Development of efficient, cost-effective in vitro micropropagation technique for threatened ethnomedicinal plant Clerodendrum indicum (L.) O. Kuntze

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Abstract

Clerodendrum indicum (L.) O. Kuntze (Verbenaceae) is a threatened ethnomedicinal plant with many bioactive secondary metabolites that could alleviate chronic diseases like cough, asthma, jaundice, leprosy, syphilitic rheumatism, and septic wounds. Their natural growth has been severely challenged due to habitat loss and massive exploitation for medical applications, leading to the threatened status. Therefore, an *in vitro* micropropagation technique has been trialled to be used for eco-restoration and metabolite exploitation. Micropropagation via direct and indirect shoot organogenesis had been established from the different explants of this plant. Murashige and Skoog (MS) media supplemented with variable concentrations of NAA, IAA, and BAP produced callus, organogenesis and whole plant. The study revealed that nodal explants resulted in more significant responses than others. The shoot and root regeneration through callus was observed in the MS media supplemented with 4.0 mg/L 6-BA and 0.5 mg/L NAA with an 84% response rate after two weeks of incubation and an average 5.6 number of shoots per callus. Only root and shoot regeneration was observed using half-strength MS media with 2.0 mg/L 6-BA and 1.0 mg/L NAA and 6.0 mg/L 6-BA and 0.5 mg/L NAA, respectively, after two weeks. The plantlets acclimatization had an average of 80% survival rate. These *in-vitro* regenerated plants by direct shoot organogenesis and through callus induction methods might aid in harvesting a bulk amount of secondary metabolites without destroying the native habitat. Thus, the methods would lead to environmental restoration sustainably.

Introduction

Clerodendrum L. (Verbenaceae) is a well-documented genus with many bioactive secondary metabolites having medicinal and pharmacological properties (Nataraj et al., 2016). Among the different species, *C. indicum* (L.) O. Kuntze is tall, erect, and robust with a sparsely branched shrub with a typical morphologically ridged, fluted, hollow stem and toughened strong tap. In contrast, the roots are light brown, more than 2.5 cm in diameter. Leaves have variable phyllotaxy-like opposite decussate to the whorl of 2 to 6 leaves (Mitra et al., 2022). The plants grow throughout peninsular India. Besides the natural habitat, this plant is often cultivated as an ornamental plant. The entire plant possesses several highly valued secondary metabolites, such as cleroindicins (phenolic compound) and flavonoids, such as hispidulin and scutellarein, scutellarein-7-O-β-D-glucuronide, and pectolinergenin (Mitra et al., 2022). These compounds are used to alleviate several diseases, like cough, asthma, scrofulous infections, jaundice, leprosy, syphilitic rheumatism, septic wounds, etc (Ghosh and Pal, 2012). However, due to the massive exploitation of the plants and the unexpected habitat loss, the plant has become a 'threatened' status (Ghosh and Pal, 2012). Therefore, it is essential to conserve this plant through *ex-situ* and *in-situ* conditions to save it from extinction from its natural habitat. Developing efficient micropropagation techniques for large-scale clonal propagation is of utmost need for creating plantlets of threatened/endangered plants. However, this *in vitro* propagation technique requires optimization of clonal culture parameters because each plant species has a unique metabolite profile and requires variable factors to regulate their cell proliferation and regeneration (Dhaka and Kothari, 2005; Sarasan et al., 2006; Behera et al., 2023). By optimizing different plant growth regulator(s) in the culture system, the meristematic cells can be switched to the embryonic and organogenic phases (Sukla et al., 2012; Haque et al., 2018; Mosoh et al., 2023). Moreover, this technique can also produce only the shoot or root system based on the requirement of medicinal attributes (Hasnain et al., 2022; Moraes et al., 2021). Axillary shoot multiplication is a rapid, reliable, reproducible technique for germplasm conservation and clonal propagation through *in vitro* conditions (Chaudhuri et al., 2007; Behera et al., 2023; Warner et al., 2023). Several scientific groups have worked out on the micropropagation technique in different species of the *Clerodendrum* genus (Mao et al., 1995; Baburaj et al., 2000; Kothari et al., 2006; Sharma et al., 2009; Jirakiattikul and Boonha, 2009; Goyal et al., 2010; Mukherjee and Bandyopadhyay, 2012; Vidya et al,. 2012; Kamdi et al., 2014; Haque et al., 2018). However, the *in vitro* culture condition of the *C. indicum* plant has not been explored in great detail except for a report on indirect shoot development through callogenesis (Ghosh and Pal, 2012). The present study aimed to develop an efficient and rapid *in vitro* culture method through direct shoot induction, callus formation, and root induction of the threatened ethnomedicinal plant *C. indicum* (L.) O. Kuntze.

Materials and Methods

Chemicals and reagents

6- benzylamino purine (BAP), Indole acetic acid (IAA) , Indole butyric acid (IBA), Napthanilic acetic acid (NAA), and 2,4-Dichlorophenoxyacetic acid (2,4-D) were produced from HiMedia, MB grade, Mumbai, India. Fungicide Bavistin, Mercuric chloride ($HgCl_2$) was produced from Merck, India, Tween-20. Ethyl alcohol was produced from HiMedia, MB grade, Mumbai, India.

Collection of plant samples, surface sterilization, and initial culture establishment

The plant samples were freshly collected from the natural habitat of Malda township, West Bengal, India (25°0' 39.0276" N and 88° 8' 27.9528" E). They were washed with running tap water followed by 2% (w/v) systematic fungicide (Bavistin) for 15 min followed by 2.5% (v/v) Tween-20 solution for 2 min. Finally, the explants were surface-disinfected with mercuric chloride ($HgCl_2$) solution (0.1%, w/v) for 5 min, rinsed five times with sterile distilled water, and used for *in vitro* culture.

Composition of culture media and culture conditions

The culture medium consisted of Murashige and Skoog basal medium (HiMedia, Mumbai, India) (containing supplemented with varying concentrations of BAP, IAA, IBA, NAA, 2,4-D as plant growth regulators and 1.8% (w/v) agar powder (w/v) adjusted to pH 5.6±0.2. The cultures were maintained at 25±2 °C under a 16h photoperiod at a photosynthetic photon flux density of 35 µmol.m^{-2}.s^{-1} provided by cool white fluorescent tubes (36W TL-D Super 80; Philips, Anand, India) in a plant growth chamber (Lab-X, Biotechnique Resources, Kolkata, India).

In vitro callus induction

According to standard protocol, the callogenesis was performed from both leaf and stem explants (Haque et al., 2018; Swamy et al., 2015). The explants were transformed to MS basal medium with the combination of 6-BA and 2-4-D in a varying concentration gradient (Table 2) for callus induction at 24±2 °C temperature and 80±5% relative humidity under 16:8 hours light and dark photoperiod and incubated for four weeks.

In vitro shoot organ culture

Surface sterilized apical nodes containing vegetative buds were inoculated in MS basal medium (Murashige and Skoog, 1962) supplemented with different concentrations of IAA, IBA, and NAA in different combinations for direct organogenesis. The MS medium supplemented with different concentrations in a combination of 6-BA and NAA was used for shoot regeneration from the callus (Table 1). After four weeks of culture, the growth and multiplication of axillary shoots were recorded.

In vitro root induction

Induction of roots from callus was performed according to standard protocol (Haque et al., 2018). Callus was excised and transferred to a half-strength MS basal medium amended with different combinations of 6-BA and NAA(Table 3) for root initiation at 24±2 °C temperature under 80±5% relative humidity under 16 h photoperiod. After four weeks of incubation, the percentage of responses of roots was recorded. The number of roots per shoot and root length was assessed with an average of 5 explants. The control set was prepared without any plant growth regulators and observed according to the treatment sets.

Preparation of sterile sand and acclimatization

The multiplied *in vitro* plantlets were gently washed in running tap water to remove the adhering medium and subsequently transferred to pots containing sterile soil with perlite and cocopeat 1:2:1 (v/v). Initially, in-vitro plants were wrapped in plastic bags to control transpiration. After 30 days, the plastic bags were opened, and the uncovered plants were maintained at 25 °C under a 16-h photoperiod. Then, the plants were transferred to an open environment for acclimatization for another month, and the percentage of survivability was calculated. The growth and vigour of the plantlets were compared with non-micropropagated plants.

Statistical analysis

All the experiments were conducted thrice with five replicates of explants per treatment and evaluated after 30 days. One-way ANOVA study used to analyze the data was at the 95% significance level ($p<0.05$) using Past 4.0 software. The response surface analysis and graph were run through Origin Pro and Design Expert software through the central composite model. All the results were expressed as means ± standard error (SE).

Results

Callus induction responses from explants

The micropropagation of *C. indicum* was established in the MS medium. The plant growth regulators have greatly influenced the rate of targeted tissue regeneration. A comparative study of callus induction ability and growth on the nature of explants, such as leaf and stem segments, were observed, and remarkable differences were found (Table 1). Browning of the callus was more profound in the leaf than in the stem explants, resulting in a lower callus induction rate. In the same media conditions (MS+ 2 mg/L BAP +1 mg/ L 2,4-D), the browning rate of leaf explants was about 33.33%, whereas in stem explants, the callus browning rate was approximately 13.33%. The callus formation was induced in the basal media supplemented with 2.5 mg/L 6-BA and 0.5 mg/L 2,4-D in both stem and leaf after 7 days. After 30 days of induction, the highest callus induction was observed with MS +2 mg/L BAP +0.5 mg/ L 2,4-D (80%) in stem explant and MS +3.0 mg/L BAP +1.0 mg/ L 2,4-D (53.33%) in leaf explant (Table 2). A green compact callus was found in MS media supplemented with 2 mg/L BAP and 0.5 mg/ L 2,4-D in the stem (Fig 1). The one-way ANOVA analysis followed by the Kruskal-Wallis test showed that the callus induction rate of both stems and leaves against different treatment conditions was statistically significant ($p<0.05$). A substantial increase in callus induction rate from the stem was observed in 2 mg/L 6-BA and 0.5 mg/L 2,4-D, maximizing the callus induction (Fig 2a). Whereas from the leaf, more than 1mg/L 2,4-D and 3 mg/L 6-BA can maximize the callus induction according to the response surface methods(p,0.05) (Fig 2b, Table 2). The R^2 and adjusted R^2 values were statistically acceptable (Table 2).

Root and bud induction from callus

Adventitious bud formation was induced from both the nodal and leaf segments. However, more browning of callus was found in leaf explants (Fig-3a) than bud explants (Fig-3b), which are the most totipotent part of the plant for micropropagation because of the presence of meristematic tissue essential to maintaining the clonal fidelity of the daughter clones. Though the leaf and stem were the most totipotent parts, various differentiation abilities were shown by the callus of the stem and leaves. Shoot regeneration from the callus was induced by MS medium adding with 6-BA (3-6 mg/L) and NAA (0.5-1.0 mg /L) (Fig. 3). Adventitious shoot buds formation in MS media supplement with 6-BA and NAA were shown in Fig. 3. Swelling of bud from calli were found in within 5 days and further development into the shoot happened within 10 days. The best response was observed when callus derived from stems cultured on MS medium supplemented with 4.0 mg/L 6-BA + 0.5 mg/L NAA (84%) (Fig. 3d). In such conditions, both shoot and root regeneration happened, and the highest number of the shoot was also observed with the shoot length of 2.1±0.5 cm in 30 days of incubation. Only shoot formation was observed with MS media addling with 5.0 mg/L 6-BA + 0.5 mg/L NAA with an average shoot length of 5.7 cm (Fig 3g). This is followed by a 5.4 cm length of shoot on MS+6.0 mg/L 6-BA + 0.5 mg/L NAA after 30 days of callus induction. These shoot and root induction and their vigour against the two plant growth regulators were statistically significant ($p<0.05$) according to one-way ANOVA analysis followed by the Kruskal-Wallis test. The response surface analysis showed that in all the treatment conditions, the shoot and root induction and their length were statistically insignificant ($p<0.05$) with the experimental datasets (Data not shown). Fully developed calli were transferred to half-strength MS medium amended with different PGRs. At first, within 10 days, small root primordia were observed(Fig 3c), which developed into adventitious roots after 20 days (Fig 3e).The root length of *C. indicum* (L.) O. Kuntze was directly proportional to the rooting responses. The highest rooting frequency (74.33 %) with a root length of approximately 3.9 cm and on average 7.43 roots were observed after 30 days of culturing on half-strength MS media added with 2 mg/L 6-BA+0.5 mg/L NAA (Fig 3h). The basal MS media without plant growth regulators did not induce roots from the shoot explants (Table 3).

Axillary shoot induction and multiplication

Axillary shoot formation was induced from the nodal segments, the most totipotent part of the plant for micropropagation. The excised nodal segment did not respond to shoot induction when grown in the basal MS medium as a control set. It also did not respond to axillary shoot formation below the 0.5 mg/L (w/v) IAA concentration, while the highest response (54%) was observed with the application of 1 mg/L (w/v) sole IAA. However, the response rate was increased with varying BAP concentrations amended with the basal MS media supplemented with 1 mg/L (w/v) IAA. The response rate was highest (81.12%) in MS basal media supplemented with 1 mg/L (w/v) IAA and 1 mg/L (w/v) BAP. The response rate to induce axillary bud was further enhanced up to 89.11% with the application of 0.5 mg/L (w/v) NAA along with 1 mg/L (w/v) IAA and 1 mg/L (w/v) BAP in the MS basal media (Fig 4; Table 3). There was a significant increase of shoot induction per node in the nodal segment when the basal media supplemented with both IAA and BAP than the sole IAA (Table 3). The highest number of axillary shoots per nodal (3.5/node) segment was observed in the basal media supplemented with 0.5 mg/L (w/v) NAA along with 1 mg/L (w/v) IAA and 1 mg/L (w/v) BAP. The shoot length was also the highest in this media combination (2.25 cm) (Fig. 4a, b). These explant responses, number of shoots/node, and shoot length differences in every variable plant growth regulator treated conditions were statistically significant ($p<0.05$) in one-way ANOVA analysis followed by the Kruskal-Wallis test. The response surface plots demonstrated the optimized response against the three plant growth regulators on %explant response, number of shoots/node, and shoot length of the targeted plants were also statistically significant ($p<0.05$), similar to the experiments (Fig 5, Table 5). Both R^2 and adjusted R^2 values were statistically good, indicating the robustness of experimental data (Table 4) where 1 mg/L IAA, 1 mg/L BAP, and 0.5 mg/L NAA induce maximum explant response (%), number of shoots/node, and shoot length (cm).

Plant transplantation

The most difficult step in micropropagation is the hardening of the micropropagated plantlets. The *in vitro* plants were very vulnerable to biotic and abiotic stresses. So, 1 g/L mancozeb (fungicide) was used (Fig-4 e &f) to minimize the fungal infections. The average success rate of the micropropagation after hardening and acclimatization was 80%. Finally, fully acclimatized plants were transferred to the wild habitat to rehabilitate the habitat and recover the species richness of the targeted medicinal plant.

Discussions

Due to the loss of habitat and massive exploitation, this medicinal plant is becoming endangered (Nataraj et al., 2016). So, the establishment of an efficient micropropagation technique may help in developing new clones for *ex-situ* conservation of the medicinal plant and also to provide an alternative approach to producing secondary metabolites of medicinal attributes (Murashige and Skoog, 1962). Callus induction is necessary for the establishment of a plant regeneration system as well as genetic change. It has the potential to be widely used in rapid reproduction, breeding, transgenic, and virus-free seedling production (Kausch et al., 2021; Hajati et al., 2016). The capacity of different plant tissues for organogenesis differs substantially. The selection of explants is an important aspect of the tissue culture system. Swamy et al. (2015) reported the micropropagation of *C. serratum* to rebuild their habitat either by callus regeneration or axillary shoot induction (Swamy et al., 2015; Vidya et al., 2012). PGRs are critical in the induction and differentiation of callus in *Clerodendrum* species because induction rates and plant growth

differ substantially amongst explants. 2-4D and 6- BA are the most important PGRs for in vitro test-tube seedlings to generate shoots and roots. However, organogenesis in cell culture is determined by the auxin-cytokinin ratio (Zhang et al., 2020). Cytokinin like Kn (Kinetin) and 6-BA have been proven to enhance cell division, bud proliferation, bud morphogenesis, and root formation, whilst auxins like NAA and 2,4-D are often utilized to stimulate callus generation and cell expansion, as well as induce somatic embryogenesis (Guo *et al*., 2021). The development of explants through direct shoot organogenesis leads to the rapid regeneration of explants. The *in vitro* propagation study of *C. indicum* revealed that the basal MS medium supplemented with one mg/L (w/v) IAA and 1 mg/L (w/v) BAP, 0.5 mg/L (w/v) NAA has a response rate of 89.11% with an average shoot length was 2.25 cm. (Fig. 3a) and axillary shoot per node was 3.5, similar to the other *Clerodendrum* species viz. *C. aculeatum* (L.) Schltdl., *C. phlomidis* L. f. and *C. Serratum* (Haque et al., 2018; Vidya et al., 2012; Baburaj et al., 2000). According to Srivastava et al. (2004), the basal MS medium supplemented with 0.5 mg/L (w/v) IAA and 5 mg/L (w/v) BAP and 0.5 mg/L (w/v) NAA induced maximum shoot of *C. aculeatum* from the leaf explants with 83% response rate. Whereas, Vidya et al. (2012) reported that application of 0.5 mg/L BAP in the basal medium induces the shoot regeneration of *C. serratum* L. of 77.8% with an average axillary shoot per node of 4.5 and average highest shoot length (4.58 cm) than the other treatment conditions. In *C. phlomidis* L. f., the highest direct shoot regeneration from the nodal explant was 33.3% with the treatment of zeatin in 0.11 mg/L concentration. The average shoot per node was 1.9, with an average shoot length of 1.2 cm (Kher et al., 2016). So, it appears that each species within a genus was a unique requirement of PGPs for its cell division and differentiation.

The culture of green compact callus development was observed in stem explants on MS medium supplemented with 2.0 mg/L BAP in combination with 0.5 mg/L 2,4-D after four weeks. However, a green friable callus could be done by lowering the BAP concentration from 3.0 mg/L to 2.0 mg/L. At the higher concentration of 2,4-D, the slower the callus grew, and more rapid senescence occurred. (Table 1). In leaf explants, callus became colourless as the concentration of 2,4-D increased gradually. Swamy et al. (2015) also reported that at a higher BAP concentration, the callus becomes brown in *C. serretum* L. and the callus growth rate slows down with the increasing rate of 2,4-D concentration.

In contrast, a combination of reduced cytokinin and negligible to without auxin concentration shifts the explant from the embryonic phase to the organogenic phase (Shukla et al., 2012). However, maintenance of the embryonic condition, rather than in organogenic condition, for a long time would be beneficial for the regeneration of many explants in a short period. The study revealed that PGRs at 25 ℃ maintained the embryonic phase for a long time with a repeat of subculture at every 28-day interval. Haque et al. (2018) also reported the two combinations of two auxin derivatives, such as 2-4-D and NAA, in MS basal media, could induce callus formation from bulb scale, root and leaf explant, from nodal explants of *Ledebouria revoluta* (Shukla et al., 2012). Thus, it is observed that the application of 2-4-D could produce the non-morphogenic friable calli, while other plant growth regulators such as IAA and 2-4-D could induce the quality of morphogenic callus.

Root induction is an essential part of the *in-vitro* regeneration of a plant. The shoot explants generally cause root induction. However, the development of roots is an organogenic process controlled by various endogenous and environmental factors (Mukherjee et al., 2012; Shukla et al., 2012). The *in vitro* root induction of various explants was caused by exogenously supplemented auxins, viz. IAA, IBA, and NAA of economically important plants such as *Allium hookeri*, *Leucocoryne* spp., *Zephyranthes* spp. (Shukla et al., 2012). In the case of root induction of *C. phlomidis* through *in vitro* conditions, the individual application of NAA and IBA in 0.8 mg/L and 0.7 mg/L produced maximum rooting, respectively (Vidya et al., 2012). The *in vitro* root induction in *C. serratum* and *C. indicum* also occurred with the treatment of 0.5 mg/L (w/v) NAA in an MS basal medium (Vidya et al., 2012). In contrast to the above finding, the present study, three auxin derivatives such as 0.5 mg/L (w/v) IAA, 1 mg/L (w/v) IBA, and 0.5 mg/L (w/v) NAA in combination have induced more robust and higher root growth in the basal media than the previous observations (Fig. 3c). Therefore, it is observed that the PGRs requirement for optimum root organogenesis is species-dependent. However, the reasons for variations of hormonal inductions among the species of this genus are still unknown.

Hardening and acclimatization of the micropropagated plants is an essential part of establishing an efficient micropropagation technique. The plant could survive in the open-air experimental garden of the institution, and no phenotypic variations were observed from the wild vegetation. Similar hardening and acclimatization approaches were also observed in the micropropagated plantlets of *Aloe* spp., *Artemisia abrotanum* L., *Clerodendrum* spp., *Bacopa* spp., *Ledebouria* spp. *Spathoglottis* spp. *Tylophora* spp., and in their natural habitat (Shukla et al., 2012; Vidya et al., 2012; Haque et al., 2018; Alihodzic et al., 2023).

Conclusion

The direct shoot organogenesis from the nodal explants of *C. indicum* showed potential approaches of regeneration of plantlets with an average regeneration potential of 73%, which is superior to other species of the genus. The *in vitro* indirect callus-mediated organogenesis regenerated many plantlets that could be used for harvesting the secondary metabolites in bulk for medicinal and pharmaceutical purposes. These *in vitro* methods could help in the restoration of threatened ethnomedicinal plant *C. indicum* (L.) O. Kuntze, in their native habitat, to conserve their biodiversity. Thus, the study establishes a most comprehensive, efficient, and cost-effective approach either to exploit their potential secondary metabolites from the targeted plant organs or to develop a large number of saplings for their ecological restoration.

Declarations

Competing Interests: The authors declare that they have no conflict of interest.

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Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author's Contribution

Ashutosh Kundu, Bikram Sahani and Rajsekhar Adhikary did the Methodology, investigation, data curation, validation, and writing - the original draft of the manuscript, formal analysis (lead); statistical analysis, validation, and visualization; **Anindita Chakraborty:** Funding acquisition**;** resources and the infrastructural facility for some experimental work; **Vivekananda Mandal:** Conceptualization (lead); formal analysis (lead); statistical analysis, validation, visualization; funding acquisition**;** resources, writing – review and editing; software; supervision and corrected the manuscript for article submission and communication.

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Tables

Table 1: Effects of different concentrations of plant growth regulators on callus induction of C. indicum from nodal and leaf explants cultured on MS medium.

PGRs (mg/L)		Explant number	Callus induction rate (after 30 days)		Morphogenetic response of callus	
6-BA	2,4-D		Stem	Leaf	Stem	Leaf
3.0	0.5	15	6.66±0.09	0	Brown friable	-
2.5	0.5	15	26.66±0.26	13.33±0.15	Green brown friable	Yellow-green friable
2.0	**0.5**	**15**	**80±1.68**	46.66±0.88	Green Compact	Yellow-green
2.0	1.0	15	20±0.16	40±0.96	Whitish brown friable	Whitish brown friable
3.0	**1.0**	**15**	**53.33±1.02**	53.33±0.98	Yellow-green compact	Whitish brown friable
1.0	1.0	15	33.33±0.52	40±0.76	Whitish brown friable	Whitish brown friable
1.0	1.5	15	13.33±0.26	13.33±0.19	Whitish brown friable	Brown friable
1.0	2.0	15	0	6.66±0.11	-	Brown friable

Here, the values are the average of triplicate trials ±SE. Here the symbol '-' indicates no response of callus.

Table 2: Summarized statistical details of RSM models of callus induction rate by PGRs.

Predictor variables	6-BA, 2,4-D							
Response variables	Callus induction rate from stem and leaf							
Coefficients of variables and interactions								
	Callus induction rate							
	Stem				**Leaf**			
	Estimate	**Std. Error**	**F value**	**p-value**	**Estimate**	**Std. Error**	**F value**	**p-value**
Intercept	34.78	21.7	1.82	0.3918	49.71	5.62	19.44	0.0496
6-BA	38.42	22.29	2.97	0.2269	34	5.78	34.65	0.0277
2,4-D	36.68	29.85	1.51	0.3441	38.62	7.73	24.94	0.0378
6-BA×2,4-D	111.37	48.73	5.22	0.1496	85.64	12.63	46	0.0211
6-BA^2	17.01	24.06	0.4998	0.5528	7.39	6.23	1.41	0.3574
2,4-D^2	67.12	38.22	3.08	0.2212	31.38	9.9	10.04	0.0868
R^2	0.8196				0.9798			
Adjusted R^2	0.3687				0.9294			
Total df	7				7			

Table 3: Effects of different concentrations of plant growth regulators on shoot and root induction of C. indicum cultured on MS medium after 30 days.

PGRs (mg/L)		Shoot induction %	Number of shoots	Root induction %	Number of root	Shoot length (cm)	Root length (cm)
6-BA	NAA						
3.0	0.5	33.3±0.26	2.5±0.03	26.4±0.09	1.5±0.09	1.2±0.09	1.0±0.06
4.0	0.5	84±1.9	5.6±0.16	71.6.±1.6	3.6.±0.6	3.2.±0.6	2.1±0.5
5.0	0.5	80±2.26	1.66±0.06	5.2.±0.2	0.4±0.05	5.7±0.9	0.2±0.05
6.0	0.5	74±2.13	1.0±0.09	0	0	5.4±0.05	0
1.0	1.0	0	0	12±0.2	0.5±0.01	0	0.5.±0.02
2.0	1.0	0	0	74.33±1.02	8.43±0.98	0	2.9±0.15
3.0	1.0	7.43±0.51	0.68±0.18	0	0	0.35±0.18	0

Here, the values are the Mean±SE.

Table 4: Effects of Auxin and Cytokinin concentrations for direct organogenesis on MS medium from C. indicum nodal explants.

PGRs (mg L^{-1})			Explant response (%)	Number of shoots/node	Shoot length (cm)
IAA	BAP	NAA			
0.00	0.00	0.00	0.00	0	0
0.25	0.00	0.00	0.00	0	0
0.50	0.00	0.00	21.00±1.18	1±0.014	0.5±0.012
0.75	0.00	0.00	38.12±1.35	1.5±0.017	0.75±0.015
1.00	0.00	0.00	54.00±0.69	2±0.011	0.8±0.02
1.25	0.00	0.00	52.04±0.97	1.75±0.015	0.77±0.019
1.00	0.25	0.00	52.12±1.21	2±0.015	0.8±0.02
1.00	0.50	0.00	66.50±0.72	2.5±0.17	1.25±0.021
1.00	0.75	0.00	73.00±0.34	2.75±0.21	1.5±0.022
1.00	1.00	0.00	81.12±0.39	3±0.019	1.75±0.019
1.00	1.25	0.00	76.15±0.69	2.75±0.021	1.5±0.021
1.00	1.00	0.25	87.00±0.35	3.25±0.019	2±0.021
1.00	**1.00**	**0.50**	**89.11±0.14**	**3.5±0.015**	**2.25±0.017**
1.00	1.00	0.75	84.16±0.15	3±0.019	1.75±0.019
1.00	1.00	1.00	76.22±0.32	2.75±0.021	1.6±0.2
1.00	1.00	1.25	71.00±0.39	2.6±0.21	1.4±0.019

Here, the values are the average of triplicate trials ±SE.

Table 5: Summarized details of RSM models of Explant response (%), Number of shoots/node, and Shoot length (cm) with the induction of plant growth promoters.

Predictor variables	2-4D, 6-BA
Response variables	Explant response (%), number of shoots/node, Shoot length (cm)

Coefficients of variables and interactions

	Explant response (%)				Number of shoots/node				Shoot length (cm)			
	Estimate	Std. Error	F value	p-value	Estimate	Std. Error	F value	p-value	Estimate	Std. Error	F value	p-value
Intercept	73.84	3.4	71.51	<0.0001	2.79	0.14	56.11	<0.0001	1.48	0.1144	32.54	<0.0001
2-4D	23.4	4.49	27.29	0.0004	0.8521	0.1848	21.26	0.001	0.4492	0.1511	8.84	0.014
6-BA	16.65	2.95	31.91	0.0002	0.6332	0.1214	27.21	0.0004	0.5775	0.0993	33.86	0.0002
2-4D × 6-BA	-5.36	14.23	0.1417	0.7144	0.3109	0.5861	0.2814	0.6074	0.867	0.4792	3.27	0.10005
2-4D ^{2}	-29.53	12.88	5.26	0.0448	-1.54	0.5304	8.47	0.0156	-1.34	0.4337	9.48	0.0117
6-BA ^{2}	-7.4	5.26	1.98	0.1896	-0.3646	0.2167	2.83	0.1234	-0.1915	0.1772	1.17	0.3052
R^2	0.9728				0.9656				0.9411			
Adjusted R^2	0.9592				0.9484				0.9132			
Total df	15				15				15			

Figures

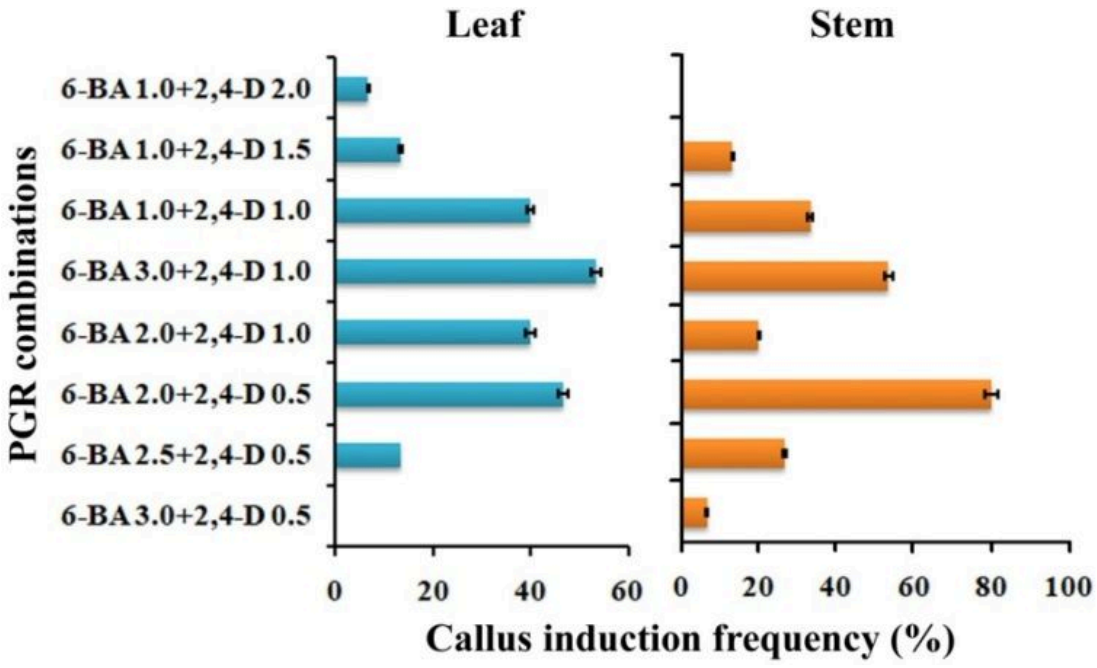

Figure 1

Graph shows changes in callus induction frequency from stem and leaf explants against different PGR concentrations. Here, the bars on the column indicate the error bars of SE.

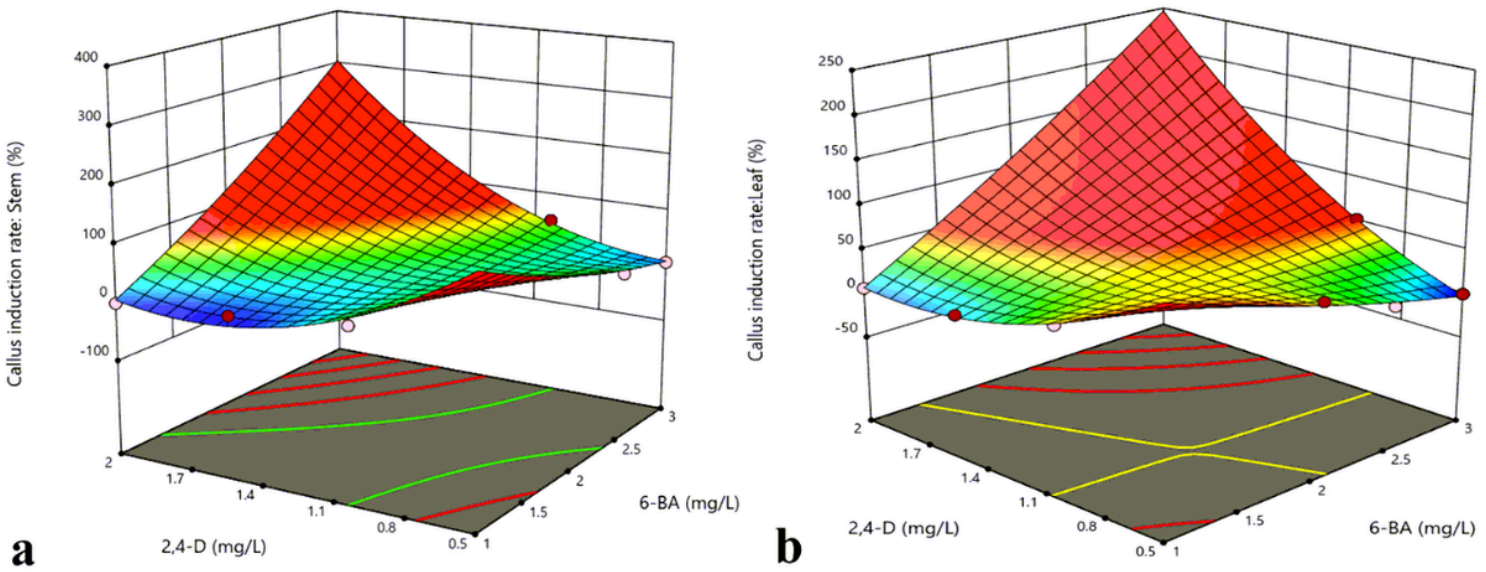

Figure 2

Response surface plots on the effects of different concentrations of PGRson callus induction from stem (a) and leaf (b) explants cultured on MS medium.

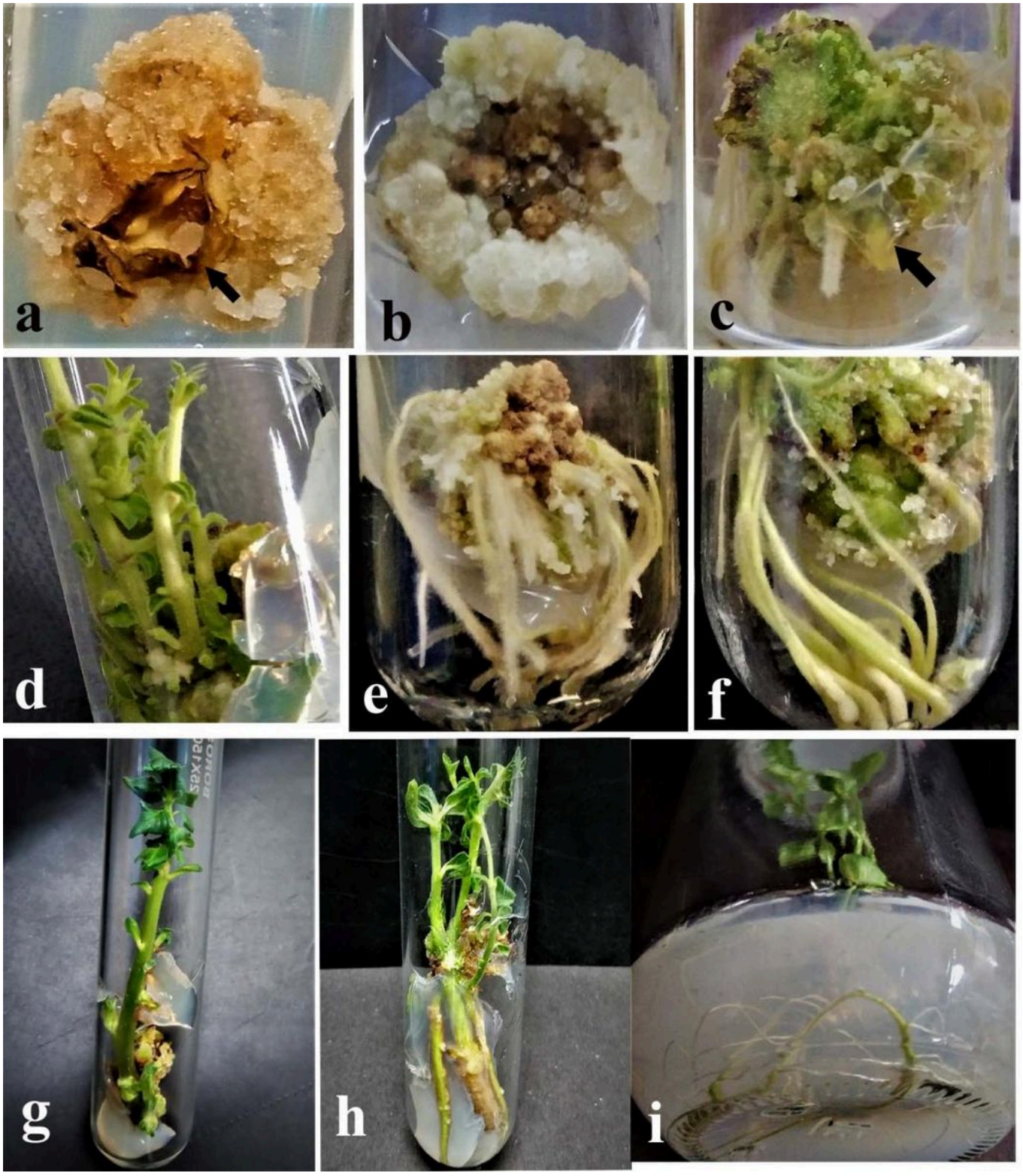

Figure 3

In vitro regeneration of C. indicumthrough callus induction from different explants leaf and stem. (a) Brown callus formed from leaf explants after 30 days in MS+1.0 mg/L 6-BA+1.5mg/L 2,4-D medium (b) Whitish brown callus from both leaf and stem after 30 days in combination MS+2.0 mg/L 6-BA+1.0mg/L 2,4-D medium; (c) Green compact callus after 30 days from stem explants in MS+2.0 mg/L 6-BA+ 0.5mg/L 2,4-D medium; (d) Shoot induction from callus after 20 days in MS+4.0 mg/L 6-BA+ 0.5mg/L NAA medium. (e-f) Only root induction in MS+2.0 mg/L 6-BA+ 1.0mg/L NAA after 30 days and 45 days; (g) Only shoot induction from callus after 60 days in MS+5.0 mg/L 6-BA+ 0.5mg/L NAA medium; (h)Both root and shoot induction in MS+4.0 mg/L 6-BA+ 0.5mg/L NAAmedium; (i) and whole plant from callus after 90 days.

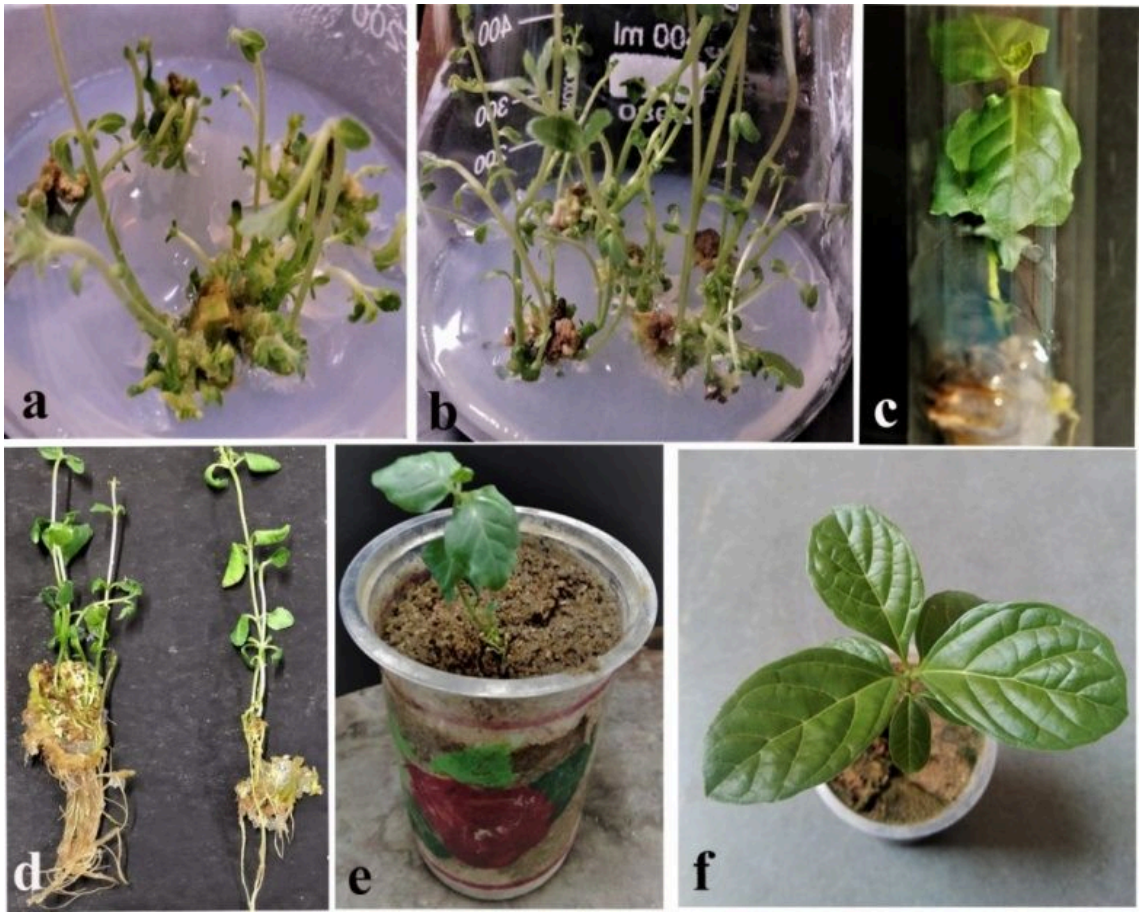

Figure 4

In vitro regeneration, multiplication and acclimatization of C. indicum. (a-b) Shoot multiplication from callus after 20 days and 30 days, respectively; (c) Whole plant with large leaf was ready for acclimatization;(d) *In vitro*rooting; (e)1 week old hardened plantlet on sterile soil with sand and cocopeat (1:2:1); and (f) 4 weeks old hardened plantlet.

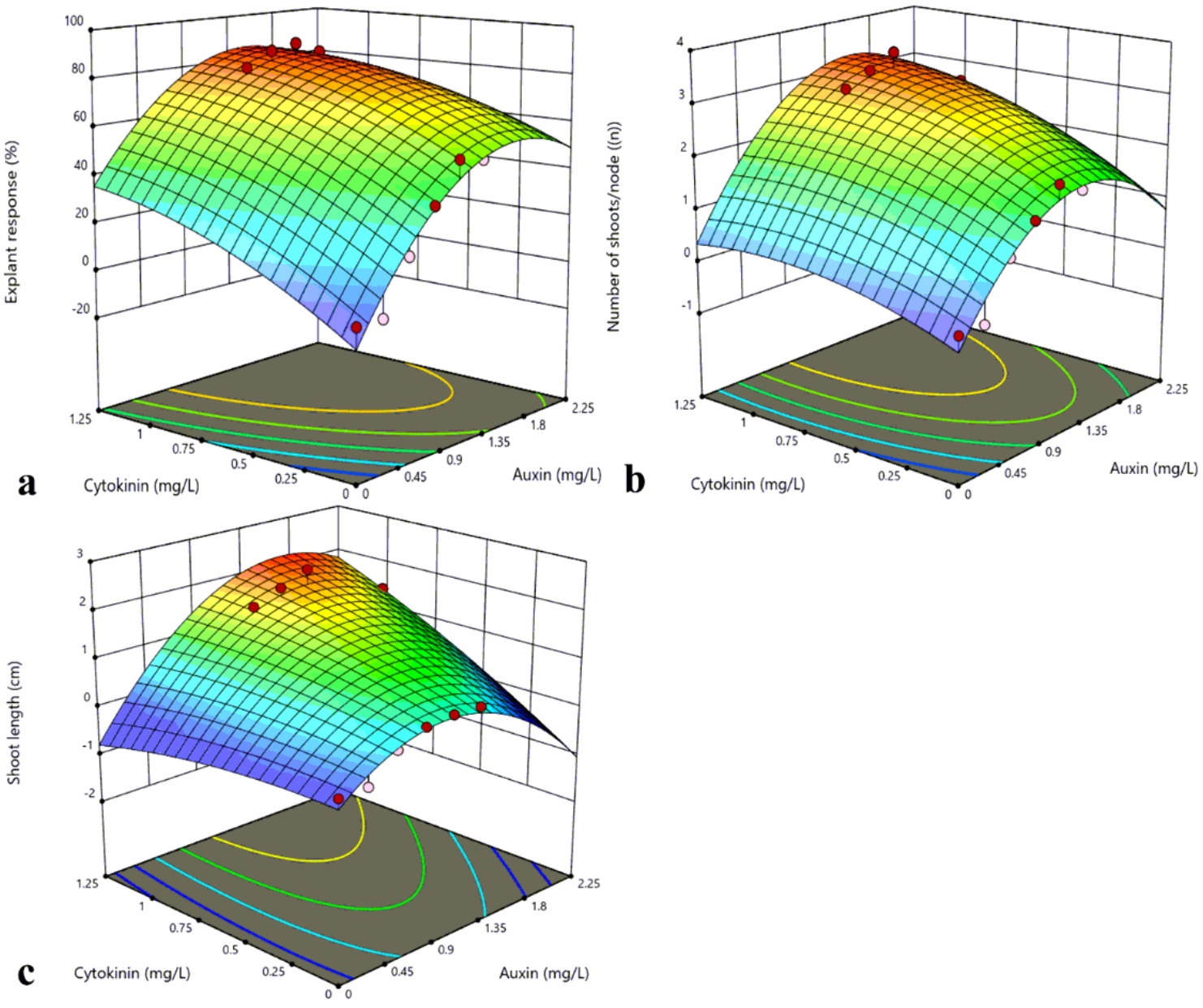

Figure 5

Response surface plots on the effects of Auxin and Cytokinin on their concentration on C. indicum nodal explants cultured for direct organogenesis. Here, (a) represents explant response, (b) the Number of shoots/node, and (c) Shoot length, respectively.