Culture Media Optimization through Response Surface Methodology for *in vitro* Shoot Bud Development of *Solanum melongena* L. for Micropropagation

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Abstract: *Response surface methodology was used for the optimization of shoot bud response and shoot bud induction in leaf explants of Solanum melongena cultivar Arka Shirish. Three independent variables were evaluated for shoot bud response and shoot bud induction. The variables include the concentrations of nitrogen (N_2), sucrose and growth regulator thidiazuron (TDZ). The shoot bud response for cultured explant was optimized at 4.34 g/l of total nitrogen, 2.65% of sucrose and 0.67 mg/l of TDZ with 95% response. The optimum medium conditions for shoot bud induction was found to be Murashige and Skoog (MS) basal medium supplemented with 4.02 g/l of total nitrogen, 2.36% of sucrose and 1.0 mg/l of TDZ with 10 number of bud per explant. The shoot buds so formed were elongated in 0.5 mg/l 2,3,5-Triiodobenzoic acid (TIBA) and 0.1 mg/l Gibberellic acid (GA_3). The elongated shoots were rooted in MS with 1 mg/l Indole-3-butyric acid (IBA). The rooted plants were transferred to pots with farmyard manure upon hardening. This study has validation value for optimization of micropropagation protocol and is further useful in genetic transformation studies for Solanum melongena variety Arka Shirish to maximize regenerative response for automation.*

Keywords: *Arka Shirish, Brinjal, Eggplant, Leaf segments, Shoot buds, Response surface methodology.*

Introduction

Eggplant (*Solanum melongena* L. 2n = 24) is an economically important crop of Solanaceae family grown mostly in tropical and temperate regions of the world [28]. It is popularly called as brinjal in India its place of origin and is also called as Eggplant in the USA and Aubergine in Europe. Although a native of India eggplant is also cultivated in Japan, Indonesia, China, Bulgaria, Italy, France, the United States and many African countries [31]. Eggplant can be consumed raw, boiled, cooked stuffed. It can be used in variety of preparations like soups,

pickles etc. [2]. It is a good source of vitamins and minerals [31]. It is low in calories and high in potassium and so could be used to control diabetes, hypertension and obesity [31]. Superoxide anion radical scavenging and iron chelating activities of nasunin a major component of anthocyanin pigment in eggplant peels were demonstrated by electron spin resonance by Noda *et al.* [26]. Recently it was reported, by Azevedo *et al.* [3] that purified anthocyanin from eggplant protected mice against cyclophosphamide mutagenicity *in vivo*.

For genetic transformation studies in eggplant, *in vitro* propagation of eggplant is a prerequisite. A number of protocols on eggplant regeneration [10, 12, 19, 20, 21] and transformation [7, 8, 9, 29] have been reported earlier. Even though several protocols on eggplant organogenesis have been reported, it is general experiences that the regeneration efficiency of eggplant is influenced by genotype, explant type, and also morphogenetic response varying within the same explant [30]. The morphogenetic response of an explant in tissue culture media depends on the interplay of various media components and plant growth regulators supplied along with the medium [5]. Different salts may be required at different stages of tissue culture which influences the response of explant to various plant growth regulators in the medium and this requirement of salts vary from species to species [5]. Therefore optimization of nutrients in *in vitro* propagation of eggplant will be a practical approach for specific genotypes and species. Medium optimization using one factor at a time is time consuming and usually results in misinterpretation of results [14]. Response surface methodology is a useful statistical tool for design of experiments, analysis of results and finding the optimal conditions [14]. RSM is needed to determine the level of a factor or combination of factors that will give maximum yield or response by minimizing the number of experimental trials [18]. Studies on tissue culture media optimization has been carried out in *Centella asiatica* [27], *Dianthus caryophyllus* L. [13] *Citrus sinensis* L. [24, 25], *Decalepis hamiltonii* Wight. & Arn [11]. Response surface methodology has also been used for optimization of lycopene extraction from tomato cell suspension culture [17] as well as for optimization of capsaicinoid production by immobilized cell cultures of *Capsicum frutescens* [32].

The present study is aimed at optimizing the concentrations of various factors like total nitrogen content, sucrose and thidiazuron, a plant growth regulator for *in vitro* shoot bud development from leaf explants of *Solanum melongena* cv Arka Shirish employing response surface methodology (RSM). The efficiency of the process has been evaluated in terms of shoot bud response and shoot bud induction followed by optimization of the process.

Materials and methods

Germplasm

Seeds of *Solanum melongena* L. cv Arkha Shirish were obtained from Indian Institute of Horticultural research, Bangalore. Seeds were thoroughly washed in running tap water then surface sterilized with 0.1% $HgCl_2$ followed by washing in sterile distilled water and soaked in sterile distilled water overnight. The seeds were again washed in sterile distilled water and inoculated on to sterile petridish with filter paper. The germinated seeds (85-90%) were inoculated into half strength MS basal medium. Leaf segments of 45 day old seedlings were used as explants for media optimization studies.

Culture medium

The total nitrogen content (Potassium nitrate, ammonium nitrate), sucrose in MS medium [22] and the growth regulator thidiazuron (TDZ; Sigma USA) were varied for media optimization studies. The shoot buds obtained from the optimized media were elongated on medium

comprising of MS salts and vitamins supplemented with 0.5 mg/l 2,3,5-Triiodobenzoic acid (TIBA; Sigma USA) and 0.1 mg/l Gibberellic acid (GA_3; Sigma USA) (Under Communication). The elongated shoots were rooted in medium having MS salts, vitamins and 1 mg/l Indole-3-butyric acid (IBA; Sigma USA). The pH of the medium was adjusted to 5.7 ± 0.2 before autoclaving at 1.06 kg/cm^{-2} at a temperature of 121ºC for 15 min. All the growth hormones were added prior to autoclaving.

Culture conditions

The cultures were incubated at 25 ± 2ºC light under 16/8 h of photoperiod with 25 µmol/m^2/s light intensity. Explants were inoculated into glass jars of 110 mm×60 mm with 40 ml medium for all the experiments. Shoot bud response was expressed based on percent of explants responding to shoot bud formation. Shoot bud induction was recorded in the explants showing shoot bud response.

Shoot bud induction, elongation and rooting

Leaf segments (4 mm×5 mm) were excised from seedlings inoculated into MS media with varying levels of nitrogen, sucrose and TDZ concentrations for shoot bud formation. Shoot buds formed after 30 days interval along with the mother explant were transferred to MS media supplemented with 0.5 mg/l TIBA and 0.1 mg/l GA_3 for elongation of shoot buds. The elongated shoots formed after 30 days intervals were excised and placed on MS medium supplemented with 1 mg/l Indole-3-butyric acid.

Hardening of the elongated shoots

The rooted plants were washed off their agar under running tap water and transferred to plastic cups having sand: compost mixture of 1:2. These cups were covered with polyethylene bags with holes. The plantlets were hardened for 60 days and then transferred to pots with farmyard manure.

Experimental design and analysis of data

The experimental design employed was a 3-variable (5 levels of each variable), second order central composite design with 5 replications at the centre points (0, 0, 0) in coded levels of variables (-1.682, -1, 0, 1, 1.682) [1]. The three independent variables for shoot bud response and shoot bud induction were concentrations of nitrogen (X_1), sucrose (X_2) and TDZ (X_3). The experimental design in the actual (X) and coded (x) levels of variables is shown in Table 1. The response functions (Y_{ijk}), i.e., shoot bud induction and shoot bud response in the culture was approximated by a second degree polynomial (Eq. 1) with linear, quadratic and interaction effects (in coded level of variables) using the method of least squares [16].

$$Y_{ijk} = b_0 + \sum_{i=1}^{n} b_i x_i + \sum_{\substack{i=1 \\ i \le j}}^{n} \sum_{j=1}^{n} b_{ij} x_i x_j + \varepsilon_{ijk} \quad (1)$$

The number of variables, denoted by n, and i, j and k, are integers. The coefficients of the polynomials are represented by b_0, b_i and $b_{ij,}$ and ε_{ijk} is the random error; when $i < j$, b_{ij} represents the interaction effects of the variables x_i and x_j. The response surface graphs were obtained from the regression equations in actual level of variables. The detailed analysis of variance (ANOVA) was conducted in coded level of variables to know the effects of individual variables. Stepwise deletion of individual non-significant ($p \le 0.10$) terms were conducted followed by recalculation of the coefficients of the regression equation, to arrive at

the final regression equation in coded level which is better converted to actual level of variables.

Table 1. Experimental design in coded and actual level of variables

Exp. No	Concentration of N_2 (g/l)		Concentration of sucrose (%)		Concentration of TDZ (mg/l)	
	Coded level (x_1)	Actual level (X_1)	Coded level (x_2)	Actual level (X_2)	Coded level (x_3)	Actual level (X_3)
1	-1	1.967	1	2.590	1	0.899
2	0	3.550	0	2.000	0	0.750
3	0	3.550	0	2.000	0	0.750
4	0	3.550	0	2.000	0	0.750
5	0	3.550	0	2.000	0	0.750
6	-1	1.967	-1	1.405	-1	0.601
7	-1	1.967	-1	1.405	1	0.899
8	1	5.133	-1	1.405	1	0.899
9	1	5.133	1	2.590	1	0.899
10	0	3.550	0	2.000	1.682	1.000
11	0	3.550	0	2.000	0	0.750
12	1.682	6.212	0	2.000	0	0.750
13	-1	1.967	1	2.590	-1	0.601
14	0	3.550	-1.682	1.000	0	0.750
15	1	5.133	1	2.590	-1	0.601
16	0	3.550	1.682	3.000	0	0.750
17	0	3.550	0	2.000	0	0.750
18	-1.682	0.887	0	2.000	0	0.750
19	1	5.133	-1	1.405	-1	0.601
20	0	3.550	0	2.000	-1.682	0.500

N_2 – Nitrogen; TDZ – thidiazuron

Optimization

Optimization was done by employing canonical analysis [15, 23] wherein the levels of the variables (x_1, x_2, x_3) (within the experimental range) were determined to obtain the shoot bud response and shoot bud induction individually. Optimization of the response functions consists of the translation of the response function (y_k) from the origin to the stationary points (x_0) [23].Then the response function was expressed in terms of the new variables, the axes of which correspond to the principal axes of the contour system. Further the roots (λ_1, λ_2, λ_3) of the auxiliary equation ($\lambda^2 - \lambda + 1 = 0$) were calculated initially to know the nature of optimum. The response function is maximum if all the roots have negative values, and minimum if all roots have positive values. If some of the roots have positive values and some negative, then it is the situation of a saddle point [15, 23].

Results and discussion

In micro propagation of plants, response surface methodology (RSM) has been used for optimization of media constituents for organogenesis and embryogenesis as well as for increasing the yield of metabolites from *in vitro* cell cultures. RSM was applied to *Decalepis hamiltonii* Wight. & Arn for development of multiple shoots, increasing shoot length by

either decreasing or increasing the concentrations of growth regulators and sucrose [11]. Response surface methodology studies in *Decalepis hamiltonii* Wight. & Arn was also useful for understanding the interaction between the various parameters employed [11]. Response Surface Methodology adopted for *Centella asiatica* cell suspension cultures showed that sucrose concentration influenced the dry cell weight of the callus [27]. In *Dianthus caryophyllus* L., various combinations and concentrations of growth regulators were optimized to give rise to callus and for the initiation of shoots and roots from the callus [13]. Various extraction parameters were optimized for extraction of lycopene from tomato cell cultures; up to 3.7 fold increase in lycopene was obtained when compared to that of the original method [17]. Similarly, RSM was applied for increasing the yield of capsaicinoid from immobilized cultures of *Capsicum frutescens*. A yield of 220 µg/g capsaicinoids was obtained in 1 to 3 days after culturing the immobilized beads on to suitable medium [32].

The present experiment has been aimed at optimization of shoot bud response and shoots bud induction from the leaf segments of *Solanum melongena*. The use of statistical methods based on experimental design such as response surface methodology enabled the evaluation of MS medium components and plant growth regulator TDZ in shoot bud response and shoot bud induction from leaf explants of eggplant. The experimental results on the effect of the three independent variables (concentrations of nitrogen, sucrose and TDZ) on the two response functions or targeted parameters (shoot bud response and shoot bud induction) are shown in Table 2. The analysis of variances (ANOVA) (in coded level of variables) is shown in Table 3 for the two response functions. The response surfaces (Figs. 1-2) are presented to aid in visualizing the effect of the variables on the response functions.

Shoot bud response

The buds response percent for shoot bud development (Y_1) shows a wide variation (20.7-93.0%) due to the various experimental conditions (Table 2). A high multiple correlation coefficient ($R = 0.96$, $p \leq 0.01$) for buds response indicates the suitability of the second order polynomial to predict the Y_1 values in terms of the three independent variables (Table 3). The total linear and quadratic effects dominate (significant at $p \leq 0.01$) over the interaction effect ($p \leq 0.05$). Among the linear effects, sucrose concentration has the maximum linear positive effect (significant at $p \leq 0.01$) on Y_1 followed by the linear negative effect of nitrogen ($p \leq 0.05$). Both nitrogen and sucrose have significant quadratic negative effects ($p \leq 0.01$) meaning curvilinear effects of these variables (Fig. 1). It also indicates that a high concentration of these variables suppress the buds response. However, TDZ concentration has no significant effect ($p \geq 0.10$) on buds response. Among the individual interaction terms nitrogen x sucrose concentration has the maximum positive effect ($p \leq 0.01$) meaning that the effect of nitrogen on shoot bud response depends on the level of sucrose (Fig. 1b). The optimum (maximum) condition for buds response is 4.34 g/l of total nitrogen content, 2.65% of sucrose and 0.67 mg/l of TDZ (Table 4).

Shoot bud induction

The number of shoot buds (Y_2) varies between 2.0 and 14.7 at different combinations of variables (Table 2). A high multiple correlation coefficient ($R = 0.93$, $p \leq 0.01$) indicates the suitability of the second order polynomial to predict the bud number (Table 3). Sucrose concentration shows the maximum linear positive effect ($p \leq 0.01$) on Y_2 followed by the linear negative effect of TDZ concentration ($p \leq 0.05$). Negative quadratic effect of nitrogen and sucrose are significant ($p \leq 0.01$). This means a curvilinear effect of the variables where TDZ possesses a marginal effect compared to nitrogen and sugar (Fig. 2).

Table 2. Experimental results for the response functions

Exp. No	Buds response %	Buds number
1	74.75 ± 4.17	5.81 ± 2.23
2	85.75 ± 3.05	9.08 ± 2.19
3	85.38 ± 4.87	9.01 ± 1.74
4	85.85 ± 1.95	9.08 ± 1.27
5	85.57 ± 1.72	9.01 ± 1.02
6	51.75 ± 3.77	5.03 ± 0.5
7	62.59 ± 3.57	2.61 ± 0.82
8	28.10 ± 1.6	3.97 ± 0.29
9	90.40 ± 6.12	8.47 ± 0.61
10	60.42 ± 3.61	8.89 ± 1.02
11	85.85 ± 2.03	9.02 ± 2.23
12	41.09 ± 1.06	2.67 ± 0.29
13	63.50 ± 2.08	7.63 ± 0.51
14	20.73 ± 1.27	2.33 ± 1.15
15	89.98 ± 4.04	8.47 ± 1.1
16	84.97 ± 4.37	8.75 ± 1.72
17	85.80 ± 5.05	9.08 ± 0.58
18	75.36 ± 2.26	6.89 ± 0.79
19	25.51 ± 1.72	2.03 ± 0.79
20	92.98 ± 3.04	14.71 ± 0.53

Table 3. Analysis of variance (ANOVA) for the shoot bud response (Y_1) and bud induction (Y_2) in coded level of variables

Source of variation	Coefficient of polynomial for Y_1	F value	Coefficient of polynomial for Y_2	F value
Constant	85.712		9.104	
x_1	-5.582	5.67^{**}	-0.383	0.79^{NS}
x_2	18.943	65.26^{***}	2.016	22.07^{***}
x_3	-2.172	0.86^{NS}	-0.885	4.25^{*}
x_1^2	-9.790	18.40^{***}	-1.851	20.28^{***}
x_2^2	-11.690	26.24^{***}	-1.613	14.91^{***}
x_3^2	-3.260	0.01^{NS}	0.600	2.06^{NS}
x_1x_2	12.857	17.61^{***}	0.643	1.31^{NS}
x_1x_3	-2.385	0.61^{NS}	0.772	0.89^{NS}
x_2x_3	-0.220	2.04^{NS}	-0.167	0.08^{NS}
TLE	5391.2	23.93^{***}	68.2	9.04^{***}
TQE	3079.6	13.67^{***}	93.0	12.32^{***}
TIE	1368.4	6.07^{**}	8.3	1.10^{NS}
R	0.964^{***}		0.933^{***}	

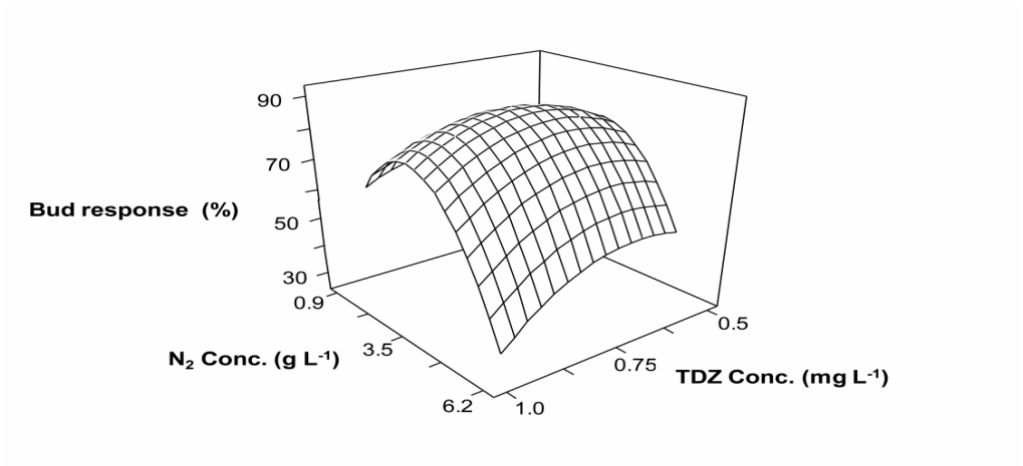
Variables: Concentrations of nitrogen (x_1), sucrose (x_2) and thidiazuron (x_3)

* Significant at $p \leq 0.10$ TLE: Total linear effect

** Significant at $p \leq 0.05$ TQE: Total quadratic effect

*** Significant at $p \leq 0.01$ TIE: Total interaction effect

^{NS} Non-significant at $p = 0.10$



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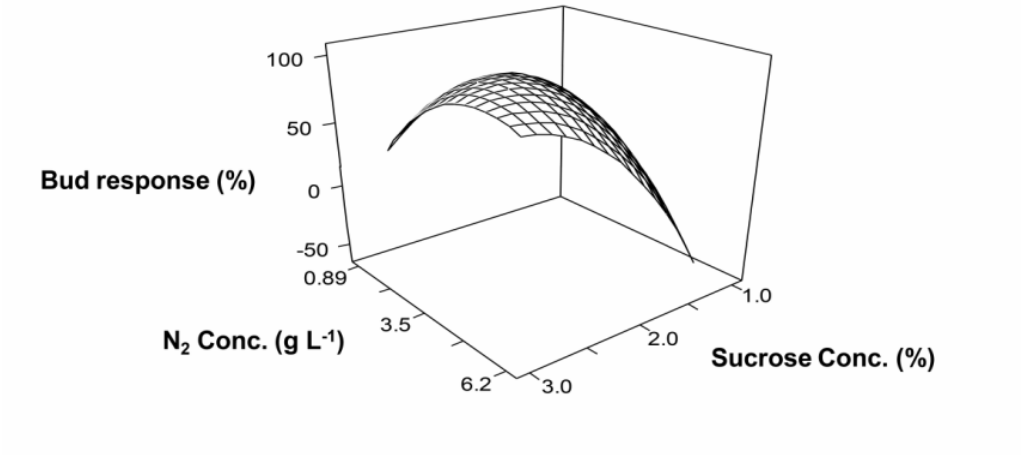

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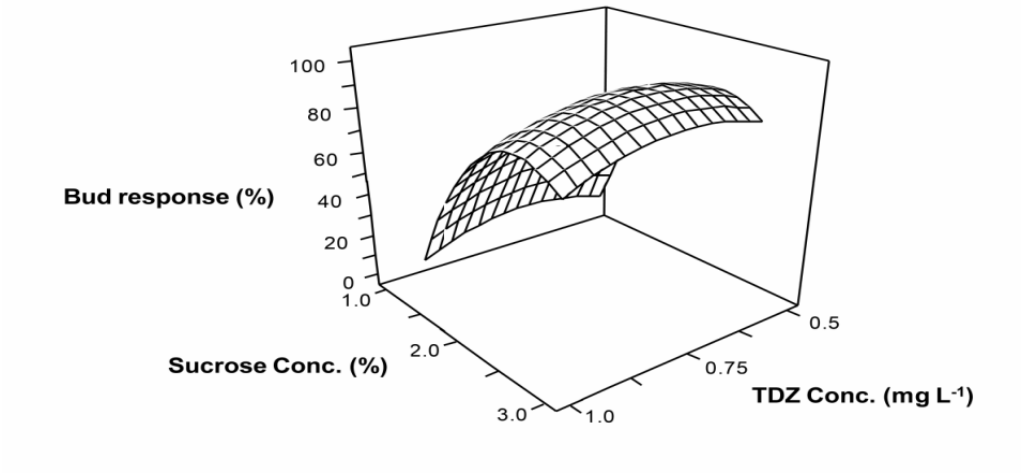


c)

Fig. 1 Bud response as a function of (a) nitrogen and TDZ concentration when sucrose concentration was maintained at 2%, (b) nitrogen and sucrose concentration when TDZ concentration was maintained at 0.75 g/l, and (c) sucrose and TDZ concentration when nitrogen concentration was maintained at 3.5 g/l

However, all the three interaction effects are non-significant at $p \leq 0.10$). The linear effect of nitrogen is negligible but its quadratic effect is highly significant at $p \leq 0.01$ and possesses a negative effect. This means that a low level of nitrogen concentration, it has a marginal effect on bud number but at a high level, it decreases the number of buds markedly (Fig. 2a). The optimum medium condition for obtaining highest number of buds can be achieved with 4.02 g/l of total nitrogen content, 2.36% of sucrose and 1.0 mg/l of TDZ (Table 4).

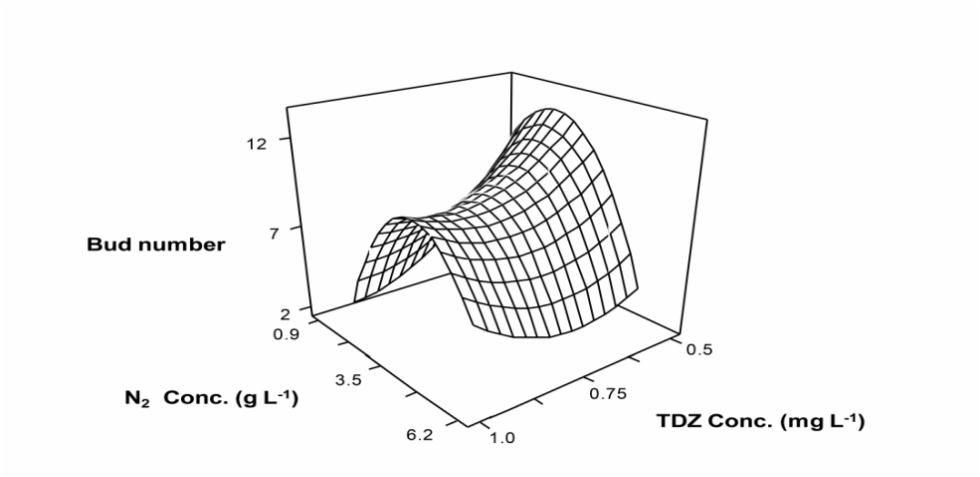
Optimization

The process of optimization (maximization) of the percent buds response (Y_1) and number of buds (Y_2) has been conducted separately. The roots (λ_1, λ_2, λ_3) of the auxiliary equation have been also determined and are shown in Table 4.

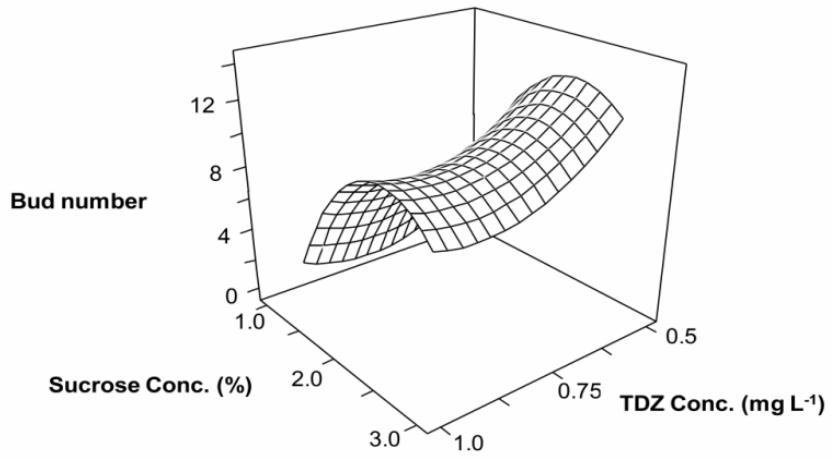
During the process of optimization of number of buds (Y_2), the roots (λ_1, λ_2, λ_3) of the auxiliary equations are positive and negative in their magnitudes indicating the optimum condition to be a saddle point. Hence, the canonical method was employed to obtain the optimum condition (Table 4) within the range of the present experimental range of variables for maximum number of buds. Maximum number of buds has been achieved with higher level of nitrogen and sucrose concentration (4.02 g/l and 2.36%, respectively) and maximum level of TDZ concentration (1.0 mg/l) with 10 number of buds per explant (Fig. 3a and 3b). Table 4 also shows that maximum percent buds response can be achieved with higher level of nitrogen and sucrose concentration (4.34 g/l and 2.65%, respectively) and minimum level of TDZ concentration (0.67 mg/l) with a yield of 95% buds response (Fig. 3a and 3b).

The shoot buds formed on this media upon transferring to 0.5 mg/l of 2,3,5-Triiodobenzoic acid and 0.1 mg/l of gibberellic acid offers the maximum elongated shoots and shoot length (Fig. 3c). No marked increase in the number of shoot buds has been observed when the cultures are transferred from TDZ to MS supplemented with 0.5 mg/l of TIBA, 0.1 mg/l of GA_3 media. The elongated shoots are rooted in 1 mg/l of IBA (Fig. 3d). The rooted plants have been transferred to small plastic cups and are covered with polyethylene sheet with holes for hardening in the green house condition. They have been hardened under the green house condition for two months and later are transferred to pots containing farm yard manure. About 80% of the plants have been able to survive after hardening. These plants are able to grow into mature fruit bearing plants (Fig. 3e).

The ability of eggplant to respond well in tissue culture has facilitated the use of genetic transformation studies in eggplant. *Agrobacterium* mediated transformation of eggplant has resulted in the development of Bt brinjal resistant to insects [4]. Genetic transformation studies have resulted in the production of eggplant tolerant to various abiotic and biotic stresses as well as production of parthenocarpic fruits [6]. Further research into the post harvest trait improvements like supplying vitamin and mineral content, increasing shelf life and improving the nutritive value of eggplant has to be taken up [6]. For efficient use of genetic engineering technology for improvement of eggplant traits, an efficient micropropagation system will be valuable.



a)



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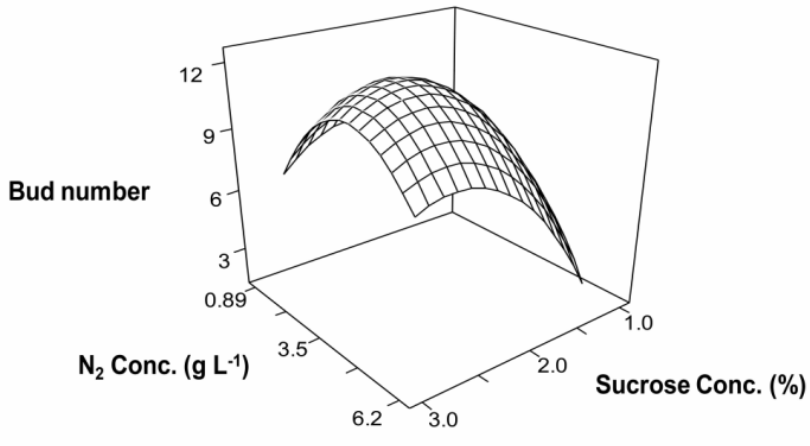

c)

Fig. 2 Bud number as function of (a) nitrogen and TDZ concentration when sucrose concentration was maintained at 2%, (b) nitrogen and sucrose concentration when TDZ concentration was maintained at 0.75 g/l, and (c) sucrose and TDZ concentration when nitrogen concentration was maintained at 3.5 g/l

Table 4. Results of the optimization study

Parameters		Buds response Y_1	Bud Numbers Y_2
Roots of the auxiliary equation	λ_1	-2.635	0.659
	λ_2	-4.832	-1.408
	λ_3	-17.274	-2.145
Optimum conditions in coded level	x_1	0.497	0.296
	x_2	1.088	0.603
	x_3	-0.552	1.681
Optimum conditions in actual level	x_1	4.34 g/l	4.02 g/l
	x_2	2.65 %	2.36%
	x_3	0.67 mg/l	1.0 mg/l
Optimized level of response function		95.2%	10.0

Variables: Concentrations of nitrogen (x_1), sucrose (x_2) and thidiazuron (x_3)

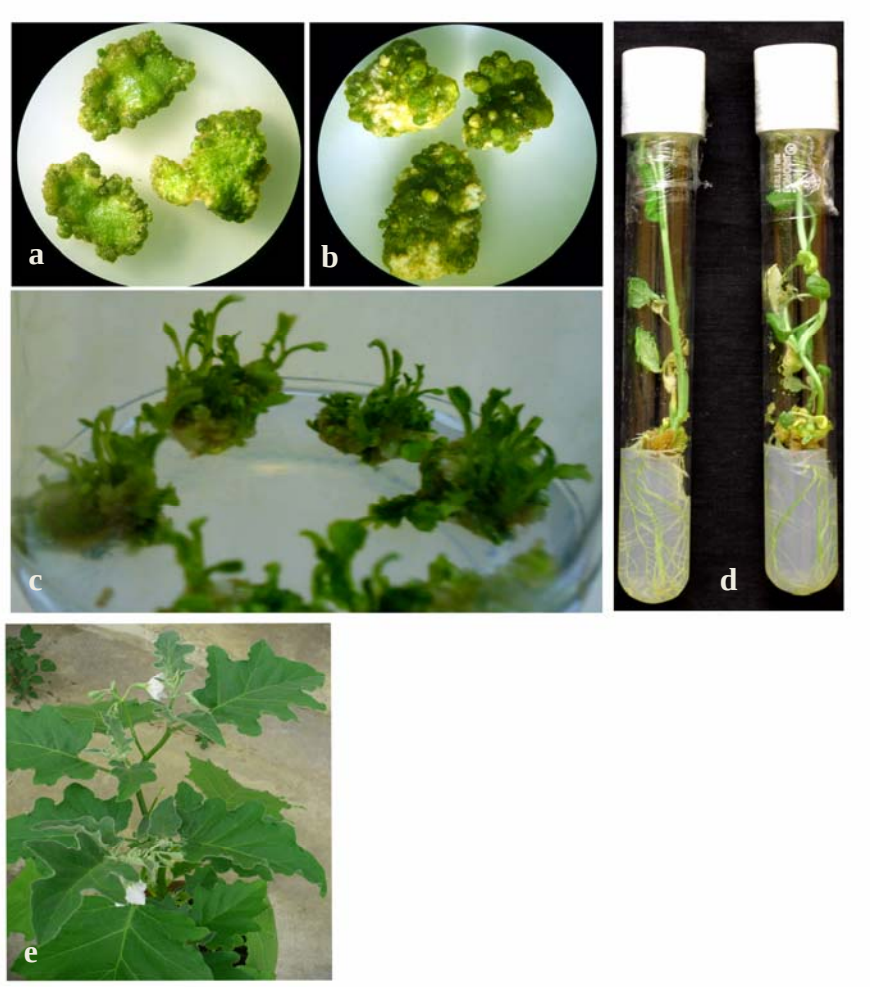

Fig. 3 Regeneration from leaf segments of *Solanum melongena* L. (a and b): Shoot bud induction from leaf segments in optimized medium (c): Shoot bud elongation in MS medium supplemented with 0.5 mg/l 2,3,5-Triiodobenzoic acid and 0.1 mg/l Gibberellic acid (d): Rooting of elongated shoots MS media supplemented with 1 mg/l Indole-3-butyric acid (e): Hardened plant in green house

Conclusion

Despite the numerous micropropagation protocols available for eggplant regeneration most of them are genotype and explant specific. However when a statistical methodology like RSM is adopted for studies of micropropagation repeatability could be expected. Eggplant is not a recalcitrant system like some of the Solanaceous members like *Capsicum*. The current paper describes the use of basic media requirements of micropropagation rather than explaining the effect of diverse hormonal usage which reduces the cost of overall protocol developed. The protocol developed here will be effective in induction of shoot buds from leaf explant of *Solanum melongena* variety Arka Shirish and can be used in genetic transformation studies. Moreover, process optimization is useful in large scale production of *in vitro* shoots through high efficiency automation in future facilitating micropropagation.

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