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OPTIMIZING SOMATIC EMBRYOGENESIS THROUGH RESPONSE SURFACE MODEL (RSM) AND CLONAL FIDELITY OF PLANTS IN GUAVA (*PSIDIUM GUAJAVA* L.)

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ABSTRACT
Response surface methodology (RSM) was employed for the optimization of various nutritional and other physicochemical parameters for somatic embryogenesis in guava (*Psidium guajava* L.). Murashige and Skoog (MS) (1962) basal salts was used for optimization of various media and other factors for induction of somatic embryogenesis from zygotic embryo explants. Eight different factors *viz.* the concentration of 2,4-dichlorophenoxy acetic acid (2,4-D), the treatment period of zygotic embryo explants, the age of explants, the concentrations of sucrose both for induction of the process and the development of somatic embryos, concentration of glutamine, sodium chloride and polyethylene glycol were initially screened using Plackett–Burman design. The contrast coefficient value with more than 95% confidence level identified variables 2, 4-D concentrations, treatment period, age of explants, sucrose in induction and in development medium having significant influence on the process of somatic embryogenesis were further optimized using Box-Behnken design generating a value of 0.9105 for "R^2" (determination coefficient) and a high value of 0.52 for "Lack of Fit" for quadratic model. On experimental validation within tested range the optimal combination of the physicochemical factors as 1.0 mgl [1] of 2,4-D in 8 days treatment of 10-weeks post-anthesis (age of zygotic embryo explants) cultured in the presence of 5% (w/v) sucrose in induction and 5% (w/v) sucrose in development medium induced highest efficiency of somatic embryogenesis. Thus application of response surface methodology resulted in a good optimization of somatic embryogenesis with genetically uniform plants formation in guava and could find application for micropropagation in other species.

KEYWORDS: Clonal fidelity, *Psidium guajava,* Response surface methodology, Somatic embryogenesis, Zygotic embryo.

INTRODUCTION

During recent years, immense efforts have been made to maintain the process under optimum condition, which can significantly increase the production of various biotechnological products. Several statistical designs are currently available to predict the behavior of a reaction through response surface methodology (RSM). Basically this optimization process involves three major steps: performing statistically designed experiments, estimating the coefficients in a mathematical model and predicting the response and checking the adequacy of the model (Annaduari and Sivakumar, 2000). The use of appropriate experimental designs and statistical analyses in plant cell and tissue culture studies is necessary to ensure unbiased and precise estimates of treatment effects and to provide proper interpretation of results (Nas *et al*., 2005). Most of the plant cell and tissue culture studies are conducted under controlled environmental condition of light, temperature and are set up as factorial experiments in completely randomized designs, randomized complete block designs or split-plot designs (Compton, 1994; Compton and Mize, 1999). These types of experimental designs are useful when the researcher has previously identified a few factors to study and there is sufficient amount of explants material to properly replicate. However, in some tissue culture research, these types of experimental designs may not be appropriate, or even feasible (Compton, 1994; Mize and Chun, 1988; Mize *et al*., 1999; Ibanez *et al*., 2003).

As central composite designs, Box–Behnken designs (BBD) (Box and Behnken, 1960) are response surface methods (RSM) used to examine the relationship between one or more response variables. Box-Behnken is a spherical, revolving design viewed as a cube, it consists of central point and the middle points of the edges. However, it can also be viewed as consisting of the three interlocking 2^2 factorial designs and a central point. Response surface methods are often used once preliminary screening has been carried out using factorial designs such as Plackett-Burman design to determine which factors significantly affect the response. Plackett–Burman designs (Plackett and Burman, 1946) is a special type of fractional factorial analysis where up to n-1 factors can be evaluated in n runs and when n is a multiple of four (Box *et al*., 1978). If the primary objective is to determine the important factors to study in further experiments, small fractions such as PBD can be quite effective in plant cell and tissue culture research (Nas *et al*., 2005).

Somatic embryogenesis process

Somatic embryogenesis is widely adopted regeneration system for the large volume high efficiency micro propagation of many plant species belonging to both

angiosperm and gymnosperms (Akhtar and Jain, 2000; Jain *et al*., 1995, 2000; Jain and Gupta, 2005; Sato, 2012). Somatic embryogenesis is frequently expressed as discrete phases or steps characterized by distinct biochemical and molecular events (Mujib and Samaj 2006; Suprasanna and Bapat, 2006) influenced by a number of factors (Akhtar *et al*., 2000; Suprasanna *et al*., 2005). Among these factors plant growth regulator has been the most important for induction of somatic embryogenesis. In general auxins, and 2, 4-D in particular have been found as the exogenous inducer of the process of somatic embryogenesis (Akhtar, 2013a, b; Akhtar, *et al*., 2000; Singh *et al*., 2007). The inducing concentration of various growth regulators varies according to the species, explants types, age and maturity status as a function of their nutritional demand for the proper progression of somatic embryogenesis (Jain *et al*., 2000; Prakash and Gurumurthi, 2010). For large volume micropropagation of any species, high efficiency of physiologically normal and convertible somatic embryos is essential. Unfortunately, such data have not been well-defined in many reports (Akhtar, 2010). In general, most embryogenic systems have not been optimized for high efficiency conversion and commercial exploitation of the process.

Common guava (*Psidium guajava* L.; Family, Myrtaceae) is a diploid species (2n=22). In guava the process of somatic embryogenesis has been studied under the six different parameters *viz*. frequency of embryogenesis (FE), intensity of embryogenesis (IE), frequency of elongated stage somatic embryos (ET), frequency of short stage somatic embryos (ST), frequency of lower stage somatic embryos (CHG) and efficiency of embryogenesis (EE) (Akhtar 1997, 2010, 2011) and followed in two different stages i. induction and ii. development (Akhtar, 1997, 2013a, b) following one-at-a-time strategy. In the present study, eight factors namely the concentration of 2, 4-D, the treatment period, the age of the explants, the concentrations of sucrose at both induction of the process and the development of somatic embryos, concentration of glutamine, NaCl and PEG have been subjected to PBD and BBD experimental analysis to optimize the most important factor(s) affecting somatic embryogenesis in *P. guajava*.

At present PBD and BBD are not being applied for the optimization of somatic embryogenesis in plant cell and tissue culture studies. The objective of this study is to demonstrate the applicability of the PBD and BBD in identifying the most important medium and treatment factors affecting the process of somatic embryogenesis for optimization of high efficiency micropropagation and to test the clonal fidelity of the plants through response surface experimental model.

True-to-type clonal fidelity is one of the most important pre-requisites in the *in vitro* propagation of plants. A serious limitation to commercial applicability of the micro propagation system is the occurrence of cryptic genetic defects due to somaclonal variation (Salvi *et al*., 2001). Hence, it is imperative to establish genetic fidelity of the regeneration system in order to maintain the quality of somatic embryogenesis derived plantlets for its commercial utility.

Polymerase chain reaction (PCR)-based techniques such as random amplified polymorphic DNA (RAPD) and inter-simple sequence repeat (ISSR) are used to test clonal fidelity of *in vitro*-regenerated plantlets in many crop species (Martin *et al*., 2004). The use of two RAPD and ISSR markers amplify different regions of the genome, allows better chances for the identification of any genetic variations in the clones. The techniques are very simple, fast, cost-effective, highly discriminative, reliable and reproducible. There is no need of any prior sequence information to design the primer and only a small quantity of DNA sample is required for the assessment of the genetic uniformity of plantlet regenerated through somatic embryogenesis. These PCR based techniques are used to demonstrate the genetic fidelity of the plantlets through optimization of somatic embryogenesis process using response surface methodology.

MATERIALS & METHODS

The process of somatic embryogenesis in guava has been induced following the protocol reported in Akhtar (1997, 2010, 2012). The effects of various treatments on the process of somatic embryogenesis have been evaluated under the six different parameters according to the scheme described previously (Akhtar, 1997, 2010). The eight different factors studied in the present experimental design are presented in table 1. The efficiency of embryogenesis (EE) which has been identified as the single most important calculated response (Akhtar, 1997, 2010) as other five experimental values have not identified the same treatment factor for high efficiency somatic embryogenesis in guava (*Psidium guajava* L.).

TABLE 1. Factors and their level employed in the Placket-Burman and Box-Behnken design for the screening of the main condition affecting the process of somatic embryogenesis in guava (*Psidium guajava* L.).

Sl. No.	Factors (Independent Variables)	Units	Variable Number	Coded symbol	Coded Values		
					-1	0	1
1	2,4-Dichlorophenoxy acetic acid	mg/l	x_1	X_1	0.5	1.0	1.5
2	Treatment Period	days	x_2	X_2	4	8	12
3	Age of Explants	weeks	x_3	X_3	8	10	12
4	Sucrose in induction medium	%	x_4	X_4	2.5	5	7.5
5	Sucrose in development medium	%	x_5	X_5	2.5	5	7.5
6	Glutamine	mg/l	x_6	X_6	50	75	100
7	NaCl	mM	x_7	X_7	50	100	150
8	PEG	%	x_8	X_8	0.5	1.0	1.5

Somatic embryogenesis experiments were performed using Murashige and Skoog (MS) (1962) basal salts modified for various treatment factors as shown in Table 1. The zygotic embryo explants dissected by collecting

fruits from 10-15-year-old guava genotypes Allahabad safeda after 8-, 10- and 12-weeks of anthesis to optimize age of the explants. Surface disinfection, sterilization of seeds and dissection of zygotic embryo explants were carried out following the protocol of Akhtar (1997, 2010, 2013a, b,c, 2018). To optimize various factors for induction of somatic embryogenesis, the media were supplemented with different levels of sucrose, modified with 2,4-D, added with glutamine, sodium chloride, poly ethylene glycol and the zygotic embryo explants were treated for different days as per the scheme presented in Table 1. The pH of the medium was set at 5.2 ±0.2 prior to media modifications and autoclaving. All media and other requirements were sterilized by autoclaving at 121 °C and 1.1 kg cm^{-2} pressure for 15 min. All cultures were incubated in an air-conditioned culture room maintained at 25° ±2°C temperature, 60–65% relative humidity, and 16 h photoperiod receiving a photon flux density (PFD) of 50–70 µmol m^{-2} s^{-1}.

The Plackett –Burman optimization of somatic embryogenesis

Plackett–Burman designs is a special type of fractional factorial analysis where up to *n*-1 factors can be evaluated in *n* runs and when *n* is a multiple of four (Box *et al.*, 1978; Plackett and Burman, 1946). Plackett-Burman design is based on the first order model:

$$Y = \beta_0 + \Sigma\beta_i X_i \quad \textbf{(1)}$$

This model was used to screen the important variables that influence the process of somatic embryogenesis in guava. Each variable was represented at two levels, high and low, which were denoted by (+1) and (-1) respectively. The coded level of each of the 8 - variable was used as given in Table 1. All experiments were performed in duplicate and the mean values were given. The variables having confidence levels greater than 95% were considered to significantly affect the somatic embryogenesis and used for further optimization.

The standard error (S.E.) of the mean was the square root of the variance of an effect and the significant level (*P*-value) of each variable was determined using the Student's *t*-test:

$$t(xi) = \frac{E(Xi)}{\text{S.E.}}$$

where $E(X_i)$ was the effect of variable x_i

The Box-Behnken optimization of somatic embryogenesis

The Box-Behnken design and response surface methodology (RSM) were used to optimize the process by finding interactions among significant factors obtained from the Plackett-Burman design. The basic strategy for RSM had four steps: procedures to move into the optimum region, behavior of the response in the optimum region, estimation of the optimal condition and verification. According to the Box-Behnken experimental design, the total number of experimental combinations was $2^k + 2k + n_0$, where k was the number of independent variables and n_0 was the number of repetitions of the experiments at the centre point. For statistical calculation, the experimental variable x_i had been coded as X_i according to the following transformation equation:

$$X_i = \frac{x_i - x_0}{\delta x} \quad i = 1, 2, 3 \qquad \textbf{(3)}$$

Where *Xi* was the dimensionless coded value of the variable x_i, x_0 the value of x_i at the centre point and δx the step change.

In this study, the Box-Behnken experimental design with five factors and three levels (low, medium and high), including six replicates at the centre point, was used for fitting a second order response surface for the efficiency of somatic embryogenesis. This methodology allowed the modeling of a second order equation that described the process. The process of somatic embryogenesis was analyzed by multiple regressions through the least squares method, and final fitting a response surface model based on a second-order design in the new region by following equation:

$$Y = \beta_0 + \sum_{i=1}^{t} \beta_i X_i + \sum_{i=1}^{t} \beta_{ii} X_i^2 + \sum_{\substack{i=1 \\ i<j}}^{t} \beta_{ij} X_i X_j + \varepsilon \qquad \textbf{(4)}$$

where *Y* was the predicted response variable and x_1, x_2,…… x_t were the input factors which influence the response *Y*; β_0 β_z, β_{zz}, β_{zj} were constant regression coefficients of the model, and X_i, X_j (i = 1,2,…t), β_{ij} (i = 1,2,…t; *j* = 1,2,…t) represented the independent variables in the form of coded values and ε was the random error. The coefficients, which should be determined in the second order model, were obtained by the least square method. Assuming that all factors were quantitative and are denoted by X_1, X_2... X_t, the second-order model was given where β_i was the linear main effects, β_{ii} was the quadratic main effects, β_{ij} was the linear-by-linear interactions, and ε was the error term. The accuracy and general ability of the above polynomial model was evaluated by the coefficient of determination R^2. In the present study Box-Behnken experimental design was chosen for findings out the relationship between the response function (six somatic embryogenesis parameters, (Akhtar, 1997, 2000, 2010, 2013a, b, c) and the variables designated as X_1, X_2, X_3, X_4 and X_5 (Table 1).

Genetic Fidelity:

DNA extraction and PCR amplification conditions:

Six to eighteen month old plantlets regenerated through somatic embryogenesis and in the process of acclimatization and soil established were tested for their genetic fidelity to the mother plants. Eleven such plantlets were randomly selected for collection of leaf tissues. DNA was extracted from young leaves using the N-cetyl-N, N, N-trimethylammonium bromide (CTAB) method described by Doyle and Doyle (1990) with modifications. A sample of 100mg of fresh young leaf material from mother plant and regenerated somatic plants were washed in 80% (v/v) ethanol and then grounded to fine powder in liquid nitrogen. Two ml of preheated (60°C) extraction buffer [2% CTAB (w/v), 0.2% β-mercaptoethanol (v/v), 100mM Tris-HCl (pH 8.0), 2mM EDTA, 1.4M NaCl] was added for DNA extraction with equal volume of Phenol: Chloroform: Isoamyl alcohol (25:24:1) by gentle swirling mixing and pelleted with chilled ethanol. The DNA pellet was re-suspended in 50–100 µl of Tris-EDTA solution. The quality of DNA extract was assessed spectro-photometrically by A260/280 ratio. DNA quantifications

was performed by visualizing under UV light, after electrophoresis on 0.8% agarose gel. The re-suspended DNA was then diluted in sterile distilled water to 5 ng/µl concentration for use in amplification reactions.

A set of ten random decamer oligonucleotides were selected from Operon Technologies Inc. (Alameda, California, USA) and synthesized from ILS (Imperial Life Science, India) for the amplification of RAPD fragments as single primers. Polymerase Chain Reactions (PCR) were carried out in a final volume of 25 µl containing 20 ng template DNA, 100µM each deoxynucleotide triphosphate, 20 ng of decanucleotide primers, 1.5mM $MgCl_2$, 1 × taq buffer [10mM Tris-HCl (pH 9.0), 50mM KCl, and 1 % Triton X-100 with 15 mM $MgCl_2$] and 2 U Taq DNA polymerase (GeNei™, Bangalore Genei, India). Amplification was achieved in a PTC 100 thermal cycler (MJ Research, USA) programmed for a preliminary 4 min denaturation step at 94°C, followed by 45 cycles of denaturation at 94°C for 1 min, annealing at 36 °C for 1 min and extension at 72°C for 2 min, finally at 72°C for 10 min. Amplification products were separated alongside a 100 bp molecular weight marker (GeNei™, Bangalore Genei, India) by electrophoresis on 1.2% agarose (A9539, Sigma) gels run in 0.5X TAE (Tris Acetate EDTA) buffer, stained with ethidium bromide and visualized under UV light. Gel photographs were scanned through Gel Doc System (Alpha-Imager Mini System, USA) and the amplification product sizes were evaluated using the software provided with the system.

Six ISSR primers (Integrated DNA Technologies Inc., Coralville, IA) used to verify the results of RAPD markers produced clear and reproducible bands. Primers were synthesized from ILS (Imperial Life Science, India). The PCR reaction was performed in a 25 µl volume containing 25 ng of template DNA, 1x PCR buffer (10 mM Tris (pH 9.0), 50 mM KCl, and 1 % Triton X-100 with 15 mM $MgCl_2$), 100 µM dNTP mix (GeNeiTM, Bangalore, India), 2.5mM $MgCl_2$ and 2U Taq DNA polymerase (GeNeiTM, Bangalore, India) and 0.5 µM primer. PCR was performed by initial denaturation at 94 °C for 4 min, followed by 35 cycles of denaturation at 94 °C for 45s, annealing at 45.7 to 49.0 °C for 1 min and extension at 72 °C for 1 min with a final extension at 72 °C for 8 min. The amplification products were resolved on 1.4 % agarose (A9539, Sigma). The size of the amplicons was estimated by comparing with 100 bp DNA ladder (GeNeiTM, Bangalore, India).

Statistical analysis

The six embryogenesis parameters (Akhtar, 1997, 2010) were analyzed using Design Expert Software 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA) and SPSS 10 package for Window (SPSS Inc., USA) in the present study. However, only the efficiency of embryogenesis (EE) is presented in the present study as other five experimental values have not identified the same treatment to get high efficiency of response. The Plackett–Burman fractional factorial design of the whole experiment consisted of eight factor variables (2,4-D concentrations, treatment period, age of explants, sucrose in induction and in development medium, glutamine, NaCl, PEG). For significant variables, the quadratic model was generated following Box-Behnken experimental design and represented as response surface curve using Design Expert Software 8.0.7.1 (Stat-Ease Inc., Minneapolis, USA).

RESULTS

Induction of somatic embryogenesis

There was a sudden and fast change in shape, size, colour and extent of callus formation within a week of subculture of the zygotic embryo explants on to PGR free medium (Fig. 1A-D) after different days of inductive treatment with 2,4-D as described by Akhtar (2010, 2012, 2013a, b). Globular transparent white somatic embryos (Fig. 1D) were observed under stereozoom microscope (SMZ – 2T; Nikon, Tokyo, Japan) after 3-weeks of culture initiation. Development and maturation of somatic embryos followed morphological trajectory similar to those described in earlier report (Akhtar 2010, 2013a, b,c). The somatic embryogenesis responses were observed usually after 8-10 weeks of culture initiation (Fig. 1E). The result of this experimental design is presented in table 2.

Plackett-Burman model of somatic embryogenesis

Plackett-Burman design matrix used for the screening of various factors affecting the somatic embryogenesis in guava is shown in table 2. The result of the analysis is presented in table 3. Thus among the factor tested only 2,4-D concentrations, treatment period, Age of explants, sucrose in induction and development medium shows a significant influence on the somatic embryogenesis as evaluated by their respective contrast coefficient value (Table 3). Since, a linear approach is considered to be sufficient for screening as per the model represented in equation 1. Neglecting the terms which were insignificant on the basis of regression coefficients and *P*- value, the linear regression equation for variable that has an important influence on the efficiency of somatic embryogenesis from Plackett-Burman design can be written as:

$$\mathbf{Y = 14.964 \quad 6.279 \times X_1 \quad 5.930 \times X_2 + 6.098 \times X_3 + 10.027 \times X_4 + 8.036 \times X_5} \qquad \mathbf{(5)}$$

where Y was the predicted response (efficiency of somatic embryogenesis) from the Placxkett-Burman design and X_1-X_5 were coded values of 2,4-D concentrations, treatment period, age of explants, sucrose in induction and in development medium respectively.

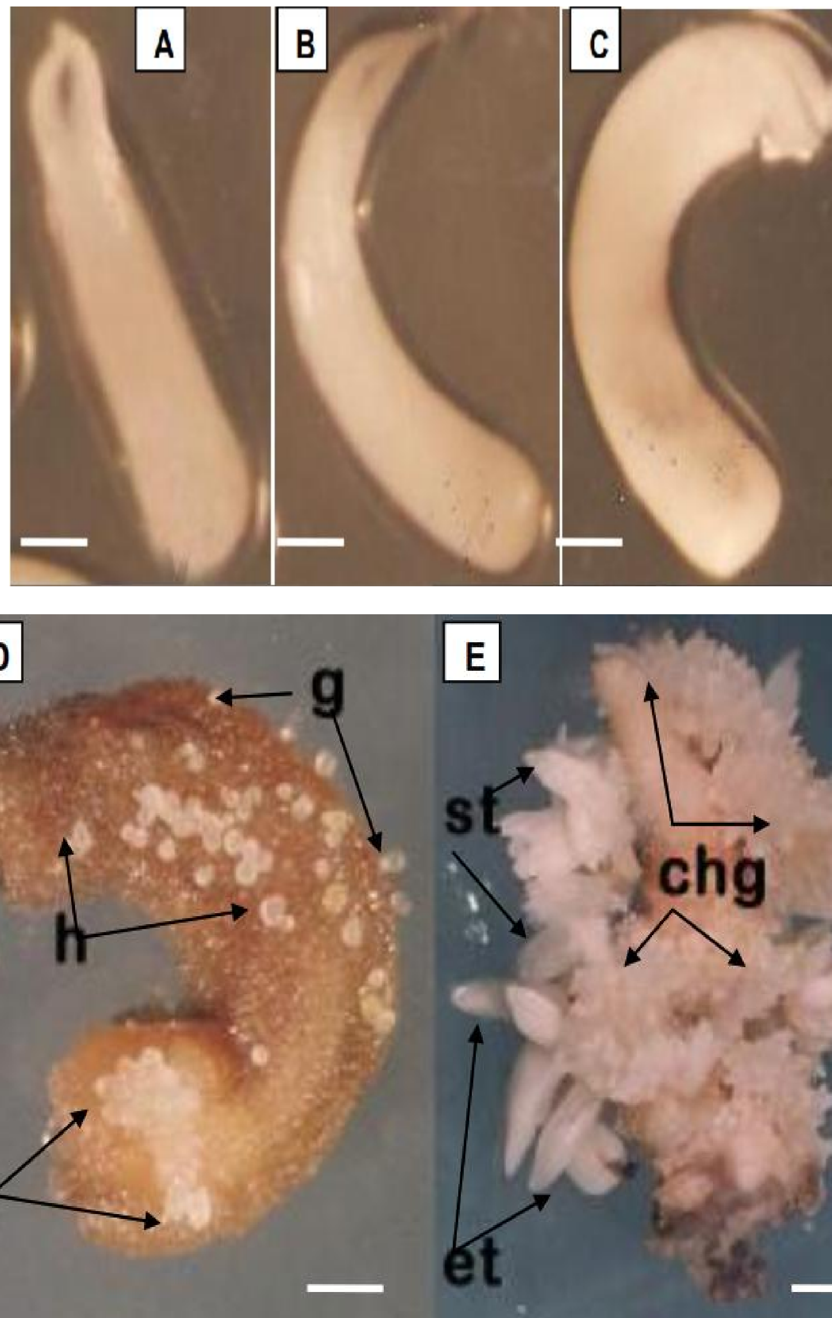


FIGURE 1: Somatic embryogenesis from immature zygotic embryo of guava (*Psidium guajava* L.). A, a zygotic embryo (8 weeks post-anthesis) (Scale bar = 1.0 mm). B, a zygotic embryo (10 weeks post-anthesis) (Scale bar = 1.0 mm). C, a zygotic embryo (12 weeks post-anthesis) (Scale bar = 1.0 mm). D, a zygotic embryo explant after 3 weeks of sub-culture on 5% (w/v) sucrose containing full-strength MS development medium following an initial 8- days treatment with 1.0 mg l^{-1} 2,4-D showing the development of globular, heart and cotyledonary stages somatic embryos (Scale bar = 0.75 mm). E, development of different-stages somatic embryos from entire hypocotyle region of a zygotic embryo explants at 10-weeks of culture initiation (Scale bar = 1.5 mm). g, globular-stage somatic embryo; h, heart-shaped somatic embryo; c, cotyledonary-stage somatic embryo; st, short torpedo-stage somatic embryo; et, elongated torpedo-stage somatic embryo

Box-Behnken model of somatic embryogenesis

The five significant variable *viz*., 2, 4-D concentrations, treatment period, age of explants, sucrose in induction and in development medium influencing somatic embryogenesis were further optimized using the Box-Behnken experimental design by generating a total of 46 experimental runs as shown in Table 4. The somatic embryogenesis responses for this entire design matrix were analyzed by Box-Behnken statistics and presented in Table 5.

TABLE 2. The Plackett-Burman experimental design matrix for the screening of the main factor affecting six embryogenesis parameters

Trial No.	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	FE (%)	IE (ANEPC)	ET (%)	ST (%)	CHG (%)	EE (Relative)
1	-1	-1	-1	-1	-1	-1	-1	-1	16.67	12.50	4.00	16.00	80.00	0.42
2	1	-1	1	-1	-1	-1	1	1	41.67	24.53	10.89	33.25	55.86	4.51
3	-1	1	-1	-1	1	-1	-1	1	66.67	39.82	7.63	22.36	70.01	7.96
4	1	-1	-1	1	-1	1	-1	1	58.33	56.73	6.16	23.34	70.5	9.76
5	1	1	-1	1	1	-1	1	-1	58.33	112.35	4.16	19.88	75.96	15.75
6	1	1	1	-1	1	1	-1	1	50.00	34.56	4.12	16.78	79.1	3.61
7	-1	1	-1	1	-1	1	1	1	75.00	41.26	9.06	27.44	63.5	11.29
8	-1	-1	1	1	1	-1	1	1	83.33	195.62	7.26	22.31	70.43	48.20
9	-1	-1	1	1	1	1	-1	-1	75.00	187.65	6.16	32.54	61.3	54.47
10	1	-1	-1	-1	1	1	1	-1	41.67	46.73	12.36	28.75	58.89	8.01
11	-1	1	1	-1	-1	1	1	-1	58.33	18.34	12.35	35.46	52.19	5.12
12	1	1	1	1	-1	-1	-1	-1	50.00	55.24	10.24	27.64	62.12	10.46

X_1= Coded values of 2,4-Dichlorophenoxy acetic acid; X_2= Coded values of Treatment Period; X_3= Coded values of Age of Explants; X_4= Coded values of Sucrose in induction medium; X_5= Coded values of Sucrose in development medium; X_6= Coded values of Glutamine; X_7= Coded values of NaCl; X_8= Coded values of PEG; ANEPC = average number of embryos produced perculture; FE = frequency of embryogenesis; IE = intensity of embryogenesis; ET = frequency of elongated stage somatic embryos; ST = frequency of short stage somatic embryos; CHG = frequency of lower stage somatic embryos and EE = efficiency of embryogenesis.

TABLE 3. Regression coefficients and their significances on efficiency of somatic embryogenesis (EE) from the results of the Plackett-Burman design.

Factors	Unstandardized Coefficient		Standardized Coefficient	*t*-ratio	*P*-value	Confidence level (%)
	Effect	Standard Error	Beta			
(Constant)	14.964	1.003		14.921	.001	99.9
X_1	-6.279	1.003	-.375	-6.261	.008	99.2
X_2	-5.930	1.003	-.354	-5.913	.010	99.0
X_3	6.098	1.003	.364	6.080	.009	99.1
X_4	10.027	1.003	.598	9.998	.002	99.8
X_5	8.036	1.003	.480	8.013	.004	99.6
X_6	.412	1.003	.025	.411	.709	29.1
X_7	.517	1.003	.031	.515	.642	35.8
X_8	-.740	1.003	-.044	-.737	.514	48.6

R= 0.995; R Squire= 0.989; Adjusted R Squire= 0.961; Std. Error of the Estimates= 3.4741

ANOVA (Efficiency of Embryogenesis)

Model	Sum of Squares	df	Mean Square	F	Sig.
Regression	3334.557	8	416.820	34.536	.007(a)
Residual	36.208	3	12.069		
Total	3370.765	11			

A quadratic second-order polynomial model was suggested (Table 6) by applying multiple regression analysis on the experimental data. The response variable and the independent variables were related based on the model equation 4, and the equation (in terms of coded factors) could be written as:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X^2_1 + \beta_{22} X^2_2 + \beta_{33} X^2_3 + \beta_{44} X^2_4 + \beta_{55} X^2_5 + \beta_{12} X_1X_2 + \beta_{13} X_1X_3 + \beta_{14} X_1X_4 + \beta_{15} X_1X_5 + \beta_{23} X_2X_3 + \beta_{24} X_2X_4 + \beta_{25} X_2X_5 + \beta_{34} X_3X_4 + \beta_{35} X_3X_5 + \beta_{45} X_4X_5 \quad \textbf{(6)}$$

where '*Y*' is predicted response; model constants; X1, X2, X3, X4, X5 independent variables; β_1, β_2, β_3, β_4, and β_5 are linear coefficients; β_{12}, β_{13}, β_{14}, β_{15}, β_{23}, β_{24}, β_{25}, β_{34}, β_{35} and β_{45} are cross product coefficients, and β_{11}, β_{22}, β_{33}, β_{44}, and β_{55} are the quadratic coefficients.. The statistical model was checked by Fisher's statistical test for the analysis of variance (ANOVA) for the response surface quadratic model and the result is summarized in Table 5. The Model F-value of 12.72 implies the model was significant. This indicated that the model was suitable for use in this experiment. Values of "Prob. > F" less than 0.0500 indicated model terms were significant. In this case X_1, X_4, X^2_1, X^2_2, X^2_3, X^2_4, and X^2_5 were significant model terms. The goodness of the model was further confirmed by a satisfactory value of determination coefficient (R^2), which was calculated to be 0.9105, indicating that 91.05% of the variability in the response could be predicted by the model. The value of the determination coefficient (Adj. R^2 = 0.8389) also confirmed that the model was significant. The lack-of-fit measures the failure of the model to represent data in the experimental domain at points which were not included in the regression. The "Lack of Fit F-value" of 0.52 implied the Lack of Fit was not significant relative to the pure error. The high value of lack of fit was good if we want the model to fit. The "Pred. R-Squared" of 0.7167 was in reasonable agreement with the "Adj R-Squared" of 0.8389. "Adeq. Precision" measured the signal to noise ratio. A ratio greater than 4 was desirable.

The ratio of 12.189 indicated an adequate signal. These results ensured that approximately 91% of the variability in the efficiency of somatic embryogenesis could be explained by this model and only 9% of the total variance could not be explained by the model. The quadratic regression equation obtained for efficiency of embryogenesis using all the terms regardless to their significance are included in the following equation:

Efficiency of Embryogenesis = $+ 233.37 \quad 15.79 * X_1 + 1.98 * X_2 + 4.50 * X_3 + 23.18 * X_4 + 12.94 * X_5 \quad 1.89 * X_1 * X_2 \quad 19.76 * X_1 * X_3 \quad 10.53 * X_1 * X_4 + 0.54 * X_1 * X_5 \quad 2.37 * X_2 * X_3 + 17.13 * X_2 * X_4 + 13.38 * X_2 * X_5 + 12.23 * X_3 * X_4 + 2.32 * X_3 * X_5 + 19.46 * X_4 * X_5 - 102.59 * X_1^2 \quad 83.94 * X_2^2 - 75.64 * X_3^2 \quad 86.30 * X_4^2 \quad 74.32 * X_5^2$ **(7)**

Interaction among the factors

The response surface (3D) plot is the graphical representation of the regression equation. The main aim of response surface is to efficiently hunt for the optimum value of the factors such that the response is maximized. Response surface curve is made for changes in the efficiency of somatic embryogenesis as a function of variations in two factors and other factors being at their constant levels. From the response surface plot, it is very easy and convenient to understand the interactions among the factor and also to locate their optimum values. The fitted response for the above regression model was plotted in figures 2. The 3D response surface plot for efficiency of embryogenesis was generated for two factors viz. 2,4-D and Treatment Period (Fig.2A), 2,4-D and Age of Explants (Fig. 2B), 2,4-D and Sucrose at Induction (Fig. 2C), 2,4-D and Sucrose at development (Fig. 2D), Treatment Period and Age of Explants (Fig. 2E), Treatment Period and Sucrose at Induction (Fig. 2F), Treatment Period and Sucrose at development (Fig. 2G), Age of Explants and Sucrose at Induction (Fig. 2H), Age of Explants and Sucrose at development (Fig. 2I) and Sucrose at Induction and Sucrose at development (Fig. 2J) while values of other variables were kept constant at the central point in each of the plot.

TABLE 4. Low, medium and high levels of variables and the corresponding real values used in the Box-Behnken design matrix for optimization of effect of 5 factors on six somatic embryogenesis parameters in guava (*Psidium guajava* L.)

Trial No.	Coded Values					Real Values					Experimental somatic embryogenesis response					
	X_1	X_2	X_3	X_4	X_5	x_1 (mg/l)	x_2 (days)	x_3 (Weeks)	x_4 (%)	x_5 (%)	FE (%)	IE (ANEPC)	ET (%)	ST (%)	CHG (%)	EE (Relative)
1	-1	-1	0	0	0	0.5	4	10	5	5	66.67	175.79	15.36	36.73	47.91	61.04906
2	0	0	1	0	1	1	8	12	5	7.5	75	321.46	12.54	34.58	52.88	113.604
3	-1	1	0	0	0	0.5	12	10	5	5	58.33	205.46	14.76	40.12	45.12	65.77084
4	0	0	1	1	0	1	8	12	7.5	5	75	376.54	7.83	32.47	59.7	113.8092
5	0	0	0	-1	1	1	8	10	2.5	7.5	66.67	88.76	16.53	32.44	51.03	28.97863
6	0	1	0	-1	0	1	12	10	2.5	5	58.33	98.75	17.65	36.77	45.58	31.3464
7	0	-1	0	0	1	1	4	10	5	7.5	58.33	219.88	13.46	36.75	49.79	64.39734
8	1	0	0	0	-1	1.5	8	10	5	2.5	58.33	113.49	15.56	38.99	45.45	36.1114
9	0	0	0	0	0	1	8	10	5	5	91.67	437.26	15.67	38.75	45.58	218.1351
10	-1	0	-1	0	0	0.5	8	8	5	5	58.33	126.46	18.72	42.38	38.9	45.06988
11	0	0	-1	0	1	1	8	8	5	7.5	75	243.16	15.67	39.83	44.5	101.2154
12	0	1	-1	0	0	1	12	8	5	5	83.33	235.62	13.67	41.28	45.05	107.89
13	1	0	0	1	0	1.5	8	10	7.5	5	83.33	165.23	4.13	22.34	73.53	36.44553
14	0	0	-1	1	0	1	8	8	7.5	5	83.33	297.75	6.67	22.23	71.1	71.70526
15	0	0	1	-1	0	1	8	12	2.5	5	58.33	132.39	15.44	39.27	45.29	42.24875
16	1	0	-1	0	0	1.5	8	8	5	5	66.67	143.28	14.52	27.85	57.63	40.47385
17	0	0	0	1	1	1	8	10	7.5	7.5	91.67	758.23	4.89	19.95	75.16	172.6552
18	-1	0	0	0	-1	0.5	8	10	5	2.5	66.67	215.67	16.67	37.85	45.48	78.39278
19	0	0	-1	0	-1	1	8	8	5	2.5	58.33	143.28	15.62	38.72	45.66	45.41478
20	-1	0	0	-1	0	0.5	8	10	2.5	5	58.33	63.44	18.24	44.45	37.31	23.19815
21	-1	0	0	0	1	0.5	8	10	5	7.5	75	204.51	12.78	36.79	50.43	76.03171
22	0	-1	0	-1	0	1	4	10	2.5	5	58.33	235.61	18.19	38.72	43.09	78.21216
23	0	0	1	0	-1	1	8	12	5	2.5	66.67	132.18	17.36	37.68	44.96	48.50367
24	0	0	-1	-1	0	1	8	8	2.5	5	58.33	142.39	17.82	41.26	40.92	49.06954
25	0	0	0	0	0	1	8	10	5	5	75	489.93	16.97	40.74	42.29	212.054
26	0	1	1	0	0	1	12	12	5	5	75	238.67	14.03	32.65	53.32	83.55837

27	0	1	0	0	-1	1	12	10	5	2.5	75	124.56	17.63	37.85	44.52	51.82942
28	0	-1	1	0	0	1	4	12	5	5	58.33	183.52	15.44	36.75	47.81	55.86794
29	0	-1	0	0	-1	1	4	10	5	2.5	50	281.26	16.56	42.85	40.59	83.54828
30	0	1	0	0	1	1	12	10	5	7.5	75	229.5	14.67	35.42	49.91	86.21741
31	0	-1	0	1	0	1	4	10	7.5	5	58.33	218.73	10.12	32.18	57.7	53.96854
32	1	0	0	-1	0	1.5	8	10	2.5	5	58.33	98.64	17.63	41.32	41.05	33.91789
33	1	1	0	0	0	1.5	12	10	5	5	58.33	112.37	12.35	35.42	52.23	31.31105
34	1	0	0	0	1	1.5	8	10	5	7.5	66.67	123.57	10.32	33.26	56.42	35.903
35	0	0	0	0	0	1	8	10	5	5	91.67	428.91	15.64	36.86	47.5	206.4204
36	0	0	0	0	0	1	8	10	5	5	83.33	543.29	13.98	35.67	50.35	224.7772
37	0	0	0	0	0	1	8	10	5	5	91.67	755.82	9.37	33.58	57.05	297.5835
38	0	0	0	0	0	1	8	10	5	5	91.67	488	16.65	37.28	46.07	241.2556
39	1	0	1	0	0	1.5	8	12	5	5	58.33	102.35	15.46	36.78	47.76	31.18767
40	1	-1	0	0	0	1.5	4	10	5	5	66.67	89.02	17.68	39.88	42.44	34.16165
41	-1	0	0	1	0	0.5	8	10	10	5	66.67	253.42	5.64	34.52	59.84	67.85237
42	0	0	0	1	-1	1	8	10	10	2.5	75	298.75	6.77	36.52	56.71	96.99666
43	-1	0	1	0	0	0.5	8	12	5	5	75	276.82	16.55	38.75	44.7	114.8111
44	0	0	0	-1	-1	1	8	10	2.5	2.5	66.67	78.99	18.75	40.44	40.81	31.17101
45	0	1	0	1	0	1	12	10	10	5	75	237.65	8.95	33.48	57.57	75.62617
46	0	-1	-1	0	0	1	4	8	5	5	58.33	226.57	15.67	37.85	46.48	70.73111

X_1= Coded values of 2,4-Dichlorophenoxy acetic acid; X_2= Coded values of Treatment Period; X_3= Coded values of Age of Explants; X_4= Coded values of Sucrose in induction medium; X_5= Coded values of Sucrose in development medium; x_1= Actual values of 2,4-Dichlorophenoxy acetic acid; x_2= Actual values of Treatment Period; x_3= Actual values of Age of Explants; x_4= Actual values of Sucrose in induction medium; x_5= Actual values of Sucrose in development medium; ANEPC = average number of embryos produced perculture; FE = frequency of embryogenesis; IE = intensity of embryogenesis; ET = frequency of elongated stage somatic embryos; ST = frequency of short stage somatic embryos; CHG = frequency of lower stage somatic embryos; EE = efficiency of embryogenesis.

TABLE 5: Analysis of variance (ANOVA) for the response surface quadratic polynomial model of efficiency of somatic embryogenesis in guava (*Psidium guajava* L.)

ANOVA for Response Surface Quadratic Model

Analysis of variance table [Partial sum of squares - Type III]

Efficiency of Embryogenesis Response

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	177096	20	8854.802	12.71631	< 0.0001	significant
X_1: 2,4-D	3989.938	1	3989.938	5.729918	0.0245	
X_2: Treatment period	62.4636	1	62.4636	0.089703	0.767	
X_3: Age of explant	324.1883	1	324.1883	0.465564	0.5013	
X_4: Sucrose at induction	8598.689	1	8598.689	12.34851	0.0017	
X_5: Sucrose at development	2678.959	1	2678.959	3.847231	0.0611	
X_1X_2	14.33523	1	14.33523	0.020587	0.8871	
X_1X_3	1561.332	1	1561.332	2.242216	0.1468	
X_1X_4	443.6623	1	443.6623	0.63714	0.4323	
X_1X_5	1.158496	1	1.158496	0.001664	0.9678	
X_2X_3	22.41299	1	22.41299	0.032187	0.8591	
X_2X_4	1173.864	1	1173.864	1.685776	0.206	
X_2X_5	716.6045	1	716.6045	1.02911	0.3201	
X_3X_4	598.4076	1	598.4076	0.859368	0.3628	
X_3X_5	21.62119	1	21.62119	0.03105	0.8615	
X_4X_5	1515.194	1	1515.194	2.175957	0.1527	
X^2_1	91855.16	1	91855.16	131.9124	< 0.0001	
X^2_2	61494.55	1	61494.55	88.31181	< 0.0001	
X^2_3	49926.65	1	49926.65	71.69926	< 0.0001	
X^2_4	64996.46	1	64996.46	93.34088	< 0.0001	
X^2_5	48202.43	1	48202.43	69.22312	< 0.0001	
Residual	17408.36	25	696.3343			
Lack of Fit	11736.22	20	586.8108	0.517275	0.8665	not significant
Pure Error	5672.142	5	1134.428			
Cor Total	194504.4	45				

Std. Dev.	26.38815	R-Squared	0.910499
Mean	86.31476	Adj R-Squared	0.838898
C.V. %	30.572	Pred R-Squared	0.71665
PRESS	55112.75	Adeq Precision	12.1895

TABLE 6: Multiple regression models for efficiency of embryogenesis in guava (*Psidium guajava* L.).

A: Sequential Model Sum of Squares [Type I]

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Mean vs. Total	342710.9	1	342710.9			
Linear vs. Mean	15654.24	5	3130.848	0.700217	0.6265	
2FI vs. Linear	6068.592	10	606.8592	0.105369	0.9996	
Quadratic vs. 2FI	155373.2	5	31074.64	44.62603	< 0.0001	Suggested
Cubic vs. Quadratic	10852.8	15	723.5203	1.103675	0.4491	Aliased
Residual	6555.555	10	655.5555			
Total	537215.3	46	11678.59			

B: Lack of Fit Tests

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Linear	173178	35	4947.943	4.361618	0.0528	
2FI	167109.4	25	6684.377	5.892287	0.0286	
Quadratic	11736.22	20	586.8108	0.517275	0.8665	Suggested
Cubic	883.413	5	176.6826	0.155746	0.9689	Aliased
Pure Error	5672.142	5	1134.428			

C: Model Summary Statistics

Source	Std. Dev.	R-Squared	Adjusted R-Squared	Predicted R-Squared	PRESS	
Linear	66.86743	0.080483	-0.03446	-0.0342	201156.2	
2FI	75.89061	0.111683	-0.33248	-0.25529	244159.7	
Quadratic	26.38815	0.910499	0.838898	0.71665	55112.75	Suggested
Cubic	25.60382	0.966296	0.848332	0.667327	64706.32	Aliased

The final regression equation in terms of actual factors on the efficiency of embryogenesis as presented in table 7 were used for validation of the developed model.

TABLE 7: Final regression equation for efficiency of embryogenesis (EE) in terms of coded and actual factors in guava (*Psidium guajava* L.).

Coefficient Estimate	Coded Factors	Actual Factors
+233.371	Intercept	
-15.7915	* X_1	* 2,4-D
+1.975848	* X_2	* Treatment Period
+4.501307	* X_3	* Age of Explant
+23.18228	* X_4	* Sucrose at Induction
+12.93967	* X_5	* Sucrose at development
-1.89309	* X_1 * X_2	* 2,4-D * Treatment Period
-19.7568	* X_1 * X_3	* 2,4-D * Age of Explant
-10.5316	* X_1 * X_4	* 2,4-D * Sucrose at Induction
+0.538167	* X_1 * X_5	* 2,4-D * Sucrose at development
-2.36712	* X_2 * X_3	* Treatment Period * Age of Explant
+17.13085	* X_2 * X_4	* Treatment Period * Sucrose at Induction
+13.38473	* X_2 * X_5	* Treatment Period * Sucrose at development
+12.23119	* X_3 * X_4	* Age of Explant * Sucrose at Induction
+2.324929	* X_3 * X_5	* Age of Explant * Sucrose at development
+19.46274	* X_4 * X_5	* Sucrose at Induction * Sucrose at development
-102.592	* X^2_1	* $(2,4\text{-D})^2$
-83.9419	* X^2_2	* $(\text{Treatment Period})^2$
-75.6357	* X^2_3	* $(\text{Age of Explant})^2$
-86.299	* X^2_4	* $(\text{Sucrose at Induction})^2$
-74.3182	* X^2_5	* $(\text{Sucrose at development})^2$

Validation of the model

By solving the equation (7) using statistical software a total of 71 point solution have been generated. When a numerical and graphical optimization program is run within the tested range, the optimum values of the variables are found as 1.0 mg/l of 2, 4-D, 8- weeks treatment period, 10-weeks post-anthesis age of zygotic embryo explants, 5% sucrose in induction and 5% sucrose in development medium. With these levels the model have predicted an optimum of 87.50% frequency of embryogenesis (FE), 523.87 average number of embryos per explants per culture (ANEPC) intensity of embryogenesis (IE), 14.71% frequency of elongated torpedo stages (ET), 36.27% frequency of short torpedo stages (ST), and 48.14% frequency of lower stages (CHG) somatic embryos resulting in 233.37 relative efficiency of embryogenesis (EE). While on experimental verification a maximum of 91.67% (FE), 755.82 (ANEPC) (IE), 9.37% (ET), 33.58% (ST), 57.05% (CHG) and 297.58 (Relative) (EE) of response have been produced indicating that a reasonably significant variability of the test variables can be explained by the model generated in the present study.

Genetic Fidelity:

Initially 10 RAPD primers (Operon Technologies Inc., Germany) were selected for screening with the mother as well as regenerated plants of *P. guajava* and all of them gave clear and reproducible bands. The number of scorable bands for each RAPD primer varied from 3 (OPA-6) to 11 (OPA-4) (Table 8). The 10 RAPD primers produced 67 distinct and scorable bands, with an average of 6.7 bands per primer. No polymorphism was detected during the RAPD analysis of plantlets raised via somatic embryogenesis (Figure 3A). All six ISSR primers produced clear and reproducible bands. The number of scorable bands for each primer varied from 6 (ISSR-01) to 13 (ISSR-06), with an average of 10.3 bands per primer. The banding profiles of all the randomly selected plantlets from somatic embryogenesis were monomorphic and similar to those of the mother plant (Figure 3B).

Thus the model can be used for highest efficiency of somatic embryogenesis and true to mother type plants production by treating 10-weeks post-anthesis zygotic embryo explants for a maximum of 8-days with 1.0 mg/l of 2, 4-D in full strength MS medium supplemented with 5.0% sucrose at both induction and development phase. By present optimization model an efficient somatic embryogenesis system has been developed for cost effective large scale micropropagation of the genetically uniform plants of guava (*Psidium guajav* L.) compared to earlier reports using one-at-a time factor testing (Akhtar 2010, 2013a, b; Rai *et al*., 2007).

TABLE 8. List of RAPD primers used to verify the genetic fidelity of plantlets regenerated through somatic embryogenesis in guava

Sl. No.	Name of primers	Primer sequence (5´– 3´)	Number of scorable bands
1	OPA-01	CAGGCCCTTC	6
2	OPA-02	TGCCGAGCTG	4
3	OPA-03	AGTCAGCCAC	8
4	OPA-04	AATCGGGCTG	11
5	OPA-05	AGGGGTCTTG	3
6	OPA-06	GGTCCCTGAC	3
7	OPA-07	GAAACGGGTG	4
8	OPA-08	GTGACGTAGG	8
9	OPA-09	GGGTAACGCC	9
10	OPA-10	GTGATCGCAG	11

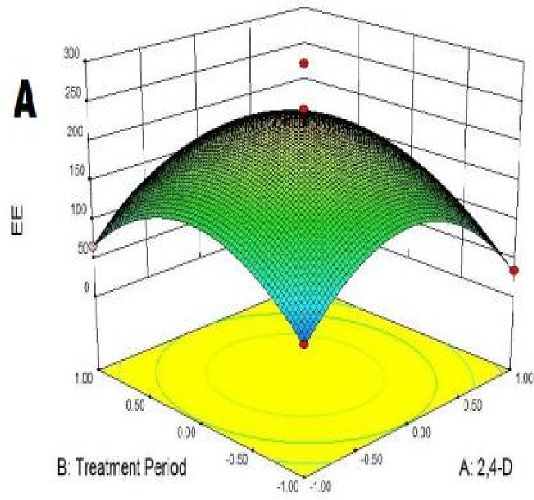
TABLE 9. List of ISSR primers used to verify the genetic fidelity of plantlets regenerated through somatic embryogenesis in guava

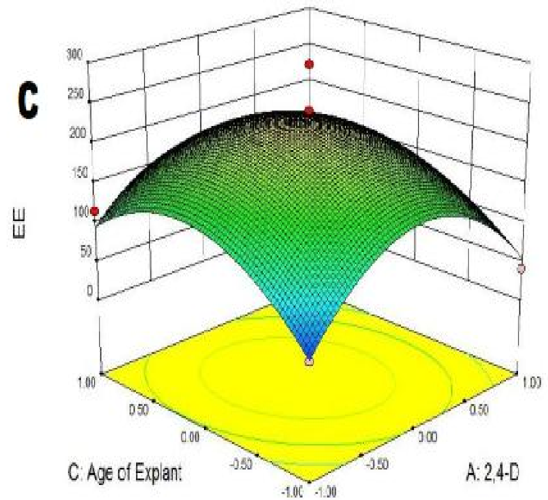
Sl. No.	Name of primers	Primer sequence (5 – 3)	Annealing Temp. (°C)	Number of scorable bands
1	ISSR-01	ACA CAC ACA CAC ACA CT	45.7	6
2	ISSR-02	ACA CAC ACA CAC ACA CG	49.0	8
3	ISSR-03	AGA GAG AGA GAG AGA GYT	49.0	12
4	ISSR-04	GAG AGA GAG AGA GAG AYC	49.0	12
5	ISSR-05	ACA CAC ACA CAC ACA CYT	49.0	11
6	ISSR-06	DBD ACA CAC ACA CAC AC	45.7	13

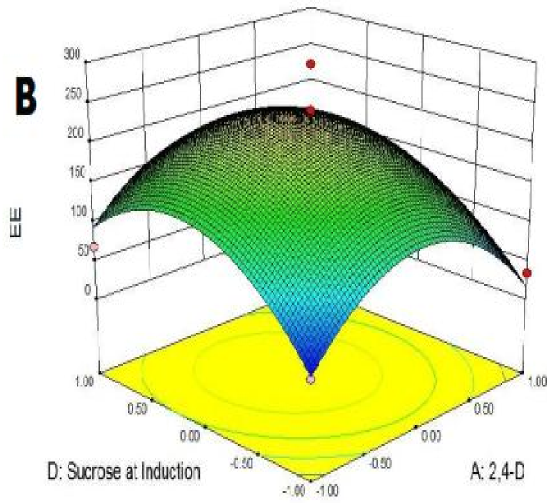
DISCUSSION

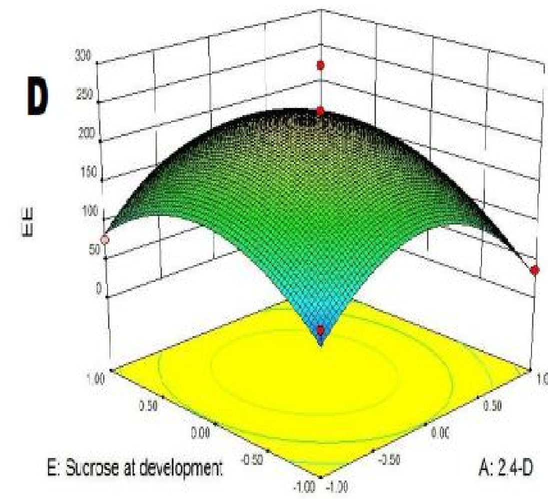
Somatic embryogenesis has been increasingly applied strategy for commercial micropropagation of plants (Jain and Gupta 2005). There is now increasing evidences that embryogenic capability of somatic plant cells exhibit a rather general feature but the appropriate conditions allowing the expression of this trait greatly varies (Fehér 2006). The factors used to induce *in vitro* embryogenesis in somatic plant cells are highly variable ranging from various plant hormones to stress treatments (Feher *et al*., 2003). In guava the one-at-times strategies for somatic embryogenesis have induces low level of response in continuous presence of 0.1 mgl^{-1} of 2,4-D (Akhtar, 2010) with slightly enhanced response in 8-days treatment with 0.5 mgl^{-1} of 2,4-D from zygotic embryo explants (Akhtar 2013a, b). The Plackett-Burman experimental design and the Box-Behnken experimental design used in the present study have induced 5 times higher efficiency of somatic embryogenesis response in the 8-days treatment from 10-week old zygotic embryo explants with a higher concentrations of 2,4-D (1.0 mgl^{-1}) and sucrose (5% w/v) both at induction and development medium. These results have clearly demonstrated that Plackett-Burman and Box-Behnken experimental design used in the present study have optimized three additional factors under considerations with much higher efficiency of somatic embryogenesis response. In many systems, following the induction, somatic embryo development proceeds under auxin free conditions (Akhtar 2013a, b; Dudits *et al*., 1991) which indicates that the cells become capable for self-supporting auxin synthesis or independent of auxins. Explants from some other species, such as grapevine, do not form somatic embryos until they are transferred from induction medium containing 2,4-D to medium without 2,4-D (Jayasankar et al. 1999). Sa´nchez *et al*. (2005) stated that 2.3 µM 2,4-D induced somatic embryos from immature zygotic embryos of *Quercus suber,* and further embryo development occurred on growth regulator-free medium. Underlying embryo development and maturations following an initial induction is established by setting up of an auxin gradient and polar transport for proper root and shoot meristem differentiation (Nawy *et al*., 2008). A high auxin dose and/or sublethal stress evokes the activation of large chromatin regions responsible for the developmental program leading to embryogenesis (Fehér, 2006). This hypothesis may explain why less differentiated cells (*e.g*. immature embryos) are more amenable for somatic embryogenesis and why various specific signals can induce similar embryogenic response.

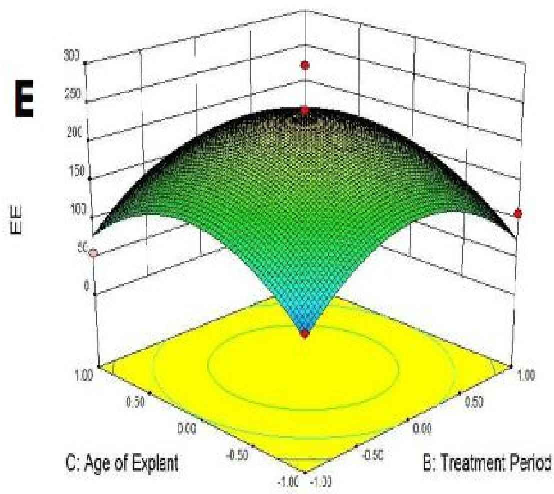
In *Acca sellowiana* the supplementation of Glutamine, Aspergine and Arginine to the culture medium enhances the rate of somatic embryogenesis induction (Dal Vesco and Guerra, 2001). Addition of glutamine in the medium has not shown any significant (Table 2) effect on somatic embryogenesis in the present study. Similarly, exogenous application of polyamines has not indicated any significant improvement of guava somatic embryogenesis (Akhtar 2013a, b). While, glutamine and casein hydrolysate have been used to promote embryogenesis in *Eucalyptus* (Pinto *et al*., 2002). An increase in the total amino acids during induction phase in the presence of 2,4-D and subsequent decrease in the course of *A. sellowiana* somatic embryo development may be related to the embryo specific protein synthesis suggested by Fehér *et al.* (2003). Similarly, in guava induction of somatic embryogenesis by 2, 4-D has been regulated by temporal modulation of endogenous polyamine metabolism involved in the synthesis of proteins specific for embryo development (Akhtar, 2013a, b).

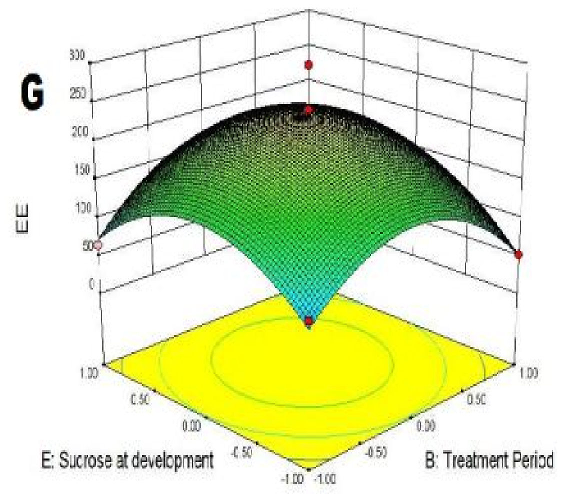

A
EE
B: Treatment Period
A: 2,4-D


C
EE
C: Age of Explant
A: 2,4-D


B
EE
D: Sucrose at Induction
A: 2,4-D


D
EE
E: Sucrose at development
A: 2,4-D


E
EE
C: Age of Explant
B: Treatment Period


G
EE
E: Sucrose at development
B: Treatment Period

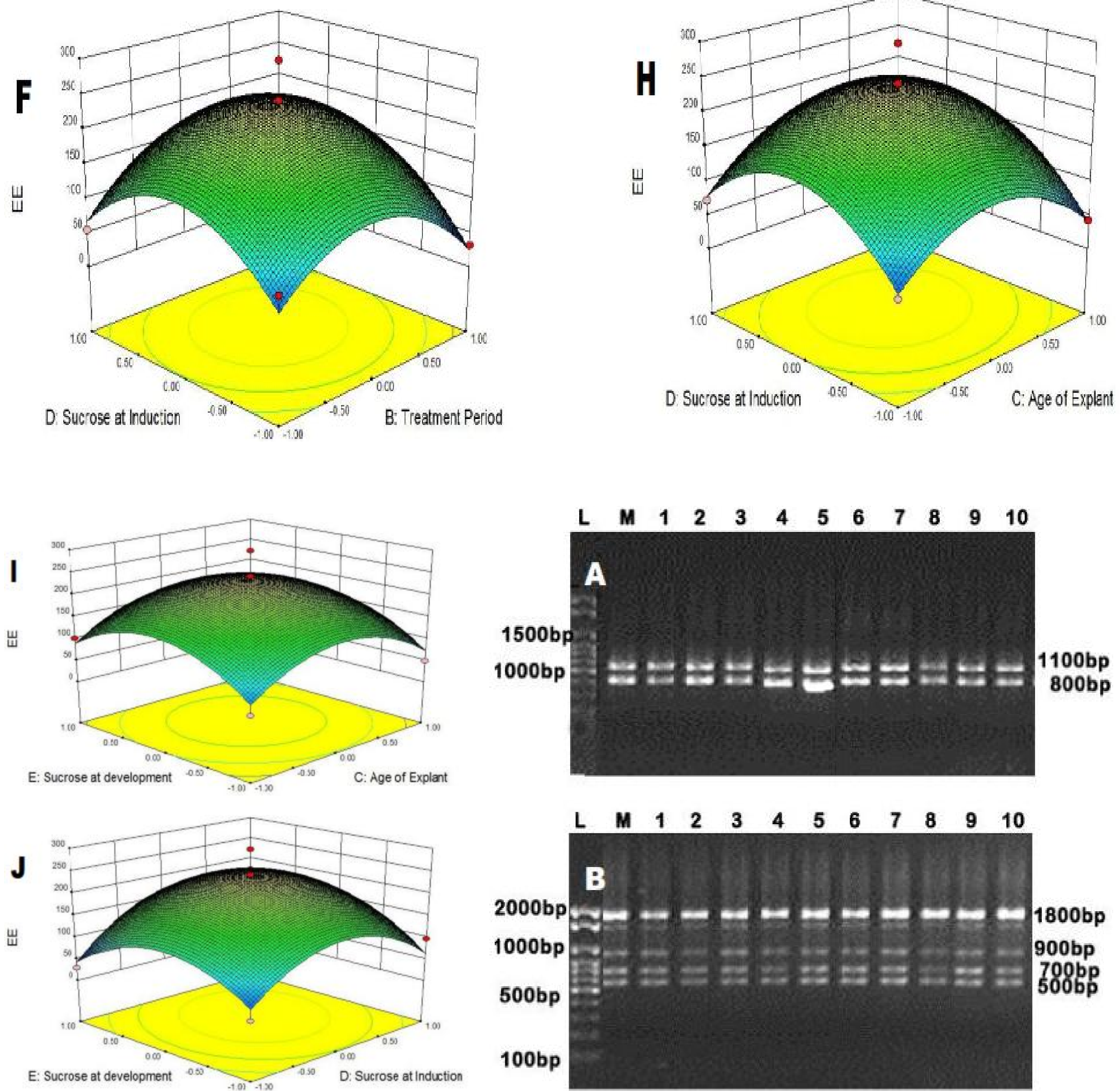


FIGURE 2: Response surface plot of efficiency of embryogenesis showing the interactive combined effects of 2,4-D and Treatment Period (Fig. 2A), 2,4-D and Age of Explants (Fig. 2B), 2,4-D and Sucrose at induction (Fig. 2C), 2,4-D and Sucrose at development (Fig. 2D), Treatment Period and Age of Explants (Fig. 2E), Treatment Period and Sucrose at Induction (Fig. 2F), Treatment Period and Sucrose at development (Fig. 2G), Age of Explants and Sucrose at Induction (Fig. 2H), Age of Explants and Sucrose at development (Fig. 2I) and Sucrose at Induction and Sucrose at development (Fig. 2J) while the concentration of other variable was kept constant at the central point in each of the plot.

FIGURE 3: Polymerase chain reaction (PCR) amplification profile with (A) random amplified polymorphic DNA (RAPD) primer (OPA-02) and (B) inter simple sequence repeat (ISSR) primer (ISSR-03). L represents 100-bp ladder; M represents the mother plant and lane 1–10 represent randomly selected plantlets raised through response surface optimization of somatic embryogenesis process.

The carbohydrate plays an important nutritional role in growth, development and maturation of somatic embryos (Scott and Lyne 1994; Reidiboym-Talleux *et al*., 1999). Sucrose serves as carbon and energy source for explants and balances osmotic pressure during expression of somatic embryogenesis (Jain and Gupta 2005; Nuutila *et al.*, 2002). Types and concentration of carbon sources have a strong effect on increasing the embryogenic expression (Cheong and Pooler 2004; Jain *et al*., 2000). Additionally, somatic embryogenesis can be induced by treatment with high osmotic stress and high temperature under a 2,4-D-free condition in carrot (Kikuchi *et al*., 2006). The much enhanced efficiency of the process at higher concentration of 2,4-D (1.0mg/l) with high level of sucrose concentration (5% w/v) both at induction and development medium from immature zygotic embryo (10-week-old) explants in the present study supports the above finding in affirmation. Hence, the 5 significant factors identified by

Plackett-Burman design has been further tested following Box-Behnken design to analyzed their interactions resulted in 5 times higher efficiency of somatic embryogenesis in present study.

The banding profiles of all the randomly selected plantlets from somatic embryogenesis were monomorphic and similar to those of the mother plant as revealed from RAPD and ISSR markers (Figure 3A, B). Working with grapevine, Khawale *et al*. (2006) have also reported the application of RAPD analysis using 30 decamer primers for adjudging clonal fidelity. Absence of genetic variation using the RAPD marker system has been reported in several cases such as somatic embryogenesis-derived plantlets of oil palm (Rival *et al*., 1998), sweet potato (Sharma *et al*., 2004). During ISSR analysis, all six ISSR primers showed monomorphic banding pattern*s* within somatic embryogenesis raised clones and with their respective mother plant. Analysis of in vitro derived plantlets of guava (*Psidium guajava*) by Rai *et al.* (2012) and Liu, X. and Yang, G. (2012) support the results obtained in the present study. They have detected genetic homogeneity and no variability among the plantlets of guava derived from in vitro cultures using ISSR as one of the molecular markers. The number of bands generated per primer was greater in ISSR (10.3) than RAPD (6.7). These differences could possibly be due to the high melting temperature for the ISSR primers, which permits much more stringent annealing conditions and, consequently, more specific and reproducible amplification. Devarumath *et al*. (2002) also revealed that ISSR fingerprints detected more polymorphic loci than RAPD fingerprinting. The genetic fidelity in tissue culture raised plant using RAPD and ISSR markers has been reported in grapevine (Nookaraju and Agrawal 2012), *Populus deltoides* (Rani *et al*., 1995), *Simmondsia chinensis* (Kumar *et al*., 2011). These results corroborate with the earlier reports on genetic stability of plantlets regenerated via somatic embryogenesis plantlets derived plantlets of banana (Lakshmanan *et al*., 2007), almond (Martin *et al*., 2004) and in *Swertia chirayita* (Joshi and Dhawan, 2007).

By validation of present response surface model it has been concluded that for optimum somatic embryogenesis response in guava (*Psidium guajav* L.), the zygotic embryo explants dissected from 10-weeks post-anthesis fruits need to be treated for 8-days with 1.0 mg/l of 2, 4-D in full strength MS medium which is supplemented with 5.0% sucrose at both induction and development phase. The RAPD and ISSR markers used to evaluate the genetic homogeneity of guava plants regenerated through somatic embryogenesis suggest that response surface methodology protocol can be used for large scale micropropagation of guava with less risk of genetic variability and can be applied in the other species also.

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