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An Alternate Cost-effective Medium for In Vitro Regeneration of Therapeutically Important *Ocimum citriodorum* Vis.

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A novel cost-effective in vitro regeneration protocol has been evolved for the therapeutically important Ocimum citriodorum Vis. In the present study, table sugar (3%) and isabgol (Psyllium husk) (3.5%) were used as an alternate source of carbon and gelling agent respectively in Murashige and Skoog's (MS) medium. The explant used in the current study was nodal segment. A significant observation revealed that all the cultures resulted in shoot induction and maximum number of shoots/ culture (6.04) and their average length (2.15 cm) was obtained on modified MS-medium supplemented with table sugar, isabgol and BAP. However, best root induction (95.83%) was obtained on ½ MS-medium augmented with table sugar (3%) , isabgol (3.5%) and NAA. An increase in average number of roots per shoot (6.91%) as well as average root length (2.73 cm) was also observed in the same modified medium. The in vitro regenerated plantlets were successfully transferred to the field and no notable variation was observed in their morphology. The overall cost of culture medium for in vitro propagation of O. citriodorum Vis. was reduced significantly by 92.69% when agar and sucrose were replaced by isabgol and table sugar, respectively.

Keywords: Cost-effective medium; Isabgol; Ocimum plants; Regeneration; Table sugar.

Ocimum citriodorum Vis. (Lemon basil) is an important medicinal plant, abundant in volatile aromatic essential oils and is known to possess culinary properties[1,2]. It is an interspecific hybrid obtained between *Ocimum basilicum* and *O. americanum*. This herb is mainly cultivated in some parts of Asia and Africa not only for its essence but also as an important source of antioxidants. It is one of the most economically important spice used for flavor in many cuisines[3]. Due to its medicinal value, it has been used as anti-inflammatory agent[4] and to treat people suffering from premature ejaculation, delayed menstruation cycle and stomach spasms[5]. The oil of *Ocimum* is valued for its therapeutic properties and possess antimicrobial, nematocidal, fungistatic and insecticidal activities[6, 7]. The essential oils of *Ocimum* species have been reported to elicit a greater escape response against

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Ades aegypti and *Anopheles minimus,* dengue and malaria vectors respectively[8].

Ideally, the plants used in the preparation of medicines should have the same genetic make-up. However, *Ocimum* cultivars show a lot of variation due to out- crossing[9, 10]. *In vitro* propagation offers an alternative method to produce genetically uniform plants[11]. The technique of tissue culture has been practiced since long for fast multiplication and conservation of medicinal plants. Despite this, the *in vitro* regeneration technique has a major disadvantage, like high cost of production[12]. Therefore, the most challenging aspect of *in vitro* propagation is to reduce the cost of production[12, 13]; without compromising the quality[14, 15] and quantity of the reproduce. As a result, several efforts have been made to reduce the cost of commercial micropropagation of plants[12, 16, 17].

Recurring cost of micropropagation includes mainly the cost of chemicals used to prepare culture medium. These include sucrose, agar, major and minor elements inorganic elements and phytohormones. Out of these components of culture medium, sucrose (source of carbon) and agar (gelling agent) are the most expensive[18]. Therefore, it is imperative to have alternative cost-effective options for *in vitro* propagation of plants. The present study reports, successful cost-cutting of MS medium by substituting agar and sucrose with isabgol and table sugar, respectively for micropropagation of medicinally important *O. citriodorum* Vis.

MATERIALS AND METHODS

Seeds of *O.citriodorum* procured from Central Institute of Medicinal & Aromatic Plants, Lucknow, India, were sown in Botanical Garden, University of Delhi, Delhi, India. Nodal segments (Fig. 1) obtained from one-year-old plants served as explant. They were surface sterilized with teepol (5%, v/v) (Rickett & Colman India Ltd.,) for 10 minutes, followed by a quick rinse with ethanol (70%, v/v). The explants were rinsed thrice with autoclaved water to wash off the sterilant.

Establishment of Cultures

Protocol for tissue culture raised plantlets of *O.citriodorum* Vis. through nodal segment has been standardized in the present study. MS medium augmented with optimal concentrations of chemicals was used for the *in vitro* regeneration of the plantlets[19]. To induce shoots, nodal segments were implanted on modified culture medium with table sugar (3%), isabgol (3.5%) and BAP (0.5 mg/l). Nodal segments inoculated on MS-medium having sucrose (3%) + agar (0.7%) + BAP (0.5 mg/l) served as control.

Similarly, for rooting of shoots modified culture medium with table sugar (3%) and isabgol (3.5%) was augmented with NAA (1 mg/l). MS-medium supplemented with sucrose (3%) + agar (0.7%) + NAA (1 mg/l) was used as control for root induction. To further reduce the cost of medium, lower concentrations of MS salts were tried for root induction. The tissue culture raised micro shoots were transferred to different strengths (1/2, 1/3 and 1/4) of modified MS medium with table sugar (3%), isabgol (3.5%) and NAA (1 mg/l).

Modified medium

T_1- MS medium+ Table sugar +Isabgol + BAP (Shoot induction)

Fig. 1. Nodal segment of *O. citriodorum Vis.* before excision. Arrow showing excised explant (x 1.2)

T_2-Medium +Table sugar + Isabgol + NAA (Root induction)

Culture Conditions

The cultures were maintained in tissue culture laboratory with controlled conditions of temperature ($25^0C \pm 2^0C$) and relative humidity (50 ± 5%). The cultures were maintained under a regulated photoperiod for 16-hour light (fluorescent tubes, 40 W) followed by 8-hour dark period.

Acclimatization and Field Transfer of Plantlets

Tissue culture raised plantlets were gently cleaned with water to remove the gelling agent. Before transplantation, the plantlets were treated with fungicide (1% Bavistin) to avoid any contamination.

Further, to acclimatize the plantlets and to maintain humidity, the pots were covered with transparent polythene bags. Once in a week, MS basal solution (salt concentration one-tenth) without the addition of sucrose and inositol was added to the potted plantlets. A week after transplantation, holes were punched in polythene bags for aeration. For further acclimatization, the plants were kept in green house for a duration of 02-weeks and then successfully transferred to the field.

Cost Analysis of the Medium with Isabgol -gelling agent and Table Sugar-carbon source

Existing cost of sucrose, agar, table sugar and isabgol was estimated and compared in Indian and US currency. The cost of sucrose and agar per kg in Indian currency is Rs. 11,404.20 and Rs. 33,620.00 and in USD 570.10 and 310.00 respectively for the current year. All the chemicals used for the preparation of the culture media were from Sigma-Aldrich. The cost was calculated by taking the weight of each component utilized for preparing 10 liter medium.

Flow Chart

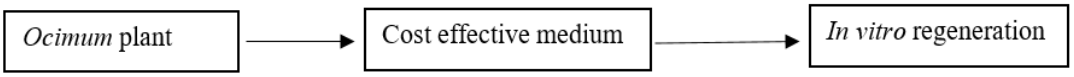

Table 1. Effect of different gelling agents and carbon sources with optimal concentration of BAP (0.5 mg/l) on the shoot regeneration frequency of the nodal segment of *O. citriodorum* Vis.

PGR	Cultures producing shoots (Percent)	Shoots / culture	Shoot-length (cm)
Table sugar (3%) + Agar (0.7%)	70.83a	5.86 ± 0.36a	2.02 ± 0.04a
Sucrose (3%) + Isabgol (3.5%)	75.00a	5.27 ± 0.27a	1.68 ± 0.04a
Sucrose (3%) + Agar (0.7%)	100.00b	5.83 ± 0.25a	2.08 ± 0.23b
Table sugar (3%) + Isabgol (3.5%)	100.00b	6.04 ± 0.21a	2.15 ± 0.06b

Table 2. Rooting of shoots from the nodal segment of *O. citriodorum* Vis. on the table sugar containing isabgol gelled MS medium of different strengths containing NAA (1 mg/l)

PGR	Cultures producing root (Percent)	Roots/ shoot	Root-length (cm)
1/2 MS	95.83b	6.91± 0.08b	2.73 ± 0.07b
1/3 MS	91.66b	6.72 ± 0.00ab	2.51 ± 0.01ab
1/4 MS	58.33a	6.41 ± 0.08a	2.42 ± 0.07a

Data record and statistical analysis

Morphogenetic responses of nodal explant were recorded based on average values 06 weeks post culture for (i) shoot induction frequency, (ii) shoots/culture and (iii) shoot length/culture. The data for rhizogenesis, was recorded on the given criteria: (i) number of shoots producing roots (ii) roots/shoot and (iii) root-length. Efficacy of the explants for in vitro regeneration has been evaluated by calculating shoots/ culture, shoot-length, roots/shoot, and root-length. The scored data was represented as the mean-values with standard-error based on twelve replicates. For reproducibility of the results, each experiment was repeated. ANOVA (Analysis of Variance) through SPSS (Statistical Package for Social Sciences) version 16.0 was used for statistical analyses. Duncan's Multiple Range Test (DMRT) at p=0.05 was used to find out the difference in the mean values to test the significance.

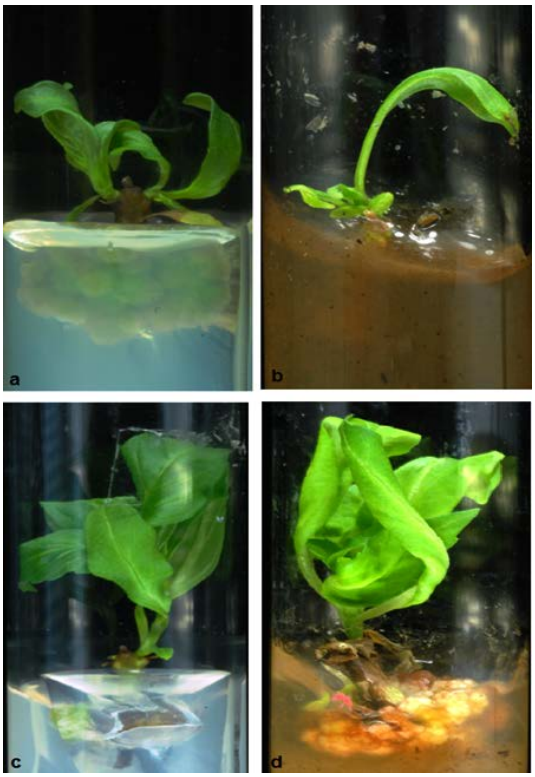

Fig. 2 (a-d). Morphogenic responses of nodal explant of *O. citriodorum* Vis. on MS nutrient medium with optimal level of BAP (0.5 mg/l) incorporated with different gelling agents and carbon source after 3 weeks of culture (a) Culture showing multiple shoots on the MS medium supplemented with table sugar (3%) and agar (0.7%) (x 2.2); (b) Explant with shoot on the medium containing sucrose (3%) and isabgol (3.5%) (x 2.3); (c) Control-medium containing sucrose (3%) and agar(0.7%) showing more shoots per explant (x 2.1); (d) Explant depicts better growth in terms of slight increase in the shoot length on the medium containing 3.5% isabgol and 3% table sugar (x 2.1)

RESULTS

Shoot induction and elongation

Table -1 presents the effect of various gelling agents and carbon sources on shoot regeneration responses of the explants. The nodal segments were implanted on MS medium containing table sugar (3%) and isabgol (3.5%) supplemented with BAP (0.5 mg/l). For control,

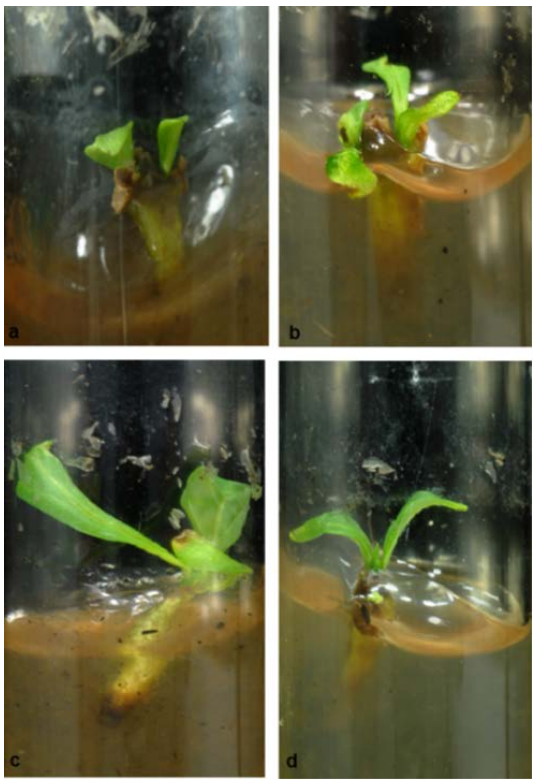

Fig. 3 (a-d). Developmental stages of *in vitro* shooting of the nodal segment of *O. citriodorum* Vis.on the MS nutrient medium + BAP (0.5 mg/l) + table sugar (3%) + Isabgol (3.5%); (a) 10 days old culture showing shoot induction (x 2.5); (b)Two-weeks old culture with multiple shoots(2.4); (c-d) 3 weeks old culture (x 2.5 and x 2.2)

MS medium with sucrose (3%) + agar (0.7%) + BAP (0.5 mg/l) was used. It was observed that sugar quality and gelling agent type did not influence the morphogenetic response of the explant (Fig. 2 a- d). No significant difference was observed in percent cultures in the morphogenic response on control MS medium (Fig. 2c) and modified MS medium containing table sugar and isabgol (Fig. 2d). On modified MS medium (table sugar and isabgol), shoot buds emerged a week after inoculation which eventually differentiated into shoots (Fig. 3 a- d). Maximum number of shoots/ culture (6.04) and shoot length (2.15 cm) was observed on modified MS medium containing table sugar 3% and isabgol 3.5% + BAP 0.5 mg/l (T_1 medium). However, shoot inducing frequency (5.86) and average shoot length (2.02 cm) declined when table sugar (3%) was used in combination with agar (0.7%) (Fig. 2a). Similarly, reduced shoot inducing frequency (5.27) and shoot length (1.68 cm) was observed when isabgol (3.5%) was used in combination with sucrose (3%) (Fig. 2b).

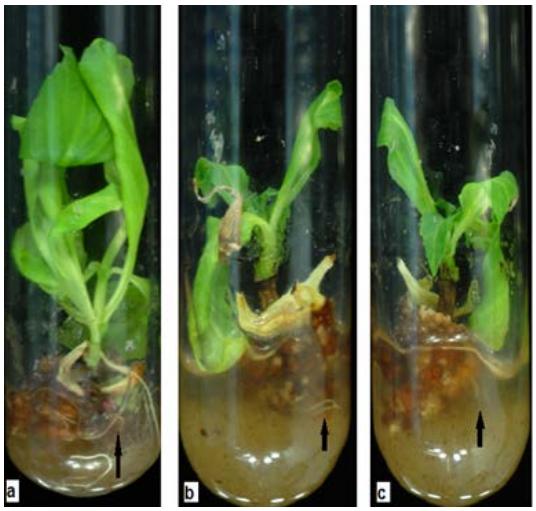

Fig. 4 (a- c). Effect of various strengths of MS salts on the root- induction of nodal segment derived microshoots of *O. citriodorum* Vis. on the MS nutrient medium + NAA (1 mg/l) + Table sugar (3%) + Isabgol (3.5%) after 4 weeks of transfer. Micro shoots with developed roots on half-strength MS nutrient medium (x 1.2) (a); Microshoots showing a significant decline in number of roots per shoot and root length on 1/3 MS medium (x 1.2) (b); Culture showing poor root growth on 1/4 MS medium (x 1.2) (c)

Rooting

Table -2 presents effect of various strengths of MS medium on root induction. Rooting was induced on all the media compositions. However, maximum root regeneration (95.83%) was obtained on modified MS-nutrient medium with table sugar and isabgol supplemented with NAA 1 mg/l (T_2 medium).

To further reduce the cost of medium, lower concentrations of MS salts were tested. The *in vitro* regenerated micro shoots were also sub-cultured on various strengths (1/2, 1/3 and 1/4) of MS medium augmented with table sugar (3%), isabgol (3.5%) and NAA (1 mg/l). There was no notable difference observed in rooting of the micro shoots at full strength MS (95.83%) medium as well as 1/2 strength MS medium (95.83%, Fig. 4a). However, there was an increase in the length of roots (2.73 cm) on 1/2 MS medium. It was observed that there was an overall decline in morphogenetic response of rooting on 1/3 MS medium (Fig. 4b, Table 2). On further decreasing the concentration of MS salts, a drastic reduction in the root inducing frequency (58.33%) of the micro shoots was observed (1/4 MS medium) (Fig. 4c). Table 3, presents results of various gelling agents

Table 3. Effect of different gelling agents & carbon sources with optimal concentration of NAA(1 mg/l) on the root induction of micro shoots obtained from nodal segment of *O. citriodorum* Vis.

PGR	Cultures producing root (Percent)	Roots/ shoot	Root-length (cm)
Table sugar (3%) + Agar (0.7%)	58.33 a	4.91±0.08 a	1.53±0.01 a
Surose (3%) + Isabgol (3.5%)	70.83 a	5.40±0.03 b	1.49±0.03 a
Sucrose (3%) + Agar (0.7%)	100 b	6.49±0.08 c	1.98±0.03 b
Table sugar (3%) + Isabgol (3.5%)	95.83 b	6.52±0.02 c	2.00±0.03 b

and carbon sources on the induction of roots from shoots. The maximum number of shoots inducing roots (6.52) as well as root length (2 cm) was higher in the modified MS medium (table sugar and isabgol) in comparison to control MS -medium containing sucrose and agar (6.49 roots per shoot and 1.98 cm).

Acclimatization and Field Transfer of Plantlets

Tissue culture raised plantlets with fully-grown roots (Fig. 5a) were washed gently to avoid any contamination. Subsequently for hardening, the plantlets were transplanted into small containers filled with autoclaved soil and humus (Fig. 5b). To maintain humidity and allow light to pass through, the plantlets were protected with transparent polythene bags. Three weeks after hardening, the polythene bags were removed. The potted plantlets were kept in green house and thereafter, transferred to the field that showed high survival rate (85%). Thus, complete plantlets were obtained successfully from the nodal segments of *O. citriodorum* Vis. on cost-effective medium (Fig. 6). The plantlets produced through tissue culture were identical to *in vivo* plants.

Cost Analysis of MS-Medium with Isabgol and Table Sugar

The estimated cost per kg of sucrose vs table sugar and agar vs isabgol in USD and Indian currency has been summarized in Table 4. There is a stark difference in the price of both sucrose (USD 570.10) vs table sugar (USD 8.05) and agar (USD 310.00) vs isabgol (USD 30.00).

Table 5, presents comparative cost analysis of sucrose vs table sugar and agar vs isabgol. It was estimated that the final cost of 10 liter modified-medium (MM) containing table sugar and isabgol was less expensive in comparison to control medium (CM) augmented with sucrose and agar. Total cost of the control medium (CM) is USD 193.99 while that of modified medium (MM) (table sugar and isabgol) is USD 14.17. Thus, 92.69% cost reduction of the medium has been achieved successfully by replacing sucrose and agar in MS medium with table sugar and isabgol respectively for in *vitro* regeneration of *O. citriodorum* Vis.

Fig. 5 (a- b). Hardening of *in vitro* raised nodal explant derived plantlets of *O. citriodorum* Vis. regenerated on cost-effective medium (a) An *in vitro* raised plantlet with well-developed shoots and roots before hardening (x 1.2); (b) Plantlets growing well in thermocol-cup filled with autoclaved soil and humus one week after transfer (x 0.7)

Fig. 6. Schematic representation of various stages of plantlet formation from the nodal explant of *O. citriodorum* Vis. A) Mature plant, (B) Explant before excision (dotted lines show the site of excision), (C) 3 weeks -old culture showing multiple shoots with callus, on the MS nutrient medium + BAP (0.5 mg/l) + table sugar (3%) + isabgol (3.5%), (D) Rooting of micro shoot on the table sugar (3%) + NAA (1 mg/l) supplemented isabgol gelled 1/2 MS medium, (E) Complete plantlet with fully grown shoots and roots

Table 4. Cost of media used for the *in vitro* regeneration of *O. citriodorum* Vis.

Item	USD	INR	Brand
Sucrose- 1 kg	570.10	11,404.20	Sigma-Aldrich
Table Sugar -1 kg	8.05	60.00	Domino pure granulated white sugar (USA) Madhur Pure and Hygienic Sugar (India),
Agar Powder – 1 kg	310.00	33,620.00	Sigma-Aldrich
Isabgol – 1 kg	30.00	1025.00	Frontier *Psyllium* Husk Powder (USA)Sat-Isabgol *Psyllium* Husk

Table 5. Comparative cost analysis of media used for the *in vitro* regeneration of *O. citriodorum* Vis.

Medium Code[a]	Cost of medium[b] 10 Liters (USD)	Percent decrease in cost in compared to CM
CM	193.99	92.69
MM	14.17	

Note: [a]CM= Control Medium, MM= Modified Medium supplemented with table sugar and isabgol
[b]Cost (/US$) were calculated as follows: sucrose = 570.10/ kg; agar = 310.00/kg; isabgol = 30.00/kg; table sugar = 8.05/kg; other components = 1.26/10 l of medium

DISCUSSION

Sucrose is widely used carbon source in plant tissue culture medium. Murashige &Skoog used sucrose (3%) in tissue culture medium preparation as the staple source of carbon [19]. It acts as a fuel during the plant tissue culture for sustaining photomixotrophic metabolism and thus ensures optimal development. It also helps in maintaining the osmotic potential of cells[20]. However, it is very costly and contributes about 34% of the total production cost[14]. There are many reports which suggest a remarkable reduction in the *in vitro* regeneration cost by using table sugar instead of sucrose[14, 21-24].

Agar used as a gelling agent imparts viscosity to tissue culture medium, due to which explants remain on surface of the nutrient medium. It is most frequently utilized gelling agent in tissue culture but is highly priced. It accounts for about seventy percent of the total cost of production[25]. Its wide use is mainly attributed to its quality for transparency, stability, and resistance to metabolism. However, some reports have raised doubt about the non-toxicity and biological inertness of agar[25, 26]. Moreover, excessive use of agar has resulted in overexploitation of this bio-resource[25, 26]. Hence, it is imperative to look for cost-effective alternatives to make plant tissue culture economically viable and sustainable. In the past, starches from other sources like potato, rice, wheat, barley, tapioca[26], cassava and sago[25] have been used as gelling agents, with varying degrees of success. Isabgol or psyllium husk derived from *Plantago ovate* Forsk seeds has been used successfully for the solidification of nutrient medium. The value addition of isabgol as a thickening factor is due to large amount of mucilage in its husk. The mucilage swells into a jelly like mass and provides mechanical support to the explant. Isabgol mucilage is a polysaccharide composed of xylose, arabinose, rhamnose, galactose and galacturonic acid[26] and therefore it is a safe gelling agent.

In the present study, isabgol and table sugar were used as an alternative gelling agent and carbon source respectively. They did not show any adverse effect on the tissue culture raised plantlets of *O. citriodorum* Vis. The results were in conformity with an earlier study[27]. They successfully used market sugar (2%) as a low-cost alternative to sucrose in the multiplication of *Chrysanthemum morifolium.* There was an increase in the number of shoots/explant (6.04) and length of shoots (2.15 cm) on nutrient medium with table sugar (3%) and isabgol (3.5%) than in comparison to MS medium with sucrose and agar (number of shoots/explants, 5.83 and length of shoots, 2.08 cm) (Table1). Such response may be due to high levels of sucrose in the medium which affect photosynthetic efficiency of tissue cultured plants[20]. Results of the present study were supported by earlier research studies which reported favourable

effect of isabgol on shoot multiplication[27, 28, 29]. The findings of present study agreed with the reports of Demo et al for micropropagation of *Solanum tuberosum* L[22]. They also used that table sugar (0.3%, w/v) in culture media that resulted in more shoot proliferation as compared to sucrose. They concluded that the difference in the response of shoot multiplication to different sources of carbon may be due their easy translocation and assimilation by the explants leading to enhanced growth. Gelling agents also affect the numbers of shoots/explant. This may be due to the difference in hardness of the gelling agents used for the preparation of culture medium. The gelling agents differ in their chemical composition and structure that eventually results in the variable diffusion rates of different elements and hormones in the nutrient medium[30].

In the current study, maximum root induction (95.83%) was obtained on medium containing table sugar as carbon source and isabgol as gelling agent supplemented with NAA (1 mg/l). These results were in consonance with the study conducted by Agrawal et.al[21]. They evolved a protocol for *in vitro* conservation at low- cost for banana utilizing isabgol and market sugar. Several researchers have reported that isabgol serves as thickening factor in culture medium used for the micropropagation of different plants like *Chrysanthemum*[31], turmeric[18], tobacco[32] and banana[21, 30]. However, it was observed that maximum number of shoots inducing roots (6.62) as well as root length (2 cm) was higher in the table sugar containing isabgol gelled medium in comparison to conventional sucrose containing agar gelled medium (6.49 roots per shoot and 1.98 cm) (Table 2). Similar results were reported in earlier studies by Dhanlakshmi and Stephan [14] who obtained higher number of roots for the Monthan variety of banana on the low-cost medium in comparison to conventional medium.

The *in vitro* regenerated micro shoots were transferred to lower concentrations of MS salts for rooting to further reduce the cost of the medium. No significant difference in the morphogenic response in terms of rooting of micro shoots at full strength MS (95.83%) medium as well as 1/2 strength MS medium (95.83%, Fig. 4a) was observed. However, average root length (2.73 cm) increased in the 1/2 MS medium. Such response might be due to the high salt concentration of culture medium that affects the osmotic potential, limits water absorption and low ionic strengths suitable for rooting [33] (Table 3, Fig. 4 a-c).

Acclimatization and high rate of survival of *in vitro* regenerated plantlets is impacted by their ability to resist the transplanting stress as well as how much readily they can adapt to autotrophic mode of nutrition [34]. In the present study, the rate of survival of tissue culture raised plantlets was 85%. The high rate of success might be due to the formation of well-developed root system of plantlets during acclimatization.

The positive role of isabgol and table sugar as substitute for agar and sucrose respectively has not been reported previously during *in vitro* culture of *O. citriodorum* Viz. In the present study, cost reduction was achieved by 92.69% in the *in vitro* regeneration of *O. citriodorum* Viz. These results were in accordance with the results of Zapata[35], who successfully reduced 90% cost of the tissue culture raised banana by substituting sucrose with commercial sugar. Similarly, Dhanlakshmi and Stephan [14] achieved 61.4% cost reduction in the medium during tissue culture of banana by substituting sucrose and agar with table sugar and isabgol respectively. Likewise, an earlier study observed 34% cost reduction by using table sugar instead of sucrose in agar gelled medium[22]. Pant et al reported that there was a cost reduction by more than six-times when isabogol and market sugar was used in the culture medium for micropropagation of *Chrysanthemum morifolium.* Likewise, Agrawal et al. reported 59% reduction in *in vitro* regeneration cost of banana by utilizing market sugar as carbon source and isabgol as thickening agent [21].

CONCLUSION

The current research work emphasized that the major obstacle in the application of *in vitro* regeneration technology in terms of high cost of production has been overcome. A novel cost-effective protocol has been established for the mass multiplication of medicinally important plant *Ocimum citriodorum* Vis. Sucrose and agar being the major components of the medium incur heavy expenditure and thus pose an economic challenge in full exploitation of tissue culture of therapeutically important plants. In the present

study, cost of culture medium has been significantly reduced by 92.69 % by utilizing table sugar and isabgol as alternate carbon source and gelling agent, respectively. Findings of the present study have helped in formulating standard culture medium and opened up possibilities for increasing the production of *O.citriodorum* Vis. many folds at comparatively low cost. There is still enough scope for further reduction in the cost of tissue-culture medium for micropropagation of medicinally important plants in healthcare system.

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Conflict of interest

No conflict of interest among authors

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