants

Abstract

Ocimum tenuiflorum var. CIM-AYU is gaining popularity for having rich source of aromatic principles of commercial importance. In the present study, an in vitro propagation method is outlined for *Ocimum tenuiflorum* using shoot buds as source of explants. For complete regeneration (callus growth, induction of roots & multiple shoots) of plants under in vitro conditions, MS media supplemented with different concentrations of growth regulators. MS media supplemented with 01mg/L of 2, 4-D and 0.5mg/L of kinetin yields high (82.22%) callus and 71.3 mg of dry biomass. On subsequent re-culturing of callus on MS media with 1.0 mg/L of BA and 0.5 mg/L of NAA showed 88.88% shooting, maximum number of shoots (9.57) and relatively better shoot lengths (4.71 cm). On careful transfer of excised shoots on to half strength MS medium supplemented with 1.0 mg/L indole-3-butyric acid (IBA) for root induction, it yields 80.1% rooting. Whereas, average root length and number of roots observed were 5.21 cm and 2.76 cm respectively per explants. Rooted plantlets were hardened and successfully established in natural soil, where they grew and matured normally. The standardized in vitro propagation protocol may helps in large-scale production of elite *Ocimum tenuiflorum* var. CIM-AYU species using nodal explants.

Key words: *Ocimum tenuiflorum* var, CIM-AYU, Nodal segments, *in vitro* propagation, 2, 4- Dichloro phenoxy acetic acid

1. Introduction

Plants make up chief source of raw materials for preparation of drugs. Due to wide spread toxicity and harmful side effects often caused by synthetic drugs and antibiotics, modern society increasingly prefers drugs of natural origin.

Thus, about three fourths of the world population depends mostly on medicinal herbs for primary health care needs [1]. The success of any healthcare programme relies on the accessibility of appropriate drugs on a sustainable basis. The genus *Ocimum* include over 170 species and almost all are aromatic in nature with a wide range of essential oils and many of which are extensively used in traditional medicine [2] and exhibit phytotherapeutic properties [3], antimicrobial [4], antifungal [5], as well as antioxidant and insect repellent activities [6, 7]. On the other hand, different *Ocimum* species are used in folk medicine for treatment of a wide range of human health problems [8]. Leaves are mainly used for the treatment of cough, bronchitis, skin disease, measles, abdominal pains and diarrhoea [9]. On the other hand, experiments further confirmed that, sweet basil treated rats showed improved forced swimming &decreased immobility scores [10].

Although reports on *in vitro* propagation studies in several *Ocimum* species are available, development of efficient micro propagation protocols for many *Ocimum* species is still in progress [11]. Rapid micro propagation protocols in young leaves, node, axillary shoot, shoot tip and inflorescence explants have been documented in many Ocimum species including *O. basilicum* [12, 13], *O. sanctum* [14], *O. Kilimandscharicum* [15], *O. gratissimum* [16], *O. americanum* and *O. canum* [17]. *O. Basilicum* [18]. In *Ocimum basilicum* Linn. var. p*ilosum* (Willd.) Benth, *In vitro* Propagation from nodal explants and phyto constituents from GC-MS Studies [19], has also been reported.

Although extensive research has been conducted in numerous *Ocimum* species, *in vitro* propagation studies in *O. tenuiflorum* var.

CIM-AYU has not been studied. In the present study we made an attempt to establish a reliable and efficient *in vitro* propagation protocol using nodal explants of *Ocimum tenuiflorum* var. CIM AYU for large-scale production and germplasm conservation.

2.0 Materials and Methods

2.1 Plant Materials

Healthy seed material was collected from CIMAP, Hyderabad, Telangana State, India. Plants were successfully planted in the University botanical garden for further experiments. Shoot tips (nodal segments of 0.8-1.0 cm with dormant auxiliary buds of about 0.6 cm) were excised from plants and used for *in vitro* propagation studies. Selected nodal segments were washed in tap water followed by washing with 10% teepol (10 min) and 1 % (w/v) Bavastin for 5 minutes. The explants were then sterilised for 20 seconds with 70% ethanol, followed by 0.1% (w/v) $HgCl_2$ for 2-3 minutes and rinsed 5-7 times in sterile distilled water in laminar air flow chamber. Thereafter, explants were trimmed at the cut ends and inoculated.

2.2 Culture Medium and Conditions

Shoot tips were cultured on modified MS basal medium [20] containing 3% (w/v) sucrose for callus initiation. The pH of the medium was adjusted to 5.7 with 1 N NaOH or 1 N HCl before gelling with 0.8% (w/v) agar. Vertically implanted explants in culture bottles were maintained at 25 ± 2°C temperatures, continuous light with a 16 hours photoperiod at 50 μ *mol* m^{-2} s^{-1} irradiance by cool white fluorescent tubes with 60-70% relative humidity.

2.2.1 Callus induction

Two week old sub cultured shoot buds were inoculated on modified MS medium supplemented with 2, 4 - D (0.1mg/L) and different concentrations of BA (0.25–1mg/L) for optimization of callus.

2.2.2 Shoot bud initiation

Nodal segments of *O. tenuiflorum* var. CIM-AYU were inoculated on MS media supplemented with different concentrations of Indole-3-acetic acid, (IAA 0.1- 3.0 mg/L), Naphthalene acetic acid, (NAA 0.1-3.0 mg/L and Benzyl Adenine (Kn 0.1-3.0 mg/L)
either independently or in combination with other growth hormones.

2.2.3 Multiple shoot initiation

Fully matured shoots (3-4 cm long) were cut at their nodal segments and transferred on to MS full strength medium (supplemented with BA 1.0+NAA 0.5).

2.2.4 In Vitro rooting

For root induction, excised micro shoots (with 3 - 4 fully expanded leaves of *in vitro* grown plants) were transferred on to half strength basal MS medium supplemented with different concentrations of IAA, IBA and NAA. The rooting results were tabulated 15 days after inoculation.

2.3 Acclimatization of Regenerated Plants

Fully rooted plantlets (5 to 6 cm in length) were carefully excised from the culture medium and washed under running sterile water to remove agar. The plantlets were transferred to poly trays containing sand, vermiculite and soil (1:1:2) and covered with transparent plastic bags to prevent loss of humidity. The set-up was maintained at 26 ± 1°C, 80 – 85% relative humidity and at a light intensity of 50 μ *mol* m^{-2} s^{-2} under a 16 h photoperiod in culture room conditions and acclimatized for a period of 3 weeks. After primary hardening the plantlets were transferred to a greenhouse with simulated habitat for improved survival.

2.4 Statistical Analysis

The experiments were performed using completely randomized design with three replications. 15 explants per replicate were used in each treatment. Data were analyzed by one way ANOVA and the mean values for treatments were tabulated for comparison. The results are expressed as means ±SE.

3.0 Results and discussion

3.1 Culture Response to Growth Regulators: Multiple Shooting

O. tenuiflorum var. CIM-AYU, plants were efficiently regenerated from nodal explants from field-grown young plants (Fig.1A), on MS medium supplemented by 0.5- 3 mg/L Benzyl Adenine with 0.5- 3.0 mg/L NAA or 0.5-3 mg/L of IAA for multiple shoot induction. The multiple shoot induction response with respect to the test concentrations of growth hormones is presented in Table 1. Of the two combinations (BA with IAA / NAA) tested in the present study, BA with NAA was found to exhibit highest shooting rate and better shoot length per explants (Fig. 1B). MS media supplemented with BA 0.5 + IAA 1.5 mg/L and BA 0.5 mg/L in combination with 1.5 mg/L of NAA gave 80.0% and 84.4% shoots respectively. On the other hand, MS media supplemented with BA 1.5 + IAA 0.5 mg/L showed 73.3% shooting with (5.27) number of shoots and shoot lengths (3.38cm) per explants. MS media supplemented with BA 0.5 and 1.5 mg/L of NAA generated 7.31 shoots per explants whereas multiple shoot production on MS media supplemented with 0.5 mg/L of BA with 1.5 mg/L of IAA showed 6.12 shoots.

Average number of shoots on MS medium supplemented with NAA was found to be higher when compared to the MS media supplemented with IAA. On the other hand, shoot lengths appeared to be almost similar in the two combinations of growth hormones tested in the present study. Overall, MS media supplemented with 1 mg/L of BA with 0.5 mg/L of NAA showed highest multiple shoots (88.8%), maximum number of shoots (9.57) and even relatively longer shoots (4.95 cm) per explants (four weeks after ideal cultural conditions). From the results, it is evident that the higher proportion of BA with half the concentration of NAA holds good for attaining rapid multiple shoots under *in vitro* conditions. BA with different root induction growth hormones greatly influences auxillary shoot regeneration from nodal explants.

In control (Medium without growth regulator), explants swelled and became necrotic two weeks after inoculation. Whereas, number and length of the multiple shoots per explants significantly reduced in all combinations of growth hormones tested either above or below optimized concentrations. Growth response (reduction in number of shoots per each node) at higher or lower than optimal concentration of cytokine has also been reported in several medicinal plants [21-23]. In addition, effect of Kinetin to overcome apical dominance, release of lateral buds from dormancy and promotion of shoot formation in dicot plant species were also reported [24]. Similarly, effect of BA on multiple shoot formation in tissue culturally propagated *O. basilicum* has also been reported [11, 12].

Table 1 Effect of Kinetin (Kn) in combination with Indole Acetic Acid (IAA) and Naphthalene acetic acid (NAA) for multiple shoot induction from nodal explants of *O. tenuiflorum* var. CIM-AYU. Values are means at least 15 multiple shoots of three different sampling bottles ± SE.

Growth regulators (mg/l)	Shooting percentage	Average no. of shoots / explant	Average shoot length/explants (cm)
MS+BA 0.5+IAA 0.5	62.2%	3.95 ± 0.2092	1.29 ± 0.1487
MS+BA 0.5+IAA 1.0	75.5%	4.68 ± 0.1887	2.53 ± 0.1368

MS+BA 0.5+IAA 1.5	80.0%	6.12 ± 0.3579	3.88 ± 0.2342
MS+BA 0.5+IAA 2.0	71.1%	5.71 ± 0.2657	2.31 ± 0.1367
MS+BA 0.5+IAA 3.0	53.3%	2.83 ± 0.2315	1.26 ± 0.1313
MS+BA 1.0+IAA 0.5	64.4%	4.19 ± 0.1856	2.74 ± 0.1486
MS+BA 1.5+IAA 0.5	73.3%	5.27 ± 0.2986	3.38 ± 0.2647
MS+BA 2.0+IAA 0.5	57.7%	2.84 ± 0.3854	1.81 ± 0.1970
MS+BA 3.0+IAA 0.5	51.1%	2.34 ± 0.3269	1.56 ± 0.1675
MS+BA 0.5+NAA 0.5	77.7%	7.65 ± 0.2929	4.18 ± 0.2063
MS+BA 1.0+NAA 0.5	88.8%	9.57 ± 0.3410	4.71±0.3601
MS+BA 1.5+NAA 0.5	73.3%	6.59 ± 0.4195	3.87± 0.3983
MS+BA 2.0+NAA 0.5	64.4%	4.25 ± 0.3891	2.52 ± 0.4275
MS+BA 3.0+NAA 0.5	55.5%	2.58 ± 0.2453	1.11± 0.2427
MS+BA 0.5+NAA 1.0	68.8%	5.38 ± 0.1641	1.91± 0.2358
MS+BA 0.5+NAA 1.5	84.4%	7.31 ± 0.2504	3.72 ± 0.3148
MS+BA 0.5+NAA 2.0	71.1%	5.92 ± 0.2431	2.61± 0.2315
MS+BA 0.5+NAA 3.0	64.4%	3.69 ± 0.2144	1.85 ± 01297

3.2 Rooting

It is a well established concept that *in vitro* root induction in many plant species at slightly higher proportions of auxin to cytokine ratio yields better results. Per cent root induction, number of roots and root lengths obtained in this study are shown in Table 2. Overall, MS media supplemented with 1 mg/L of IBA were found to be remarkable among all root induction hormones tested in the present study. The best response with optimum rooting (80.1%) was observed in MS media containing 1 mg/L of IBA which also gave the highest number of roots (5.21) per explants and an average of 2.76 cm of root length (Fig. 1C). On the other hand relatively poor rooting response was observed on MS media supplemented with 0.5 to 2 mg/L of NAA and / or IAA. Other growth regulator tested in the present study reveal that optimum rooting in shoots of *O. tenuiflorum* was achieved on half strength MS medium supplemented with 1.0 mg/L NAA and thus support the theoretical concept of root induction in media supplemented with either NAA or IBA.

Table 2 Effect of different concentrations of Naphthalene Acetic Acid (NAA), Indole butyric Acid (IBA) and Indole Acetic acid (IAA) on root induction from nodal explants of *Ocimum tenuiflorum* var. CIM-AYU. Values are means at least 15 multiple roots of three different sampling bottles ± SE.

Growth regulators (mg/l)	Rooting percentage	Average no. of roots / explant	Average root length/explants (cm)
MS + NAA 0.5	26.7	0.89 ± 0.1856	0.41 ± 0.1365
MS + NAA 1.0	42.2	1.73 ± 0.2458	0.87 ± 0.1112
MS + NAA 1.5	62.2	3.67 ± 0.1755	1.61 ± 0.1542
MS + NAA 2.0	53.3	2.55 ± 0.1266	1.14 ± 0.1486
MS + IBA 0.5	68.9	3.33 ± 0.2865	1.87 ± 0.1624
MS + IBA 1.0	80.1	5.21 ± 0.1986	2.76 ± 0.1672
MS + IBA 1.5	73.3	4.14 ± 0.1587	2.14 ± 0.1861
MS + IBA 2.0	57.8	3.69 ± 0.2854	1.13 ± 0.1901
MS + IAA 0.5	17.8	0.73 ± 0.1624	0.63 ± 0.1241
MS + IAA 1.0	24.4	1.58 ± 0.2684	1.42 ± 0.1627
MS + IAA 1.5	37.8	2.74 ± 0.2254	0.56 ± 0.1451
MS + IAA 2.0	31.1	1.67 ± 0.3245	0.31 ± 0.1328

3.3 Callus Induction

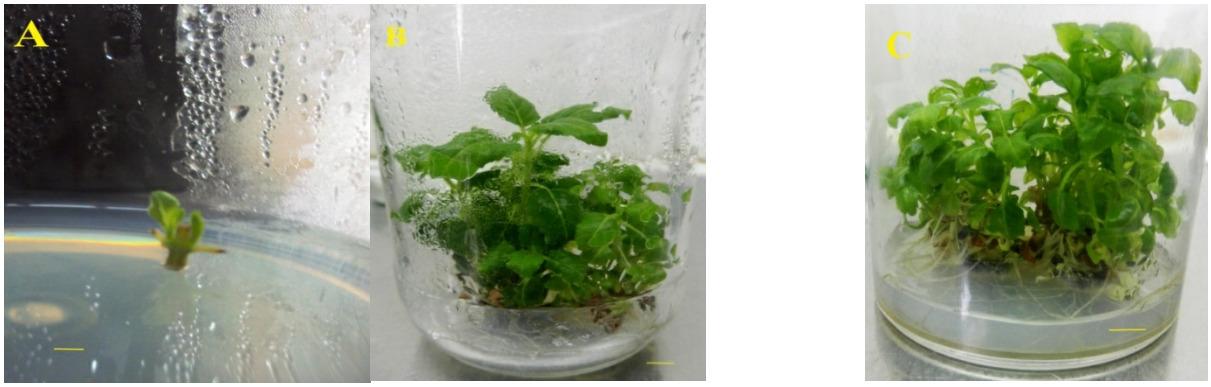
Dedifferentiated nodal explants were transferred on to the callus induction medium supplemented with 0.1 mg/L of 2, 4-D and different concentrations (0.25 – 1 mg/L) of kinetin were presented in Table 3. Growth of callus was measured in four week old cultures and expressed as dry biomass. Overall, highest callus dry biomass (71.3 mg) and callus (82.22%) was recorded in MS medium supplemented with 0.1 mg/L of 2, 4-D and 0.5 mg/L of Kinetin (Fig. 1E). Other combinations tested for callus growth in the present study were also found to be promising. Generally, callus induction studies in many *in vitro* propagation protocols reveal that equal proportions of growth hormones in culture media play a crucial role in optimal callus induction. Contrary to this trend, in the present

study it was found that at a relatively higher proportion of Cytokine to auxin, optimum callus biomass prevailed.

Table 3 Effect of Kinetin on callus induction and dry biomass of *Ocimum tenuiflorum* var. CIM-AYU from nodal explants.

S. No	Growth medium	Growth regulator concentrations (mg/L)	Callus %	Dry Biomass of Callus (mg)
1	MS Basal medium*	MS + 2,4-D (0.0) + Kn (0.0)	NA	NA
2	MS Basal medium	MS + 2,4-D (0.1) + Kn (0.25)	68.88	59.5 ± 0.4358
3	MS Basal medium	MS + 2,4-D (0.1) + Kn (0.5)	82.22	71.3 ±0.7856
4	MS Basal medium	MS + 2,4-D (0.1) + Kn (0.75)	73.33	66.8 ±0.3251
5	MS Basal medium	MS + 2,4-D (0.1) + Kn (1.0)	55.55	49.7 ±0.6584

With out growth regulators; NA Callus growth not observed; Values are means of 10 replicated calli grown in three different culture bottles ± SE.



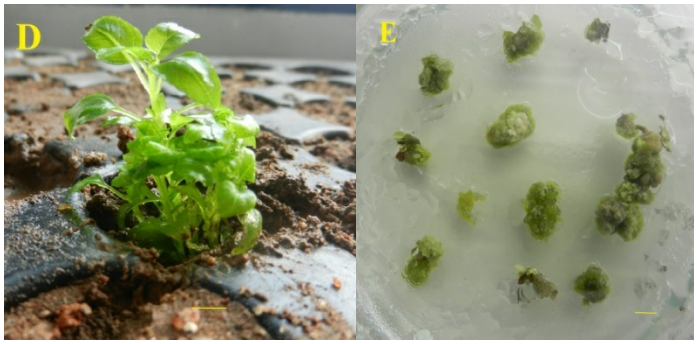


Fig.1. *In vitro* propagation of *Ocimum tenuiflorum* var. *CIM-AYU* developmental stages (A: Axillary bud proliferation; B: Multiple shoots; C: Multiple shoots with rooting; D: Transferred for hardening; E: Callus). A) Single nodal explants with induced growth of nodal buds on MS medium supplemented with 1 mg / L of Benzyl Adenine (BA) and 0.5 mg / L of Naphthalene acetic acid (NAA)-one week after inoculation; B) Shoot multiplication on MS medium supplemented with Benzyl Adenine 1.0 + NAA 0.5 mg / L 4 weeks of culture; C) Regenerated shoots with well developed adventitious roots cultured on ½ MS medium supplemented with 1 mg / L of BA; D) Hardening plant; E) Proliferation of callus from nodal segments on MS medium supplemented with 0.1 mg / L of 2,4-D with 0.5 mg/L of Kinetin.

Conclusions

In the present study, a competent and reliable micro propagation protocol for *in vitro* regeneration of *Ocimum tenuiflorum* var. CIM-AYU, from nodal ex-plants has been established. The standardized protocol ensures large scale supply of genetically uniform plants through micro propagation technique, which is important for sustainability and conservation of germplasm.

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