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RESEARCH ARTICLE

BIO TECHNOLOGY

COMPARISON OF SOMATIC EMBRYO FORMATION IN *OCIMUM BASILICUM* L., *OCIMUM SANCTUM* L. & *OCIMUM GRATISSIMUM* L.

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ABSTRACT

Cotyledonary leaves of *Ocimum* species *viz., Ocimum basilicum* L., *Ocimum sanctum* L. & *Ocimum gratissimum* L. were used for comparative studies on somatic embryogenesis. Murashige and Skoog (MS) medium with 2,4-Dichlorophenoxyacetic acid (2,4-D) and benzyladenine (BA) was used to initiate callus. MS with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA was found suitable for the development of callus with maximum weight and lesser days to induction for *O. basilicum* and *O. sanctum* whereas MS with 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA initiated callus of maximum weight with high % of response and lesser days to induction for *O. gratissimum.* High % of response to callus induction was found in MS with1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA for *O. basilicum* and *O. sanctum*. Differentiation into globular stage of somatic embryos was observed in all cultures but with variation in duration, % response and embryo colour, on transfer of sub-cultured callus to MS media containing different concentrations and combinations of BA, Kinetin (KIN) & Indole Acetic Acid (IAA). Maximum differentiation into globular shaped somatic embryos was observed in all concentration ranges of KIN with or without IAA and in BA (2.0, 3.0 mg l^{-1}) which had coconut water (CW) as an additional supplement.

KEYWORDS

Ocimum basilicum, Ocimum sanctum, Ocimum gratissimum, Cotyledonary leaves, Callus induction, Somatic embryogenesis

ABBREVIATIONS

MS	Murashige and Skoog (1962) medium
BA	Benzyladenine
2,4-D	2,4-Dicholophenoxyacetic acid
KIN	Kinetin
IAA	Indole acetic acid
CW	Coconut water

INTRODUCTION

The genus '*Ocimum*' (Family - Lamiaceae) collectively and commonly called as 'Basil' is a diverse and rich source of aromatic essential oils attributed for their pharmaceutical, culinary and aromatic properties. The genus consists of about 160 species with a geographic distribution spread over tropical, sub-tropical and warmer parts of temperate regions of the world.[18, 8, 9, 23]

The economically important parts of *Ocimum* are their leaves and tender shoots which on steam distillation yield pleasant smelling, volatile essential oils having phenylpropanoids and terpenoids as their major active compounds.[18] These essential oils are being used as pharmaceutical agents because of their anti-microbial, anti-emetic, anti-diabetic, anti-fertility, anti-asthmatic, anti-stress, insecticidal, diuretic, expectorant, analgesic, hepatoprotective properties, [20, 16, 17, 6] as flavoring agents in soups, salads, confectionaries, cheese & meat products; dental & oral products and as fragrances in perfumery, herbal toiletries & aromatherapy treatment.[22]

Ocimum is conventionally propagated through seeds. Poor germination potential restricts their multiplication and the seedling progeny of all *Ocimum* species show variability due to cross-pollinating nature of the plant.[7] *In-vitro* micropropagation is an effective alternative means for rapid multiplication of species for which conventional methods have limitations.[13, 25, 24, 10] There have been earlier reports on direct clonal propagation[15, 24, 1, 2, 6, 22] and a few on micropropagation through direct somatic embryogenesis[5] in *Ocimum* species. So far, we have not come across many reports on indirect somatic embryogenesis in *Ocimum basilicum, Ocimum sanctum & Ocimum gratissimum.* The present investigation describes a practical methodology for inducing somatic embryos through an intervening callus formation from three species of *Ocimum viz*., *Ocimum basilicum, Ocimum sanctum & Ocimum gratissimum* using cotyledonary leaves as explants; the first ever to be reported using cotyledonary leaves as explants. Somatic embryogenesis is of theoretical and practical importance because it can offer advantage for mass propagation and also combine efficient cloning with genetic manipulation[21]. The intervening callus formed can be maintained as viable pure cell cultures to be used for any of these following purposes *viz.,* suspension culture studies for optimisation of secondary metabolites, regeneration studies for mass propagation and genetic manipulation studies.[5]

MATERIALS AND METHODS

(i) Explant source

Seeds of *Ocimum basilicum, Ocimum sanctum & Ocimum gratissimum* were received from University of Agricultural Sciences (UAS), Bangalore. Sterilised petriplates were filled with absorbent cotton made wet with sterile distilled water. Seeds were washed with liquid detergent, rinsed thrice with distilled water and spread on the cotton soaked with water. All transfer was done under asceptic conditions and petriplates were kept in the culture room. Cotyledons raised from seeds were excised and washed with sterile distilled water. Cotyledons were surface sterilised under aseptic conditions with 70 % ethanol solution for 45-60 seconds, rinsed twice with sterile distilled water each for 1 min, followed by 6-8 mins in a solution of 0.1 % mercuric chloride solution. Final rinsing with sterile distilled water was done 4 times, each with duration of 1 min.

(ii) Culture medium

a) Callus Induction Media

MS culture medium[12] with sucrose (3 %, w/v), myo-inositol (100 mg/l) and varying combinations of BA and 2,4-D (0.5 mg l^{-1} BA and 0.5 – 2.0 mg l^{-1} 2,4-D).

b) Somatic Embryo Induction Media

MS media with varying concentrations and combinations of BA, Kinetin and IAA (0.5-5.0 mg l^{-1} BA, 1.0 – 5.0 mg l^{-1} KN, 1.25, 2,5 mg l^{-1} IAA). Coconut water (25 %) was used as an additional supplement to a few cultures.

pH of the media was adjusted between 5.6 and 5.8 and gelled with 0.8 % of agar (HiMedia). The media was dispensed into 60 ml boiling tubes and 100 ml conical flasks (Qualigens), capped with aluminium foil and autoclaved for 20 mins. at 104 kPa and 121˚C.

(iii) Isolation of coconut water

Fresh coconut water was drained from dehusked immature coconuts by drilling holes through two of the micropyles. Collected water from all the fruits was heated at 80-100 °C for 10 minutes with continuous stirring to precipitate out the proteins, fats and other materials. The precipitates were separated by filtration and the filtrate was stored at -20 °C for future use.[3]

(iv) Culture conditions

Cultures for callus induction were maintained in the culture room at 25 ± 1˚C with 50 – 55% relative humidity under dark whereas cultures for somatic embryogenesis were maintained same as above but under 16 h photoperiod provided by day-light fluorescent tubes (1000-2000 lux, Philips TL 40 W). The cultures were grown from 30 to 45 days for callus induction and 45-90 days for somatic embryo formation before data were collected.

(v) Callus induction

Cotyledonary leaves of the three species were explanted onto culture medium (2 per tube and 3 per flask) with 0.5 mg l^{-1} BA in combination with 1.0 mg l^{-1}, 1.5 mg l^{-1} 2,4-D and 2.0 mg l^{-1} 2,4-D. Cultures were grown for 30 to 60 days before data were collected. The raised calli were subcultured every 30 days at their respective plant growth harmone levels.

(vi) Somatic embryogenesis

Calli induced from cotyledonary leaves were transferred to somatic embryo induction media. Growth regulators such as BA, (1.0 – 5.0 mg l^{-1}), KIN (2.5 – 5.0 mg l^{-1}), IAA (1.25, 2.5 mg l^{-1}) were used in various concentrations and combinations. Coconut water (CW) at 25% was used as an additional supplement for a few cultures. Cultures were grown for 45 to 90 days before data were collected. Microscopic observations of somatic embryos were made using Hund Wetzler Fluorescence Microscope (H 600 AFL, Germany) and images were captured using Infinity Capture (Version 1).

(vii) Statistical analysis

Treatment consisted of 20 explants for individual species and each treatment was replicated thrice. All data are represented as mean ± SE. Values were recorded upto 8 weeks of culture for callus induction and 12 weeks of culture for somatic embryogenesis.

Data were analysed by one way analysis of variance (ANOVA) and means were compared using Duncan's multiple range test at 5 % probability level using SPSS software (version 16).

RESULTS AND DISCUSSION

(i) Induction of callus

Cotyledonary leaves were cultured on MS media with various levels of 2,4-D combined with 0.5 mg l^{-1} BA (Table 1). All combinations reported positive for callus initiation but showed variations among the three species in days to initiation, degree of callusing, nature and colour of the callus. Combination of 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA produced callus of maximum weight for *O. basilicum* (Fig 1a) as well as for *O. sanctum* (Fig 1b) in accordance to the report by Gopi and Ponmurugan (2006). Callus showed a higher degree of semi-friable nature in *O. basilicum* with colour being creamy white to pale green. *O.* sanctum was found to be of less semi-friable nature and was creamy in colour. *O. gratissimum* produced callus which was compact to a higher degree. However, the % response for this concentration was less for all the three species when compared to the highest % response recorded in 1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA for *O. basilicum* and *O. sanctum* and 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA for *O. gratissimum.*

Combination of 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA provided callus of maximum weight, high % response as well as lesser days to callus induction for *O. gratissimum* (Fig 1c). Callus was observed to be less compact and creamy in colour. The same combination did not produce good results for the other two species; the least been observed in *O. sanctum* (% response - 2.5 ± 0.02 and callus weight - 29 mg ± 1.67). Days to callus initiation was found to be almost the same for *O. basilicum* and *O. sanctum* for the combinations -0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA and 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA. These findings suggest that a minimal and an intermediate ratio of 2,4-D and BA is required for initiation of callus of good % response and maximum callus weight from cotyledonary leaves of *O. gratissimum.*

1.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA yielded a high % of response for *O. basilicum* and *O. sanctum* but more number of days to callus induction for all the three species. Callus from *O. sanctum* was found to be friable (the only combination to do so) and from *O. gratissimum* was found to be compact to a much lesser extent when compared to other combinations. 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA showed lesser days to callus induction (callus semi-friable) but less % response in all three species. Earlier investigation by Dode *et al*. (2003) reported only a high concentration of BA (2.0 mg l^{-1} BA + 0.2 mg l^{-1} NAA) to be effective in initiating calli from cotyledonary leaves of *O. basilicum. O. gratissimum* produced callus of compact nature (Fig 1f) after increasing levels of 2,4-D with BA whereas *O. basilicum* and *O. sanctum* produced semi-friable and friable callus (Fig 1d,e). Dode *et al.* (2003) also reported callus of *O. basilicum* to be semi-friable on lower BA concentrations and compact for higher BA concentration.

Based on these callus induction studies, MS with 1.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA is suggested to be used for the development of callus with maximum weight and lesser days to callus induction for *O. basilicum* and *O. sanctum* and MS with 0.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BA for the development of callus from *O. gratissimum* which has maximum weight, high % response and lesser days to callus induction.

Table 1

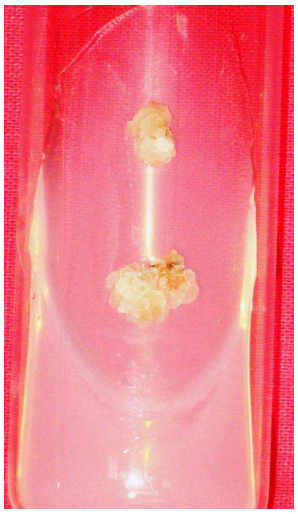
Effects of different concentrations of 2, 4-D in combination with BA for callus induction from cotyledonary leaves of Ocimum species.

Species	Growth regulators (mg l^{-1})		Duration for callus induction (days)	Response (%)	Callus weight (mg)	Callus growth and colour
	BA	2,4-D				
O.basilicum	0.5	0.5	18^{ab} ± 1.63	2.4^{b} ± 0.09	29^{b} ± 1.63	Semi-friable, Creamy white
O.sanctum			25^{c} ± 1.63	32.5^{c} ± 0.40	106.1^{c} ± 1.72	Semi-friable, Creamy
O.gratissimum			16^{ab} ± 1.52	33.30^{ab} ± 0.24	134.2^{a} ± 2.08	Compact, Creamy
O.basilicum	0.5	1.0	17^{ab} ± 1.63	33.30^{ab} ± 0.68	42.07^{b} ± 0.82	Semi-friable, Creamy white to pale green
O.sanctum			44^{c} ± 1.20	7.5^{c} ± 0.40	65.3^{c} ± 1.01	Semi-friable, Creamy
O.gratissimum			39^{abc} ± 0.08	50^{a} ± 1.63	76.55^{a} ± 1.63	Compact, Creamy
O.basilicum	0.5	1.5	39^{abc} ± 2.44	37.5^{b} ± 0.81	31.46^{b} ± 1.63	Semi-friable, Creamy white to pale green
O.sanctum			41^{abc} ± 1.53	17.5^{c} ± 0.40	67.57^{c} ± 1.36	Friable, Creamy
O.gratissimum			19^{abc} ± 0.93	17.5^{a} ± 1.63	61.85^{a} ± 0.81	Compact, Creamy
O.basilicum	0.5	2.0	16^{abc} ± 1.63	22.5^{b} ± 1.63	37.77^{b} ± 1.63	Semi-friable, Creamy white to pale green
O.sanctum			20^{abc} ±1.63	9.5^{c} ± 1.42	28.75^{c} ± 1.63	Semi-friable, Creamy
O.gratissimum			18^{ab} ± 1.63	2.4^{b} ± 0.09	29^{b} ± 1.63	Compact, Creamy

20 explants maintained for individual species in each treatment replicated thrice and data recorded up to 8 weeks of culture. Values are mean ± SE, Means followed by the same letter are not significantly different by Duncan's multiple range test at 5 % probability level.

1a

1b



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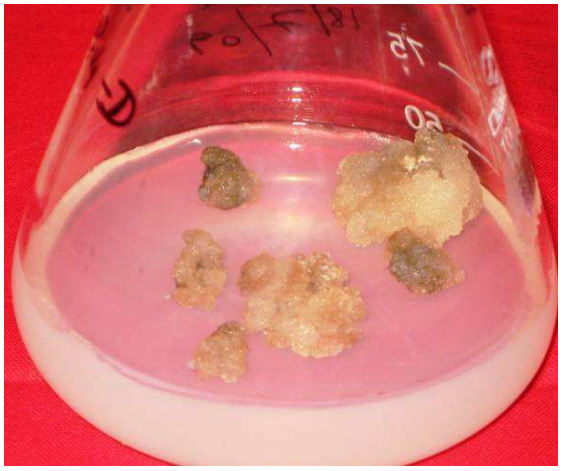

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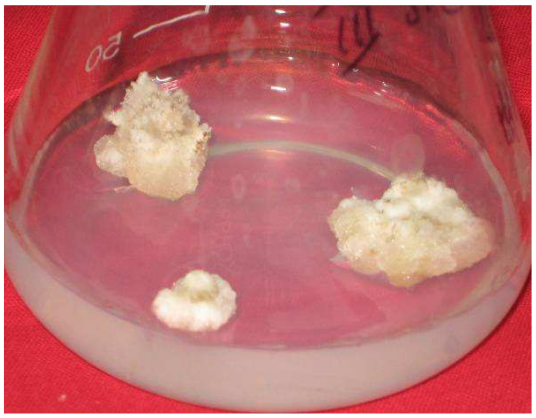


1f

Figure 1
Callus induction in Ocimum sps.
Initiation of callus from cotyledonary leaves of:
1a) O. basilicum, 1b) O. sanctum, 1c) O. gratissimum.
Proliferated callus after two subcultures: 1d) *O. basilicum,* 1e) *O. sanctum,* 1f) *O. gratissimum.*

(ii) Somatic embryogenesis from the sub-cultured callus

Induction of somatic embryogenesis was tried with MS + 2.5 mg l^{-1} BA + 1.25 mg l^{-1} IAA and MS + 5.0 mg l^{-1} BA + 2.5 mg l^{-1} IAA (Table 2). Subsequent elimination of 2,4-D (used along BA for callus induction) and gradual increase in BA concentration was reported to trigger somatic embryogenesis in bamboo by Godbole *et al.* (2002). The above mentioned combinations induced but to a lesser extent formation of somatic embryos. However, a high degree of callus proliferation was observed in *O. basilicum* for both the combinations. Observations were first made by Overbeek *et al.* (1941), using coconut water as an additional supplement for development of embryos in *Datura stramonium* Inclusion of coconut water (CW) at 25% to MS with BA (1.0, 2.0, 3.0 mg l^{-1}) enhanced the embryogenic potential of the callus for all three species. *O. gratissimum* showed the highest degree of embryogenesis (Fig 2c, 3) with callus being light to dark green in colour for all the three combinations. CW at 25% with BA (2.0, 3.0 mg l^{-1}) showed good results. The degree of embryogenesis was found to be less in *O. basilicum* when compared to the other two species (Graph 1). MS with KIN (2.5, 3.0, 5.0 mg l^{-1}) alone and in combination with IAA (1.25, 1.5, 2.5 mg l^{-1}) provided the highest percentage of embryogenic callus for all three species with *O. basilicum* being less when compared to the other two species (Fig 2a,b,3). Their degree of response was almost found to be the same in all the combinations using KIN as the cytokinin. Microscopic observations of horizontal sections of all embryogenic callus revealed embryos to be at globular stage (Fig 2d,e,f). Fast growing, well established somatic embryos have been observed in various plants using KIN as plant growth harmone *ex. Emelia zeylanica*[19], *Citrus* species.[11]

Table 2

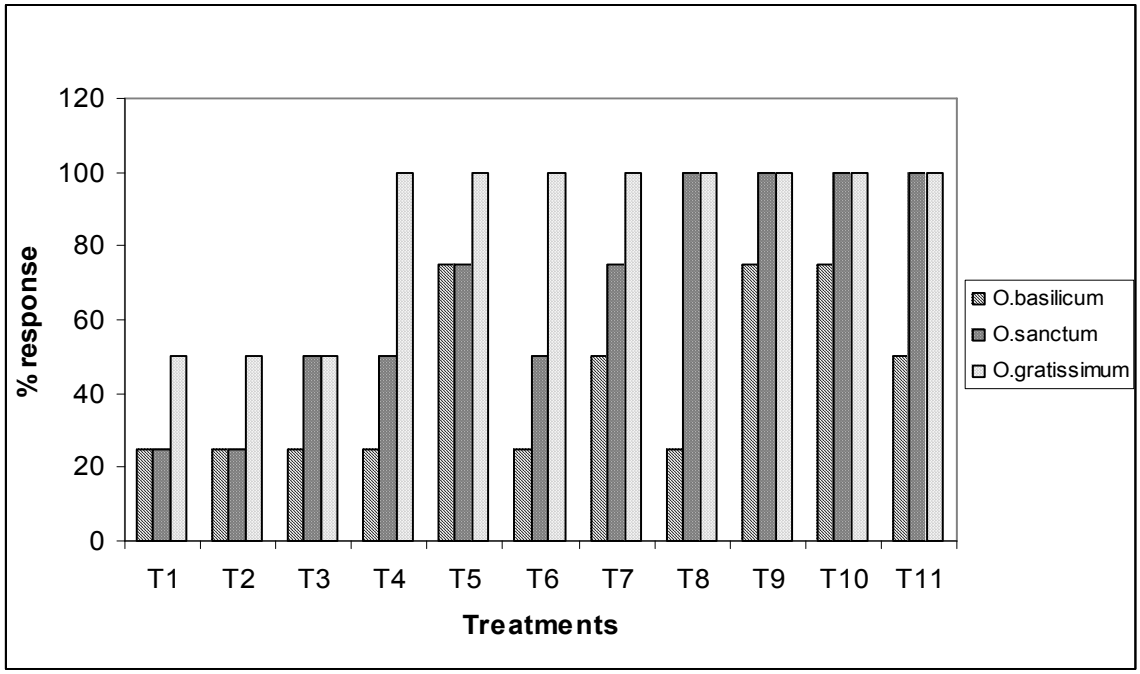
Effects of different concentrations and combinations of BA, KIN and IAA for somatic embryogenesis (Globular stage) in Ocimum species. (Coconut water (CW) at 25 % was used as an additional supplement for a few cultures).

Species	Growth regulators (mg l^{-1})			Duration (Days)	Somatic embryo colour
	BA	KIN	IAA		
O.basilicum	2.5	-	1.25	$28.6^{a} \pm 0.22$	Light green
O. sanctum				$27.1^{b} \pm 0.34$	Light green
O.gratissimum				$43.7^{c} \pm 0.36$	Intense light green
O.basilicum	5.0	-	2.5	$34.9^{a} \pm 0.17$	Creamy green
O.sanctum				$39^{b} \pm 0.42$	Light green
O.gratissimum				$79.3^{c} \pm 0.53$	Green
O.basilicum	1.0 + CW	-	-	$28.5^{abc} \pm 0.22$	Light green
O.sanctum				$27.1^{abc} \pm 0.27$	Light green
O.gratissimum				$30.3^{abc} \pm 1.10$	Green
O.basilicum	2.0 + CW	-	-	$20.6^{a} \pm 0.30$	Cream
O.sanctum				$16.3^{b} \pm 0.21$	Light green
O.gratissimum				$25^{c} \pm 0.47$	Pale to intense green
O.basilicum	3.0 + CW	-	-	$14.5^{a} \pm 0.22$	Creamy
O.sanctum				$20.3^{b} \pm 0.26$	Colourless to green
O.gratissimum				$26.6^{c} \pm 0.18$	Light green
O.basilicum	-	2.5	-	$15.3^{a} \pm 0.26$	Intense cream
O.sanctum				$11.5^{b} \pm 0.16$	Light as well as intense green
O.gratissimum				$6.5^{c} \pm 0.16$	Intense green
O.basilicum	-	3.0	-	$6.9^{ac} \pm 0.17$	Intense cream with green specks
O.sanctum				$10.4^{b} \pm 0.40$	Creamy green
O.gratissimum				$7.5^{ac} \pm 0.16$	Intense green
O.basilicum	-	5.0	-	$7.4^{a} \pm 0.16$	Creamy green
O.sanctum				$9.5^{bc} \pm 0.30$	Light green
O.gratissimum				$9.8^{bc} \pm 0.32$	Green
O.basilicum	-	2.5	1.25	$16.6^{a} \pm 0.16$	Cream
O.sanctum				$12.2^{b} \pm 0.24$	Light green
O.gratissimum				$7.5^{c} \pm 0.40$	Light green
O.basilicum	-	3.0	1.5	$12.6^{ac} \pm 0.22$	Cream
O.sanctum				$8.2^{b} \pm 0.24$	Light green
O.gratissimum				$14.2^{ac} \pm 0.46$	Light green
O.basilicum	-	5.0	2.5	$20.9^{ab} \pm 0.52$	Light to intense green
O.sanctum				$20.1^{ab} \pm 0.60$	Creamy green to green
O.gratissimum				$16.8^{c} \pm 0.38$	Green

20 explants maintained for individual species in each treatment replicated thrice and data recorded up to 12 weeks of culture. Values are mean ± SE, Means followed by the same letter are not significantly different by Duncan's multiple range test at 5 % probability level.

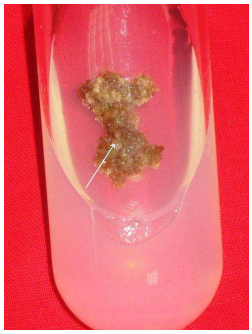
Graph 1

Percentage response to somatic embryogenesis in 3 Ocimum spp. in different embryo induction media.



T1- MS with 2.5 mg/l BA + 1.25 mg/l IAA, T2- MS with 5.0 mg/l BA + 2.5 mg/l IAA, T3- MS with 1.0 mg/l BA + 25% CW, T4-2.0 mg/l BA + 25% CW, T5-3.0 mg/l BA 25%CW, T6-2.5 mg/l KIN, T7- 3.0 mg/l KIN, T8-5.0 mg/l KIN, T9-2.5 mg/l KIN + 1.25 mg/l IAA, T10-3.0 mg/l KIN + 1.5 mg/l IAA, T11-5.0 mg/l KIN + 2.5 mg/l IAA

2a



2b

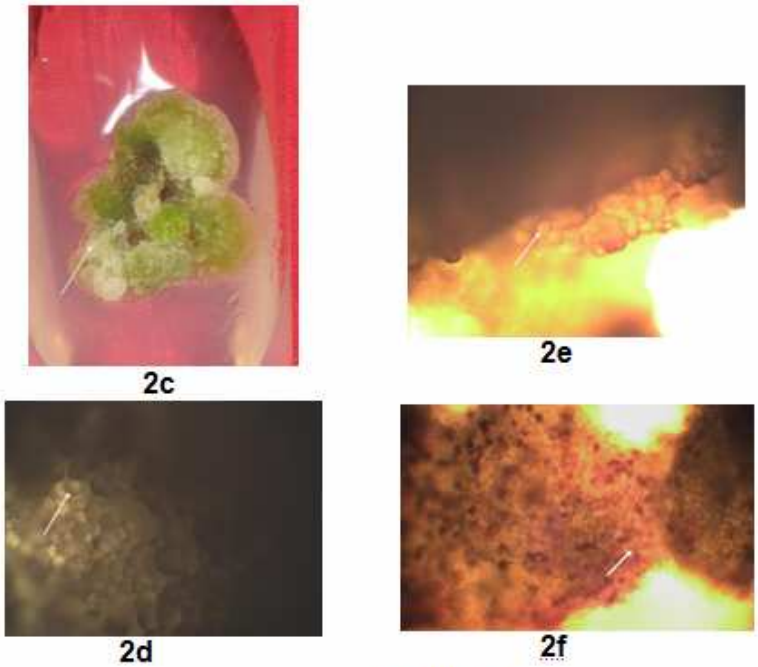


Figure 2

Indirect somatic embryogenesis in Ocimum spp.:
2a) O. basilicum from 2.5 mg/l KIN and 1.25 mg l^{-1} IAA, 2b) O. sanctum from 3.0 mg l^{-1} KIN and 1.5 mg l^{-1}, 2c) O. gratissimum from 3 mg/l BA and 25% CW.
Globular shaped somatic embryos observed microscopically: 2d) O. Basilicum, 2e) O. sanctum, 2f) O. gratissimum.

These findings suggest KIN to be used for the development of somatic embryos in *O. basilicum, O. sanctum* and *O. gratissimum.* Embryogenic callus was found to be creamy green to intense green in colour for all three species, compact for *O. gratissimum* and *O. sanctum* and semi-friable for *O. basilicum*. Microscopic observations showed embryos to be at globular stage.

CONCLUSION

The present investigation offers a protocol for development of somatic embryos in *Ocimum basilicum, Ocimum sanctum & Ocimum gratissimum.* Somatic embryogenesis is an important system in micropropagation where, on maturation of these somatic embryos into heart and torpedo stages lead to regeneration of plants. The optimal growth harmones and their concentrations observed for callus initiation and embryogenesis form the basis for further studies on somatic embryo maturation from globular into heart and torpedo stages and thereafter regeneration in the three *Ocimum* species.

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