

MICROPROPAGATION OF AN ENDANGERED MEDICINAL HERB *OCIMUM CITRIODORUM* VIS.

Anamika Tripathi¹, N.S. Abbas² and Amrita Nigam¹

¹School of Sciences, Indira Gandhi National Open University, Maidan Garhi, New Delhi

²Department of Biology, Bhaskaracharya College of Applied Sciences, University of Delhi, New Delhi

Email: dr_nsabbas@yahoo.co.in

Abstract: An efficient protocol has been developed for rapid micropropagation of *Ocimum citriodorum* Vis., an endangered medicinal herb. The cotyledons were excised from the *in vitro* germinating seedlings and used as explants for the present study. The explants yielded the highest frequency of 87.49% shoot regeneration with an average shoot length of 4.98 cm on Murashige and Skoog (MS) medium supplemented with 1 mg l⁻¹ 6- benzylamino purine (BAP) + 0.1 mg l⁻¹ naphthalene acetic acid (NAA) + 500 mg l⁻¹ casein hydrolysate (CH) + 25 mg l⁻¹ adenine sulphate (AS). Alteration from the optimal concentration of BAP resulted in the formation of callus. Regenerated microshoots were separated and rooted on MS medium containing NAA (0.5 mg l⁻¹). Well-developed complete plantlets were transferred onto plastic cups containing sterile soil and humus (1:1). Subsequently the acclimatized plantlets were successfully grown in garden. The regenerated plants were morphologically identical and exhibited similar growth characteristics as compared to the donor plants. Cytological studies of the regenerants revealed no change in chromosome numbers. Thus, regeneration protocol demonstrated in the present study provides a basis for the germplasm conservation and investigation of its medicinally active constituents.

Keywords: Cotyledonary explant, cytology, histological observations, *ocimum citriodorum*

INTRODUCTION

Ocimum citriodorum Vis. (Lemon basil) belongs to the family Lamiaceae, is rich in aromatic essential oils and valuable for its medicinal, volatile and culinary properties (Venugopal and Rao, 2011). It is a hybrid between basil (*Ocimum basilicum*) and African Basil (*Ocimum americanum*) (Janarthanam and Sumathi, 2012). Lemon basil grows upto 20-40 cm in height. It flowers in late summer to early fall and bears white flowers. Its leaves are similar to basil leaves, but narrower. Seeds form on the plant after flowering and dry on the plant itself. Lemon basil is a popular herb in Arabian, Laotian, Persian and Thai cuisine. It is primarily grown in Northeastern Africa and Southern Asia, for its strong lemon fragrance in cooking and in the preparations of antioxidant tea bags (Janarthanam and Sumathi, 2012). It is helpful to ease the people suffering from early ejaculation, late menstruation, breast milk, works as a gas cleanser in the human body, helpful in removing fever (Epriliati and Ginjom, 2012) and inhibits hepatocarcinogenesis (Tripathi, 2011).

Ocimum plants possess essential oil, which contains biologically active constituents that are insecticidal, nematocidal, fungistatic, antimicrobial or antioxidant (Janarthanam and Sumathi, 2012). These properties can be attributed to the predominant essential oil constituents, such as estragol, eugenol, linalool, citral and 1-8, cineole (Stanko *et al.*, 2010a). It was observed that essential oil obtained from the lemon basil inhibits the growth of *Staphylococcus aureus*. Its essential oil is effective against many other food borne pathogenic bacteria also such as, *Enterococcus faecalis*, *Enterococcus faecium*, *Proteus vulgaris* and *Staphylococcus epidermis* (Stanko *et al.*, 2010a).

The conventional method for the propagation of *Ocimum species* is through seed germination and stem cuttings. However, poor germination of the seeds (< 10%) due to season dependency (Saha *et al.*, 2014) and susceptibility to seedling blight and root rot diseases (Siddiqui and Anis, 2009) as well as unusually longer time (28 days or more, Sulistiarini, 1999) required for the rooting from stem cuttings, restricts its multiplication. Therefore, it is necessary to develop an *in vitro* rapid and reproducible protocol for the large scale production of such medicinally important plant (Venugopal and Rao, 2011). Ideally, the medicinal plants should have the same genetic make-up as of the selected high-yielding clones. As seedling progeny of *Ocimum* shows variability due to cross pollinating nature of the plant (Gopi *et al.*, 2006), the *in vitro* micropropagation could prove an effective tool for obtaining the species with high progeny uniformity (Asghari *et al.*, 2012). *In vitro* plant regeneration of *Ocimum citriodorum* from leaf (Venugopal and Rao, 2011) and nodal segment (Venugopal and Rao, 2011; Janarthanam and Sumathi, 2012) have been reported earlier. Janarthanam and Sumathi (2012) reported that the highest percentage of shoot formation with maximum number of shoots per culture was obtained from the nodal explants of *O. citriodorum* on Murashige and Skoog (1962) medium augmented with 1 mg l⁻¹ BAP and 0.025 mg l⁻¹ indole acetic acid (IAA) and rooting of the shoot was accomplished when 0.5 mg l⁻¹ indole butyric acid (IBA) was alone present with the basal medium (Janarthanam and Sumathi, 2012). Venugopal and Rao (2011) reported that the higher frequency of shoot formation was obtained from leaf and nodal explants in MS medium containing BAP and IBA. However, to date, there is no report on *in vitro* studies of *O. citriodorum* through cotyledonary

explant. The present study is the first report to describe a simple, rapid, reproducible, economical and high frequency regeneration protocol for the *in vitro* micropropagation of *O. citriodorum* using cotyledonary explants and the subsequent establishment of these plants in soil.

MATERIAL AND METHOD

Plant material and culture conditions

Seeds of *O. citriodorum* were collected from the two year old plants growing in the beds of Botanical garden of University of Delhi, Delhi, India. These seeds were cleaned thoroughly under running tap water, followed by washing with teepol detergent solution and then with sterile distilled water. The cleaned seeds were surface sterilized with 0.2% (w/v) mercuric chloride (HgCl_2) for 5 min and finally the traces of sterilant were removed by repeatedly washing in sterile distilled water. All subsequent operations were carried out in a laminar air flow chamber. Thereafter, seeds were germinated on MS (1962) medium supplemented with 3% sucrose and solidified with 0.8% agar. No plant growth regulators (PGRs) were added. The seeds were incubated for 1 month and cotyledons excised from these aseptically germinating seedlings were used as explants.

The explants were cultured on MS medium supplemented with one of the four cytokinins namely, BAP, kinetin (Kn), thidiazuron (TDZ) and N^6 -(-2 isopentenyl) adenine (2iP) at different concentrations (0.5, 1.0, 1.5, 2.0 mg l^{-1}) with their adaxial face in contact with the culture medium. The best PGR for the shoot regeneration was determined based on the regeneration rate. After selecting BAP as the best PGR for direct shoot regeneration, further experiments were carried out to assess the influence of NAA, CH and AS on the shoot regeneration. All plant growth regulators were obtained from Sigma Aldrich (USA). Salts and other chemicals were obtained from Qualigens, Glaxo and SRL, Mumbai (India). Sucrose (3%, w/v) as carbon source was added to the media. The pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. Approximately, 20 ml media was dispensed in each 150 × 25 cm test tubes (Borosil, India), plugged with non absorbent cotton wrapped in two-layered muslin cloth and sterilized by autoclaving at 1.06 kg cm^{-2} at 121°C for 15 min. The cultures were maintained on continuous light emitted from fluorescent incandescent tubes (40 W, Phillips, Kolkata, India) for 16 h light followed by 8 h dark period in a culture room at 25 ± 2°C temperature with a relative humidity of 55 ± 5 %.

Rhizogenesis and acclimatization of regenerated plantlets

Occasional rooting was observed in shoot proliferation cultures that were left for over 5 weeks. However, for proper rooting the microshoots with 4-6

leaves (2-3 cm) were harvested and transferred to hormone free MS medium for 2 weeks to eliminate any carry over effect of cytokinins (Shibli *et al.*, 1997). Elongated shoots (about 4-5 cm in length) were then carefully transferred to the MS medium supplemented with NAA (0.1-1.0 mg l^{-1}), IBA (0.1-1.0 mg l^{-1}) and IAA (0.1-1.0 mg l^{-1}). MS basal medium was used as control. Cultures were incubated under the same conditions as mentioned above.

Well developed plantlets were carefully removed and washed thoroughly in running tap water to remove agar. Further, they were treated with 1% bavistin (BASF, Mumbai, India) solution to prevent any fungal infection. Thereafter, they were transferred to plastic pots (5 cm diameter) containing autoclaved soil: humus (1:1). Subsequently, acclimatization was achieved by covering the plastic pots with polythene bags to maintain the humidity. The plants were irrigated with one-tenth of MS basal salt solution devoid of sucrose and inositol. After 1 week, 3-5 holes were made in polythene bags and plants were irrigated after every 4 days. The potted plants were maintained in the culture room. After 45 days, the plantlets were transplanted to earthen pots (25 cm diameter) containing garden soil. They were kept under shade in a net house for another 2-3 weeks before being transferred to field for developing into mature plants. Pre-acclimatization or gradual plant exposition to external environment could contribute to the future survival of the plant under greenhouse conditions (Dibax *et al.*, 2010).

Recording of data and statistical analysis

The morphogenetic response of explants were evaluated after 6 weeks of culture in terms of (i) shoot inducing frequency of explants, (ii) average number of shoots per explants and (iii) average shoot length per explant. The following parameters were considered for the rhizogenesis: (i) root inducing frequency of shoots, (ii) average number of roots per shoot and (iii) average root length. For *in vitro* regeneration, the shoot inducing frequency of explants, the average shoot length, root inducing frequency of shoots and average root length has been represented as mean values along with standard error (mean ± SE). The mean values were calculated on the basis of a minimum of 12 replicates and each experiment was repeated twice. The data expressed as mean ± SE was statistically analyzed using ANOVA (Analysis of Variance) through SPSS (Statistical Package for Social Sciences) version 16.0. The differences between means were tested for significance by Duncan's multiple range test (DMRT) at $p=0.05$.

Histological studies

For histological observations regenerating explants were excised and fixed in a mixture of formalin: acetic acid: ethanol (1:1: 16) for 24 h following dehydration in an ethanol/ xylene series.

The material was infiltrated and embedded in paraffin wax. Sections were cut on a rotary microtome at 7–8 µm thickness, dried onto slides, dewaxed in xylene and rehydrated in a descending ethanol series. Sections were stained with fast green and counter stained with safranin. Slides were observed under a Zeiss, Primo Star Microscope (Carl Zeiss Micro Imaging GmbH, Gottingen, Germany) and suitable sections were photographed using a Canon – G10 digital camera.

Scanning Electron Microscopy

To support the findings of *in vitro* studies scanning electron microscopy was also performed for the *in vitro* regenerated plantlets. Samples were fixed for 24 h with Karnofsky's fixative and stored in 0.1M phosphate buffer (pH- 7.2). The tissues were critical point dried and sputter coated with gold. Observations and photographs were made on Leo-435 VP variable pressure scanning electron microscope (Co-operation Zeiss- Leica).

Cytological examination of mother plant and regenerants

To determine the chromosome number of the regenerants the root tips were collected. The root tips were pre-treated with ice for 24 h at 4°C. They were fixed in freshly prepared Carnoy's solution (alcohol: acetic acid= 3:1) for 24 - 48 h and stored in 70% (v/v) ethanol at 4°C (Wang *et al.*, 2012). Fixed root tips were subsequently washed 3–4 times with distilled water and hydrolyzed in 1M HCl for 10 min at 60°C and then rinsed in distilled water (Mohammad *et al.*, 2013). Subsequently root tips were stained with Feulgen stain for 1 hr. After washing, tips were immersed in aceto-carmin and squash preparations were made for the cytological studies. Cytogenetic examination and chromosome counting were carried out with a Zeiss, Primo Star Microscope equipped with a Canon – G10 digital camera (Carl Zeiss Micro Imaging GmbH, Gottingen, Germany). The chromosome numbers of the regenerants were compared with that of mother plant to prove that they are genetically similar to their parent.

RESULT

Shoot induction and elongation

In order to establish an efficient *in vitro* regeneration protocol for the commercial exploitation of this important endangered herb, the explants were excised from the aseptically germinating seedlings (Fig.1e). The explants were inoculated on MS basal medium as well as medium supplemented with various concentrations (0.5, 1.0, 1.5, 2.0 mg l⁻¹) of cytokinins (BAP, Kn, TDZ, 2iP). The explants cultured on the MS basal medium, enlarged in size and became necrotic after 2 weeks of inoculation. Addition of cytokinin was essential for differentiation of multiple shoots. Of the four cytokinins tested BAP was found to be the best for the induction of shoots followed by 2iP, TDZ and Kn (Table 1). On MS media containing BAP (1 mg l⁻¹), the explants initially enlarged from its original size after 6 days of inoculation (Fig. 2a) followed by the appearance of protuberances like structure. Such structures differentiated into shoot buds and subsequently gave rise to shoots on the same media composition without an intervening callus phase after 3–4 weeks of inoculation (Fig. 2b). On altering the concentration of BAP from the optimal level, calli were produced. In order to enhance the morphogenic response, NAA was incorporated in the MS medium with optimum concentration of BAP. Significant increase in shoot production was observed (62.49%), when NAA was added to the medium at low concentration (0.1 mg l⁻¹), suggesting a synergistic effect of NAA on shoot proliferation (Table 2). Higher concentration of NAA (above 0.1 mg l⁻¹) in the medium resulted in the production of callus. A remarkable threefold increase in morphogenic response could be achieved (Table 2) when adenine sulphate and casein hydrolysate were incorporated in MS medium with BAP and NAA. Thus the maximum shoot regeneration frequency (87.49 %) was observed on MS medium containing BAP (1 mg l⁻¹) + NAA (0.1 mg l⁻¹) + CH (500 mg l⁻¹) + AS (25 mg l⁻¹) with an average of 6.04 shoots per explant having an average shoot length of 4.98 cm after 4–5 weeks of culture (Table 2 and Fig. 2b- g).



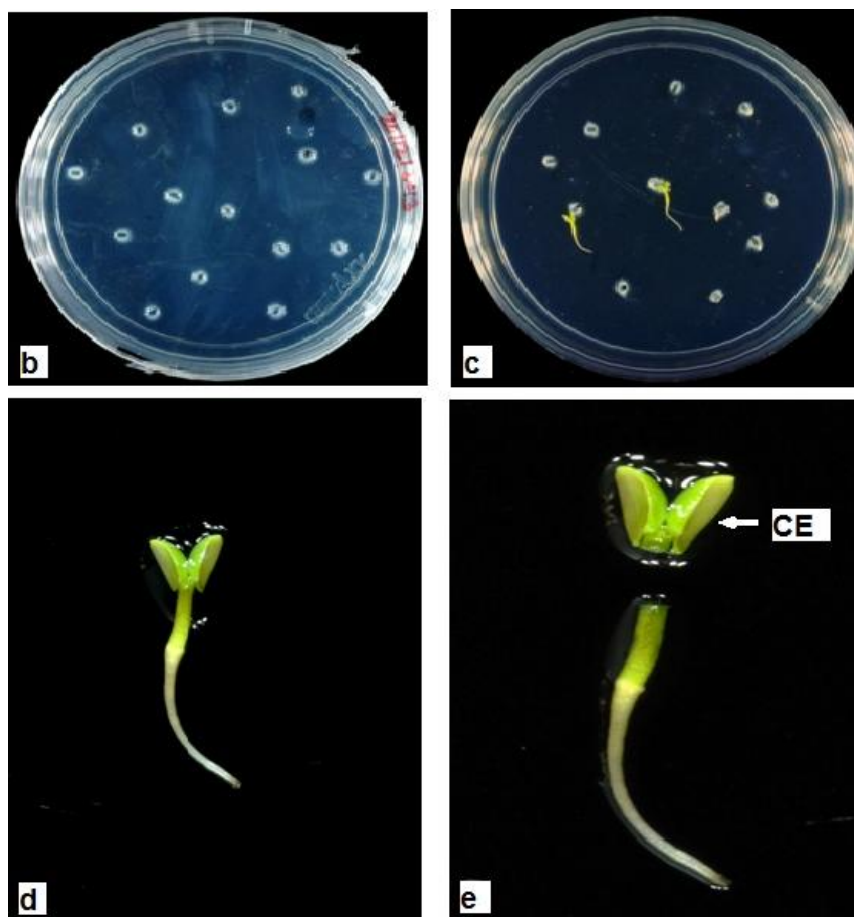


Fig. 1 (a-e): A schematic presentation of the cotyledonary explants from seedling of *O. citriodorum*: (a) An inflorescence of the mother plant from which seeds were collected; (b) Seeds on the MS basal medium; (c) Germinating seedlings after 2 weeks of inoculation; (d) Explant as it looks before excision from the seedling; (e) Excised cotyledonary explant (CE)

Table 1: Effect of different concentrations of cytokinins on multiple shoot induction from the cotyledonary explants of *Ocimum citriodorum*

Plant growth regulators (mg l ⁻¹)		No. of cultures forming shoots (%)	No. of shoots/ explant	Shoot length (cm)
BAP	0.5	0a	0a	0a
	1.0	29.16c	4.0 ± 0.0de	3.78 ± 0.31cde
	1.5	0a	0a	0a
	2.0	0a	0a	0a
Kn	0.5	0a	0a	0a
	1.0	0a	0a	0a
	1.5	20.83bc	4.41 ± 0.12e	4.20 ± 0.50e
	2.0	16.66bc	3.83 ± 1.17abc	3.65 ± 0.21bcd
TDZ	0.5	24.99bc	2.75 ± 0.35b	3.82 ± 0.03de
	1.0	12.49ab	2.75 ± 0.35b	3.45 ± 0.21bcd
	1.5	20.83bc	3.41 ± 0.12cde	3.62 ± 0.45bcd
	2.0	0a	0a	0a
2iP	0.5	0a	0a	0a
	1.0	16.66bc	3.0 ± 0.70bc	3.30 ± 0.07bc
	1.5	20.83bc	3.0 ± 0.00bc	3.40 ± 0.28bcd
	2.0	25.00bc	2.83 ± 0.24b	3.24 ± 0.16b

Mean values in a column followed by different letters are significantly different as determined by SPSS at $p = 0.05$ according to DMRT

Table 2: Effect of CH and AS on multiple shoot induction from the cotyledonary explants of *Ocimum citriodorum* in the MS medium containing optimum concentration of BAP and NAA.

Plant growth regulators (mg l ⁻¹)				No. of cultures forming shoots (%)	No. of shoots /explants	Shoot length (cm)
BAP	NAA	CH	AS			
1.0	0.1	-	-	62.49a	4.16±0.57a	4.03±0.19a
1.0	0.1	500	25	87.49b	6.04±0.06b	4.98±0.00b
1.0	0.1	700	50	79.16ab	5.95±0.007b	4.66±0.07b

Mean values in a column followed by different letters are significantly different as determined by SPSS at $p = 0.05$ according to DMRT

Root induction and acclimatization of plantlets

The *in vitro* induced shoots were placed on MS basal medium as well as on the medium supplemented with auxins (IBA, IAA and NAA) at various permutation and combinations. Shoot failed to produce roots in the MS basal medium. No significant results were obtained in the MS medium supplemented with different concentrations (0.1 – 1.5 mg l⁻¹) of IBA and IAA (data not shown). However, root primordial emerged from the base of shoots after 2 weeks of transfer on MS medium supplemented with NAA (0.5 mg l⁻¹) (Fig. 2h). A maximum of 87.49% shoots induced an average of 6.04 roots with an average root length of 4.77 cm after 3-4 weeks of transfer on MS

medium augmented with NAA (0.5 mg l⁻¹) (Table 3 and Fig. 2i, j). Higher concentration of NAA drastically reduced the root inducing frequency (Table 3). Plantlets with well developed shoots and roots were removed from the culture tubes, thoroughly washed and transferred to the thermo cups containing sterile soil and humus (1:1) (Fig. 2k). The plants were hardened by following the procedure stated in material and methods and subsequently transferred to the garden soil under natural environment (Fig. 2l). About 95% survival frequency was observed for the *in vitro* regenerated plantlets. The plants were green, healthy and phenotypically similar to the mother plant.

Table 3: Effect of different concentrations of NAA on root induction from the microshoots obtained from the cotyledonary explants of *Ocimum citriodorum*

Concentration of NAA (mg l ⁻¹)	No. of cultures forming roots (%)	No. of roots/ microshoot	Root length (cm)
0.1	16.66a	1.25±0.25a	1.20±0.10a
0.5	87.49c	6.04±0.20c	4.77±0.04d
1.0	41.66b	3.20±0.20b	4.21±0.07c
1.5	20.83a	1.80±0.37a	3.18±0.09b

Mean values in a column followed by different letters are significantly different as determined by SPSS at $p = 0.05$ according to DMRT

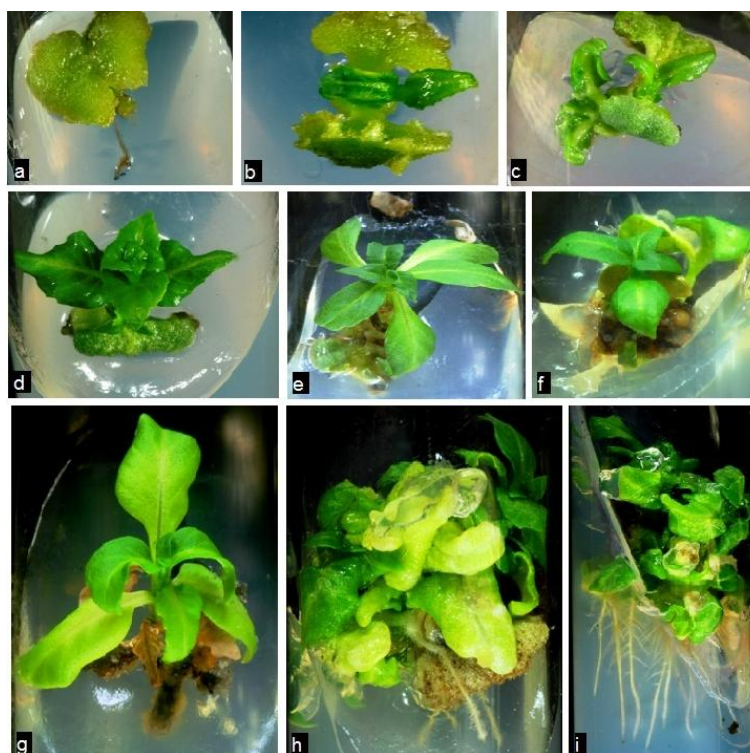




Fig. 2 (a-l): Direct organogenesis through cotyledonary explants of *O. citriodorum*: (a) Enlargement of the explant after 5 days of inoculation on the MS medium containing BAP (1 mg l^{-1}); (b) Initiation of shoot formation after 2 weeks of inoculation on the MS medium containing BAP (1 mg l^{-1}) + NAA (0.1 mg l^{-1}) + CH (500 mg l^{-1}) + AS (25 mg l^{-1}); (c) Differentiation of multiple shoots after 2-3 weeks of inoculation; (d) Enhanced shooting after 3-4 weeks of culture on the same medium; (e) Excised single shoot growing well on the same medium composition after 1 week of subculture; (f) Rapid proliferation and multiplication within 2 weeks; (g) Elongation of shoot after 3 weeks; (h) Root induction from the excised shoots after 2-3 weeks of transfer on the MS medium containing NAA (0.5 mg l^{-1}); (i) Profuse rooting after 3-4 weeks of transfer on the root induction medium; (j) Plantlet showing healthy shoots and roots just before hardening; (k) 1 month old *in vitro* regenerated plant proliferating in the pot containing sterile soil: humus (1:1); (l) Tissue culture raised plants well established in the field after 3 months of transfer

Histological observations

Histological studies were carried out during a period of two week culture to analyse the regeneration process. During the first two days no clear histological changes were detectable (Fig. 3a). The first visible changes thereafter consisted of the setting of a cell differentiation process in the epidermal and sub-epidermal layers of the explants. Epidermal cells divided periclinally and anticlinally producing both further epidermal cells and a new subepidermal layer (Fig. 3b). This process led to the formation of clusters of smaller daughter cells that differed from the

mother ones by their thinner wall and densely stained nuclei and cytoplasm. These particular cell clusters formed meristemoids (M) (Fig. 3b). The arrangement of cells in these bulges, their shape, size and development of tracheary elements (Te) (Fig. 3b) suggested that these were the site of origin of shoot primordia. After 1 week of culture, formation of shoot bud (Sb) with leaf primordial (lp) was observed (Fig. 3c). A transverse section of 2 weeks old culture showed shoot bud (Sb), first leaf primordial (lp1) and second leaf primordial (lp2) with bulges of midrib (mr) (Fig. 3d).

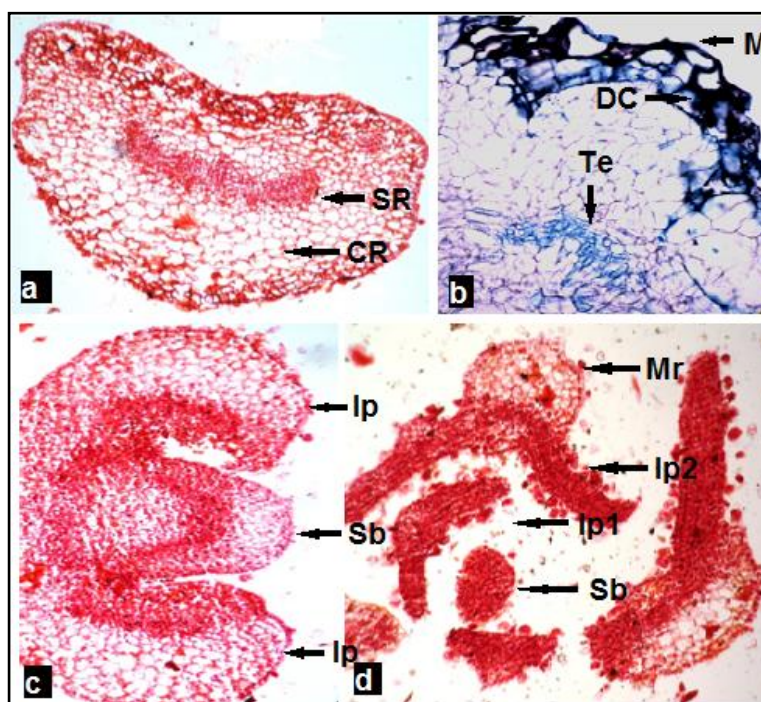


Fig. 3 (a-d): Histological evidences of regeneration of complete plantlet from cotyledonary explants of *O. citriodorum*: (a) Cotyledonary leaf explants freshly prepared from 5 day old seedling showing stellar region (SR) and cortical region (CR); (b) High magnification of 3 days old culture showing the presence of dedifferentiated cells (DC) in the epidermal and sub-epidermal tissues, initiation of formation of first meristemoids (M) and tracheary element (Te); (c) Longitudinal section of 1 week old culture showing shoot bud (Sb) and leaf primordial (lp); (d) Transverse section of 2 week old culture showing shoot bud (Sb), first leaf primordium (lp1), second leaf primordium (lp2) with bulges of midrib (mr)

Scanning Electron Microscopy (SEM)

SEM observations of the regenerating explant revealed typical shoot formation with an apex and shoot primordia. The shoot primordium was covered by epidermis, built of more or less equal sized cells. Shoot bud formation was initiated from the explants as small nodular outgrowth without any apparent callus stage. This developing outgrowth eventually led to the formation of shoot buds (Fig. 4b). The formation of buds of different size could be observed

in the developmentally active region of the cotyledonary explants, thus documenting that they have the much sought after potential for *in vitro* plant regeneration ability in this plant species. On the surface of the regenerated leaf, glandular trichomes were observed (4c). The trichomes consisted of a stalk cell, a neck cell and a pear shaped secretory cell (capitate type II). Thus, these observations confirmed the results of *in vitro* studies.

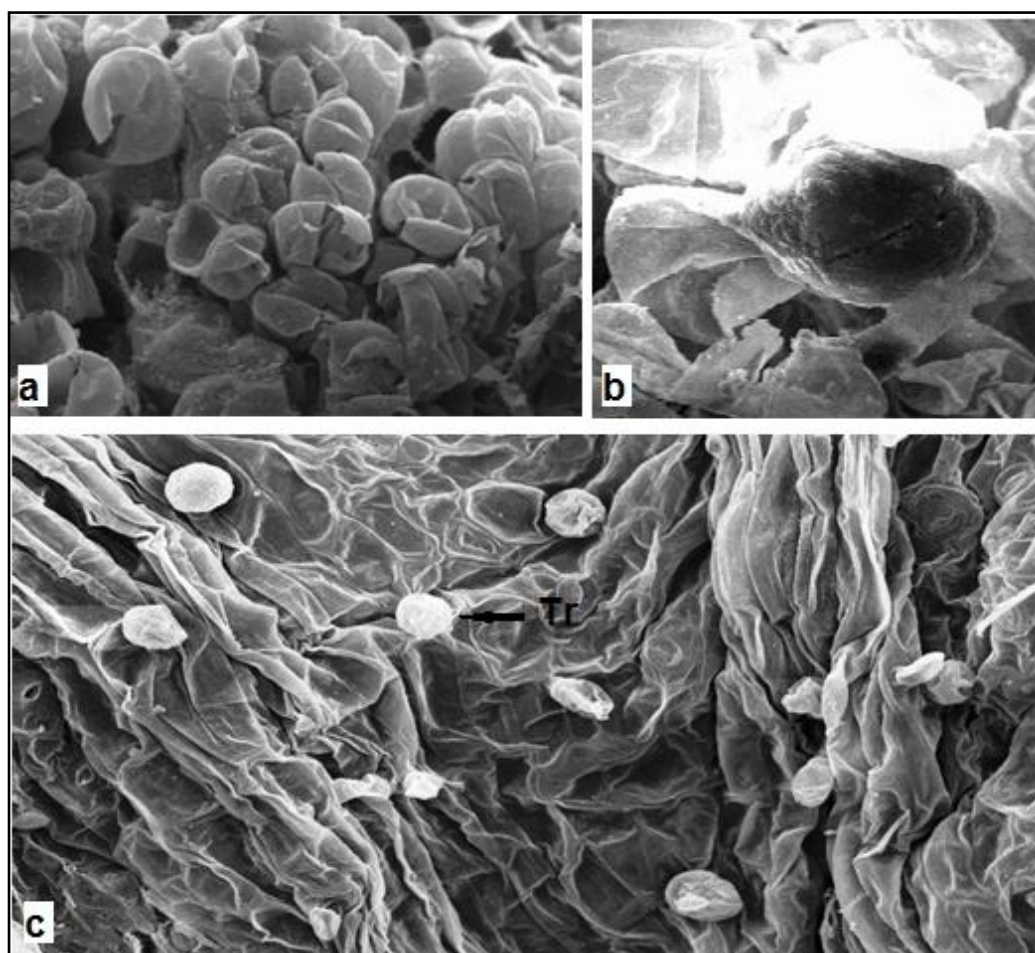


Fig. 4 (a-c): Scanning electron micrograph showing the development of shoot bud and leaf from the cotyledons of *O. citriodorum*: (a) SEM of upper surface of the explants after 5-6 days of culture showing callus like structure; (b) Shoot bud; (c) Surface of *in vitro* regenerated leaf showing trichomes (Tr)

Cytological analyses

The chromosome number has been found to be $2n = 72$ from both regenerants (Fig. 5a) and mother plant (Fig. 5b). In the present study, cytogenetic analysis did not reveal any signs of abnormal mitosis in dividing cells from the root tips of *in vitro*

regenerated plantlets. Although plants regenerating in culture medium may show variability (Chakravarty and Sen, 1992). The plantlets regenerating from the cotyledons in the present study appear to be uniform and genetically stable.

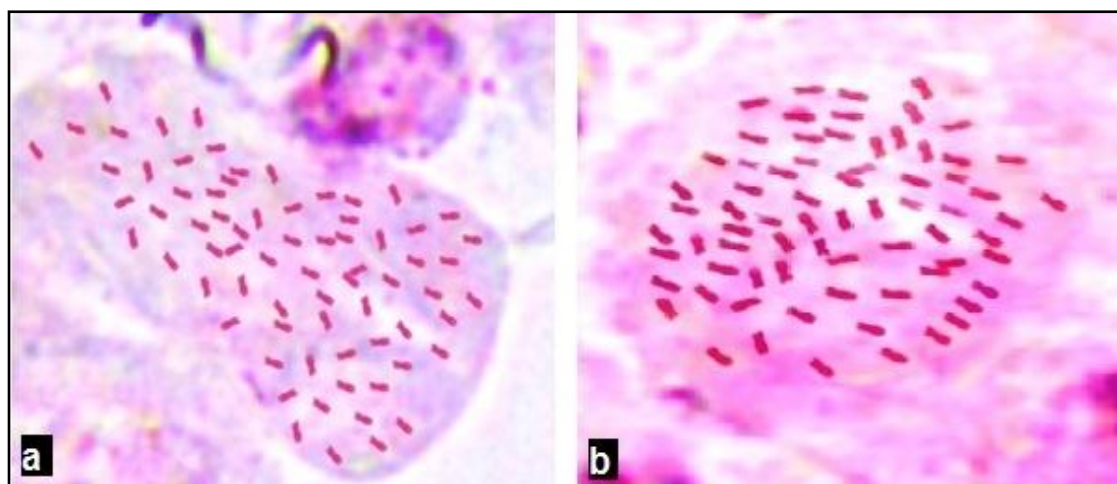


Fig. 5 (a-b): Cytological analysis of *O. citriodorum*: (a) 72 chromosomes of a metaphase cell from a root tip squash of a regenerant; (b) Root tip squash showing chromosomes of the donor plant.

DISCUSSION

Development of direct shoot regeneration protocol from cotyledons of *O. citriodorum* is one of the important requisitions toward clonal mass multiplication for commercial and pharmaceutical application. Obtaining regenerants of same genetic composition is yet another significant parameter toward this direction. In the present study, the plantlets were regenerated directly from the cotyledons of *in vitro* raised seedlings of *O. citriodorum*. MS medium was used for the *in vitro* studies as it is the most appropriate and widely used medium in dicotyledonous morphogenic processes due to high NO_3^- and NH_4^+ concentration and proportion between these nitrogen forms (Dibax *et al.*, 2010). Of the four cytokinins, viz. BAP, Kn, TDZ and 2iP tested, BAP proved to be the best both in terms of percentage morphogenic cultures as well as average shoot number per explant. Significance of BAP in inducing multiple shoots has already been reported in different species of *Ocimum* like, *O. basilicum* (Sahoo *et al.*, 1997), *O. sanctum* (Singh and Sehgal, 1999), *O. gratissimum* (Gopi *et al.*, 2006), *O. citriodorum* (Janarthanam and Sumathi, 2012) and *O. basilicum* (Shahzad *et al.*, 2012). Thus, our results are in consistence with the earlier reports. The promotory effect of BAP in the present study over other cytokinins could be due to its easy permeability, increased affinity for active cell uptake, less resistance to the enzyme cytokinin oxidase, or receptor abundance in its perception apparatus which interacts with the coupling elements in the signal transduction chain (Burch and Stuchbury, 1987). Moreover, BAP if added exogenously shortens the duration of S phase of cell division through recruitment of latent origin of DNA replication both *in vitro* and *in vivo* (Francis and Sorell, 2001). Addition of growth regulators such as auxins to the culture is extremely important, since they are able to start cell division and control the growth processes

and cell elongation (Asghari *et al.*, 2012). In the present study also the morphogenic response enhanced by the incorporation of NAA in the medium containing optimum concentration of BAP. This suggested synergistic effect of NAA on the shoot proliferation from the explant. This could be due to the fact that auxin in combination with cytokinin, leads to rapid cell division, forming a large number of relatively small and undifferentiated cells (Mendoza and Kaeppler, 2002). Besides it, auxin to cytokinin ratio modulates the cell division, cell elongation and plant regeneration (Sahu *et al.*, 2013). The number of shoot per culture was decreased when BAP concentration was increased from its optimal level (1 mg/l). This may be due to the toxicity of BAP at higher concentration which might lead to genetic, physiological and morphological changes, resulting in a reduction of the proliferation rate *in vitro* (Narayanswamy, 1977).

The multiplication and establishment of cotyledonary explant was further improved by the addition of AS and CH. This could be due to the fact that adenine in the form of adenine sulphate can stimulate cell growth and greatly enhance shoot formation (Murashige, 1974) by providing an available source of Nitrogen to the cell. This nitrogen can be taken up more rapidly than inorganic Nitrogen (Thom *et al.*, 1981). Our results confirmed the efficiency of CH in *in vitro* growth which could be due to its high concentration of glutamine that balances the shortage of amino acid synthesis when the medium has a phosphorous deficiency (Miel, 1985).

In the present study, rooting of the shoots was induced in the MS medium supplemented with NAA. Similar results were recorded in *O. sanctum* (Begum *et al.*, 2000) and *O. basilicum* also (Pattnaik and Chand, 1996). This could be probably due to fact that NAA reduces the induction period (Washida *et al.*, 2004) as well as increases the biomass (Kusmapudi *et al.*, 2010) by inducing proliferation and lateral root

formation (Nandgopal and Kumaria, 2007; Sudha and Seen, 2001).

From the histological studies it is evident that *O. citriodorum* displayed the direct pattern of regeneration. The present study revealed the time course of the organogenesis and the triggering of cell division that leads to the formation of a complete plantlet. Shoot primordia were observed after 3-5 days of culture on the inductive media, it may imply that BAP activates cell division of the competent cells (Mendoza *et al.*, 1993).

The regenerants have been further revalidated as genetically uniform and similar to the mother plant by chromosome counting. Cytogenetic analysis can provide information about abnormal mitosis or changes in ploidy levels (Radic *et al.*, 2005). In the present study cytogenetic analysis did not reveal any signs of abnormal mitosis in dividing cells from the root tips of *in vitro* regenerated plantlets. The base number of *Ocimum* has been suggested as $x = 12$ (Stanko *et al.*, 2010b). *O. citriodorum* is probably an allohexaploid ($2n = 6x = 72$) as referred by Paton and Putievsky (1996). Our results are confirmatory with the reports of Mukharjee *et al.* (2005) and Stanko *et al.* (2010b) who observed $2n = 72$. However, some researchers observed altered chromosome numbers also, like Paton and Putievsky (1996) who reported $2n = 64$ for the same species.

CONCLUSION

The present study has demonstrated the feasibility of a direct regeneration and clonal propagation protocol to produce true-to-type plants of *O. citriodorum*. To the best of our knowledge, this is the first report of successfully inducing plantlets from the cotyledons of *O. citriodorum*. The ability of reliably producing true-to-type plants, offers a promising tool for its structural and functional genomics, as well as biotechnological studies.

ACKNOWLEDGEMENT

Anamika Tripathi is thankful to Prof. Ved Pal Singh, Department of Botany, University of Delhi, Delhi, for providing laboratory facilities and Indira Gandhi National Open University, Maidan Garhi, New Delhi, for providing fellowship as Research & Teaching Assistant and Senior Research & Teaching Assistant.

REFERENCES

Asghari, F.; Hossieni, B.; Hossani, A. and Habib S. (2012). Effect of explants source and different hormonal combinations on direct regeneration of basil plants (*O. basilicum* L.). *Australian Journal of Agricultural Engineering*, 3 (1): 12-17.

Begum, F.; Amin, N. and Azad A.K. (2000). *In vitro* clonal propagation of holy basil- *Ocimum sanctum* L. *Plant Tissue Culture*, 10: 31-37.

Burch, L.R. and Stuchbury, T. (1987). Activity and distribution of enzymes that interconvert purine bases, ribosides and ribotides in the tomato plant and possible implications in cytokinin metabolism. *Physiologia Plantarum*, 69: 283- 288.

Chakravarty, B. and Sen, S. (1992). Chromosome and nuclear DNA in regenerants of *Scilla indica* (Roxb.) Baker derived from two explants sources. *Cytologia*, 57: 41-46.

Dibax, R.; Quisen, R.C.; Bona, C. and Quoirin, M. (2010). Plant regeneration from cotyledonary explants of *Eucalyptus camaldulensis* Dehn and histological study of organogenesis *in vitro*. *Brazilian Archives of Biology and Technology*, 53 (2) 311-318.

Epriliati, I. and Ginjom, I.R. (2012) Bioavailability of phytochemicals, *Phytochemicals - A Global Perspective of Their Role in Nutrition and Health*, Dr Venketeshwer Rao (Ed.). InTech.

Francis, D. and Sorell, D.A. (2001). The interphase between the cell cycle and plant growth regulators- a mini review. *Plant Growth Regulators*, 33: 1-12.

Gopi, C.; Sekhar, Y.N. and Ponnurugan, P. (2006). *In vitro* multiplication of *Ocimum gratissimum* L. through direct regeneration. *African Journal of Biotechnology*, 5(9):723-726.

Janarthanam, B. and Sumathi, E. (2012). Plantlet regeneration from nodal explants of *Ocimum citriodorum* Vis. *Bangladesh Journal of Scientific and Industrial Research*, 47(4): 433-436.

Kusmapudi, S.; Selvakkumar, C.; Senthil, A.K. and Beddireddi, S.L. (2010). *In vitro* root culture of *O. sanctum* L. and evaluation of its free radical scavenging activity. *Plant Cell, Tissue and Organ Culture*, 101: 105-109.

Mendoza, M.G. and Kaeppler, H.F. (2002). Auxin and sugar effects on callus induction and plant regeneration frequencies from mature embryos of Wheat (*Triticum aestivum* L.) *In Vitro Cellular and Developmental Biology- Plant*, 38: 39- 45.

Miel, F.B.; Guignard, J.L.; Bury, M. and Agier, C. (1985). Glutamine as an active component of casein hydrolysate: Its balancing effect on plant cells cultured in phosphorus deficient medium. *Plant Cell Report*, 4: 161- 163.

Mohammad, A.; Moieni, A. and Badi, H.N. (2013). Morphological, physiological, cytological and phytochemical studies in diploid and colchicines-induced tetraploid plants of *Echinacea purpurea* (L.). *Acta Physiologiae Plantarum*, 35: 2075-2083.

Murashige, T. (1974). Plant propagation through tissue culture. *Annual Review of Plant Physiology*, 25:135-166.

Murashige, T. and Skoog F. (1962). A revised for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant*, 15: 473- 497.

Nandgopal, S. and Kumari, B.D.R. (2007). Effectiveness of auxin induced *in vitro* culture in chicory. *Journal of Central European Agriculture*, 8 (1): 73-80.

- Narayanswamy, S.** (1977). Regeneration of plants from tissue cultures. In: Applied and Fundamental Aspects of Plant Cell Tissue and Organ Culture, *Ornamental Species*, Volume 5, McGraw-hill Publishing Company, USA, pp 3-9.
- Paton, A. and Putievsky,** (1996). Taxonomic problems and cytotoxic relationships between and within varieties of *Ocimum basilicum* and related species (Labiatae). *Kew Bulletin*, 51: 509-524.
- Pattnaik, S. and Chand, P.K.,** (1996). In vitro propagation of the medicinal herbs *O. americanum* L. syn. *O. canum* Sims. (hoary basil) and *O. sanctum* L. (holy basil). *Plant Cell Reports*, 15: 846- 850.
- Radic, S.; Prolic, M.; Pavlica, M. and Pevalek, K.B.** (2005). Cytogenetic stability of *Centaurea ragusina* long term culture. *Plant Cell Tissue and Organ Culture*, 82: 343-348.
- Saha, S.; Sengupta, C. and Ghosh P.** (2014). Molecular and phytochemical analyses to assess genetic stability in alginate- encapsulated microshoots of *O. gratissimum* L. following in vitro storage, *Nucleus*, DOI 10.1007/s13237-014-0107-y
- Sahoo, Y.; Pattnaik, S.K. and Chand, P.K.** (1997). In Vitro clonal propagation of aromatic medicinal herb *Ocimum basilicum* (Sweet Basil) by axillary shoot proliferation. *In Vitro Cellular Developmental Biology- Plant*, 33: 293-296.
- Sahu, A.R.; Rath, S.C. and Panigrahi, J.** (2013). In vitro propagation of *Aerva lanata* (L.) Juss. ex Schult. through organogenesis, *Indian Journal of Biotechnology*, 12: 260-264.
- Shahzad, A.; Faisal, M.; Ahmad, N.; Anis, M.; Alatar, A. and Hend, A.A.** (2012). An efficient system for in vitro multiplication of *Ocimum basilicum* through node culture. *African Journal of Biotechnology*, 11 (22): 6055- 6059.
- Shibli, R.A.; Ajlouni, M. M.; Jaradat, A.; Aljanabi, S. and Shatnawi, M.** (1997). Micropropagation in wild pear (*Pyrussyrca*), *Scientia Horticulturae*, 68: 237-242.
- Siddiqui, I.; and Anis, M.** (2009). Morphogenic response of the alginate encapsulated nodal segment and antioxidative enzymes analysis during acclimatization of *O. basilicm* L. *Jouranl of Crop Science and Biotechnology*, 12 (4): 233-238.
- Singh, N.K. and Sehgal, C.B.** (1999). Micropropagation of Holy Basil (*Ocimum sanctum* Linn.) from young inflorescences of mature plants. *Plant Growth Regulation*, 29: 161- 166
- Stanko, K.C.; Orli, S.; Politeo, O.; Strikić, F.; Kolak, I. and Satovic, Z.** (2010a). Composition and antibacterial activities of essential oils of seven *Ocimum* taxa. *Food Chemistry*, 119: 196–201.
- Stanko, K.C.; Liber, Z.; Besendorfer, V.; Javornik, B.; Bohanec, B.; Kolka, I. and Satovic, Z.** (2010b) Genetic relations among basil taxa (*Ocimum* L.) based on molecular markers, nuclear DNA content and chromosome number. *Plant Systematics and Evolution*, 285: 13-22.
- Sudha, C.G. and Seenii, S.** (2001). Establishment and analysis of fast growing normal root culture of *Decalepis arayalpathra*, a rare endemic medicinal plant. *Current Science*, 81: 371-374.
- Sulistiarini, D.** (1999). *Ocimum gratissimum* L. In: Oyen, LPA, NguyenXuan Dung (eds.) Plant Resource of South East Asia, No. 19: *Essential Oils Plants*. Prosea Foundation, Bogor, Indonesia, pp. 140-142.
- Thom, M.A.; Maretzki, K.E. and Sakai, W.S.** (1981). Nutrient uptake and accumulation by sugarcane cell culture in relation to growth cycle. *Plant Cell Tissue and Organ Culture*, 1:3–14.
- Tripathi, A.** (2011). Anticarcinogenic potential of some medicinal plants, *The Botanica*, 59 (61): 169-175.
- Venugopal, L.G. and Rao, A.P.** (2011). In vitro propagation and callus induction of endangered medicinal herb *Ocimum citriodorus*. World Congress on Biotechnology, *Biotechnology-2011*.
- Wang, Q.M.; Gao, F.Z.; Gao, X.; Zou, F.U.; Sui, X.; Wang, M.; Hui, Y.J. and Wang, L.** (2012). Regeneration of *Clivia miniata* and assessment of clonal fidelity of plantlets. *Plant Cell Tissue and Organ Culture*, 109: 191-200.
- Washida, D.; Shimomura, K.; Takido, M. and Kitanaka, S.** (2004). Auxins affected ginsenoside production and growth of hairy roots in *Panax* hybrid. *Biological and Pharmaceutical Bulletin*, 27: 657-660.