Research in Plant Sciences, 2013, Vol. 1, No. 2, 4-14
Available online at http://pubs.sciepub.com/plant/1/2/1

DOI:10.12691/plant-1-2-1

Endogenous Polyamines: A Temporal Cellular Modulator of Somatic Embryogenesis in Guava (*Psidium guajava* L.) cv. Allahabad Safeda

Nasim Akhtar*

Department of Biotechnology, GITAM Institute of Technology, GITAM University, Gandhi Nagar Campus, Rushikonda, India
*Corresponding author: nasimakhtar01@yahoo.co.in

Received December 27, 2012; Revised April 26, 2013; Accepted April 27, 2013

Abstract Somatic embryogenesis was improved in guava (*Psidium guajava* L.) cv. Allahabad safeda by periodic subculture of zygotic embryos explants (10-weeks post-anthesis) onto 3% (w/v) sucrose containing plant growth regulator free full-strength Murashige and Skoog's agar-solidified medium following an initial induction in the presence of 2,4-dichlorophenoxy acetic acid (2,4-D). The interaction of 2,4-D concentrations and treatment durations showed significant effects on six different embryogenesis parameters viz. (i) frequency of embryogenesis, (ii) intensity of embryogenesis, (iii) frequency of elongated torpedo-stage somatic embryos, (iv) frequency of short torpedo-stage somatic embryos, (v) frequency of lower- (cotyledonary, heart, and globular) stages somatic embryos, and (vi) efficiency of embryogenesis. The embryogenesis responses were shifted gradually from 0.01 mgl^{-1} to 0.5 mgl^{-1} concentrations 2,4-D in a temporal manner. An 8-days treatment of zygotic embryo explants with 0.5 mgl^{-1} 2,4-D induced somatic embryogenesis in highest efficiency. The role of exogenous and endogenous concentration of total, free, conjugated, and bound forms of various polyamines viz. putrescine, spermidine and spermine were studied and discussed in regulation of somatic embryogenesis as a function of 2,4-D concentration. The present study on guava (*P. guajava* L.) cv. Allahabad safeda indicated that temporal regulation of somatic embryogenesis by 2,4-D was modulated by polyamines metabolism.

Keywords: *guava, polyamines, somatic embryogenesis, zygotic embryo*

1. Introduction

Somatic embryogenesis is characterized by the production of true-to-mother type plants [1], accumulate embryo-specific proteins and mRNAs [2,3]. The process has been applied in cell and tissue culture systems for large scale production of plants, synthetic seeds, protoplast culture, somatic hybridization, cryopreservation, inducing variability and genetic transformation in different plant species [4,5]. Somatic embryogenesis have been reported from zygotic embryos as starting explants material in several species including guava [6,7,8,9,10], *Acca sellowiana* [11], *F. sellowiana* [12], *Myrtus communis* [13] and other leguminous tree species [14] and conifers [15]. Despite the successful protocols on induction and/or enhancement of somatic embryogenesis in different species [5], the regulatory mechanisms are still far from realization to generalize the process. Plant growth regulators (PGRs) especially auxins (mainly 2,4-D) is most often associated with cell division, polar transport and production of organized cell clusters leading to somatic embryo development [16]. Periodic sub-culture of explanted tissues onto auxin-free development medium leads to the normal development of somatic embryos [15,17].

Extensive studies of polyamines in various herbaceous and woody plant species showed that they might play a regulating role in somatic embryogenesis and other developmental processes [18,19]. Polyamines in plants are present as free (free cations), as conjugated (acid-soluble forms conjugated with phenolics, hydroxycinnamic acids, and other low-molecular-weight compounds), and as bound (acid-insoluble forms bound covalently to macromolecules and cell walls) [20]. Numerous reports have shown a good correlation between polyamine levels and a variety of fundamental processes such as macromolecular biosynthesis, cell division, cell and tissue differentiation, organogenesis, and embryogenesis [21,22,23]. Some investigators have suggested that polyamines viz. spermidine, spermine and putrescine are either essential as PGRs or secondary messenger in signaling pathways [24,25]. Polyamines play a crucial role during somatic embryogenesis in several important plants like *Arabidopsis sp. [26]*, *Momordica charantia* [27], *Picea abies* [28] and *Vitis vinifera* [29]. In the present study an efficient somatic embryogenesis system for rapid regeneration of guava (*Psidium guajava* L.) cv. Allahabad safeda by periodic subculture of zygotic embryo explants to PGR-free medium following an initial treatment with 2,4-D have been established. The stimulatory role of 2,4-D on temporal modulation of various polyamines regulating the process have been correlated and discussed for induction, differentiation, development and maturation

of somatic embryos. The study may be helpful in developing the regeneration protocol through somatic embryogenesis in other recalcitrant fruit trees.

2. Materials and Methods

Table 1. Effect of 2,4-D on induction of somatic embryogenesis from zygotic embryo explants of guava (Psidium guajava L.) cv. Allahabad safeda

Treatment 2,4-D (mg l^{-1}) [§]	Frequency of embryogenesis	Intensity of embryogenesis	Frequency of various stages of somatic embryos			Efficiency of embryogenesis
	FE (%)[†]	IE (ANEPC)[†]	ET (%)[†]	ST (%)[†]	CHG (%)[†]	EE (Relative)[†]
0.000	0	0	0	0	0	0
0.001	0	0	0	0	0	0
0.005	0	0	0	0	0	0
0.010	0	0	0	0	0	0
0.050	22.23 ±6.36	22.33 ±5.80	21.27 ± 2.26	42.09 ± 3.67	36.64 ± 5.39	3.19 ±1.46
0.100	51.39 ±6.36	48.13 ± 13.27	18.34 ± 2.13	39.30 ± 3.58	42.36 ± 5.54	14.52 ±5.89
0.500	80.56 ±6.36	149.12 ± 29.29	14.90 ± 2.27	36.38 ± 2.04	48.72 ± 4.30	61.23 ± 11.32
1.000	68.06 ±4.82	66.64 ±6.13	10.46 ± 2.05	35.51 ± 3.61	54.03 ± 5.45	20.83 ±3.20
1.500	40.28 ±4.82	31.77 ± 10.63	9.18 ± 0.95	28.76 ± 2.37	62.06 ± 3.30	5.11 ±2.76
2.000	26.39 ±9.62	22.25 ±8.12	2.67 ± 1.17	19.10 ± 5.13	78.23 ± 4.79	1.29 ±0.71
3.000	0	0	0	0	0	0
df (10, 22), $P \leq 0.05$	113.92	52.62	104.63	149.28	208.94	61.29

[§]Zygotic embryos (10 weeks post-anthesis) of guava were treated for 8-days with different concentrations of 2,4-D in full strength MS agar medium added with 3% (w/v) sucrose. Development and maturation of somatic embryos were observed following sub-culture after 8-days to similar 3% (w/v) sucrose containing full-strength MS agar medium but free of growth regulators. Embryogenic responses were noted 10-weeks after culture initiation.

[†]Mean values represent average of the three independent experiments ± standard deviations.

[#]ANEPC, average number of embryos per culture; CHG, cotyledonary, heart, globular stage somatic embryos; ET, elongated torpedo stage somatic embryos; ST, short torpedo stage somatic embryos; 2,4-D, 2,4-dichlorophenoxy acetic acid.

The guava genotypes Allahabad safeda (10-15-year-old tree) was tagged during the experimental period at Doddaballapur area of Bangalore district. The flower buds at fully matured state or half opened state were bagged and tagged to ensure the self pollination and physiological age of the zygotic embryo explants (Figure 1A) at the time of culture establishment. Fresh fruits were collected after 10-weeks of anthesis from one and the same plants for the three consecutive years of experiments. Surface disinfection, sterilization and dissection of zygotic embryo explants were carried out following the protocol of [6,7,8,9,10]. Murashige and Skoog (MS) [30] basal salts was selected for preparation of medium as in [6,7,8,9,10]. As per the need of the experiment various media were supplemented with 0%, 3% (w/v) sucrose concentrations. The media were modified with 0.0, 0.001, 0.005, 0.01, 0.05, 0.1, 0.5, 1.0, 1.5, 2.0 and 3.0 mgl^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) prior to autoclaving.

Zygotic embryo explants (10-weeks post-anthesis) treated with 2,4-D for 60-days were regarded as continuous treatment. Subsequently different durations of pulse treatment were provided by sub-culturing of explants to embryo development medium (full strength MS basal with 3% (w/v) sucrose) after 2-, 4-, 6-, 8-, 10-, 12-, 14-, 18-, 28-, and 38-days of inductive treatment with various concentrations of 2,4-D in 3% (w/v) sucrose containing full strength MS induction medium. A total of 121 combination permutations of treatment period and 2,4-D concentrations were used for these optimizations. Different polyamines viz. putrescine, spermidine and spermine were added either to induction medium alone or to both induction and development media at 0.0, 0.25, 0.50, 1.0, 2.0 and 4.0mM concentrations. All the experiments were repeated in three subsequent years almost at the similar reproductive growth periods of the plants in order to minimize the seasonal variations. Germination of somatic embryos was achieved on half strength MS agar-medium with 3% (w/v) sucrose without any PGR. Somatic plantlets were grown for a short period of 2-weeks in full strength MS liquid medium with 3% (w/v) sucrose. Well grown plantlets from the above medium were acclimatized initially in more porous mixture of sand:soil:compost in a ratio of 5:1:2 (w/w/w) followed by hardening in 1:2:3 (w/w/w) ratios of above mixture.

Sterilization of all the media and other requirement were performed by autoclaving at 121 ℃ and 1.1kg cm^{-2} pressure for 15min. Cultures were incubated in an air-conditioned culture room maintained at 25 ℃ ± 2 ℃ temperature, 60-65% relative humidity, and 16h photoperiod at a photon flux density (PFD) of 50-70µmol m^{-2} s^{-1} provided by 40W white fluorescent tubes from a distance of 30-35cm.

2.1. Analysis of Polyamines

The zygotic embryo explants were treated for 8-days in the presence 0.5 mgl^{-1} 2,4-D followed by sub-culture to PGR free 3% (w/v) sucrose containing MS agar-medium. Different forms (i.e. free, conjugated and bound) of cellular polyamines (putrescine, spermidine and spermine) were analyzed from 24- zygotic embryos per treatments after 2-, 4-, 6-, 8-, 10-, 12-, 14-, 18-, 28-, 38-, and 60-days of culture initiation.

2.1.1. Extraction and Dansylation of Polyamines

Extraction of polyamines was carried out by grinding the sample of 24- zygotic embryos in a small mortar with 1 ml of cold 5% (w/v) trichloro-acetic acid (TCA) and centrifuged at 1,500 × g for 15 min. The supernatant, containing free (acid soluble) and bound (acid soluble) polyamines, and the pellet (bound acid-insoluble) polyamines were separated. The pellet and 50µl of the supernatant were hydrolyzed in separate sealed vials with 300µl 12 M HCl for 20h each at 100 ℃ to analyze the bound polyamine fraction. Dansylation of polyamine was carried out according to [31,32] with slight modifications. All samples were filtered, dried, and re-dissolved in 300µl 5% (w/v) TCA for dansylation. Forty-five µl each sample was mixed with 45µl of a saturated solution of Na_2CO_3

and 90µl dansyl chloride (5mg ml^{-1} in acetone). Dansylation was carried out for 10 mins at 70 ℃. An aqueous proline solution (25µl of 100mg ml^{-1}) was added to react for 30 mins with the excess of dansyl chloride in the dark. When the reaction was completed, 500µl toluene was mixed with each sample, shaken well and left until the two phases (organic and aqueous) separated. The organic phase, containing the polyamines, was transferred to a fresh vial, dried in a heat-speed vacuum drier (Concentrator 5301, Eppendorf AG- Germany) and the residue dissolved in 500 µl acetone.

2.1.2. Separation and Quantification of the Polyamines

The dansylated polyamines were separated with TLC on 7.5cm x 2.5cm plates (300µm in thickness, Silica gel 60, E. Merck, Darmstadt, Germany). The mobile phase used was ethyl acetate: cyclohexane (3:5, v/v) mixture. The chromatography performed for about 15min on closed recipients. The plates were revealed under UV light (254nm), so the putrescine (Rf 3.2cm), spermidine (Rf. 1.9cm) and spermine (Rf under the front) bands were visible by identification of bands with those of pure standards (Sigma) treated in the same way. The bands were scraped off from the plate and re-dissolved with 750 µl of acetone. The sample were shaken and centrifuged at 6000×g for 3min, and quantified at 365nm (excitation) and 510nm (emission) with a high-resolution spectrophotofluorometer (model RF-540, Shimadzu, Tokyo, Japan).

2.2. Data Analyses and Evaluation of Somatic Embryogenesis

Completely randomized designed were used for each and every experiments. For all the above described treatments 12, 15, 18, or 24 zygotic embryos explants samples were used and each experiment was repeated to a minimum of 3-times over 3-cosecutive years. The evaluation of somatic embryogenesis was performed under the six different embryogenic parameters as in [6,7,8,9,10]. The data for all the six embryogenesis parameters were subjected to full factorial design for one-way as well as multivariate analysis of variance (ANOVA) with P≤0.05 and results are presented in Table 2. The mean value for entire data were plotted in 3-D interactive graphs using SPSS 10 package for Window (SPSS Inc., USA) and presented as Figure 2. Concentrations (mg l^{-1}) of 2,4-D were transformed to log to the base 10 values and presented on X-axis in Figure 2. The values presented in Table 1 represent means ± standard deviations. The error bar on each chart in Figure 3, Figure 4 represented mean ± standard deviation.

3. Results

3.1. Induction of Somatic Embryogenesis

Zygotic embryo explants (Figure 1A) showed a rapid change in shape, size, colour and extent of callus formation within a week of subculture onto 3% (w/v) sucrose containing PGR-free medium after different durations of inductive treatment with 2,4-D as compared to continuous treatment described earlier [7,10]. Development of somatic embryos was observed as globular structure under stereozoom microscope (SMZ - 2T; Nikon, Tokyo, Japan) at the end of the third week of culture initiation irrespective of the 2,4-D concentrations and the pulse treatment of zygotic embryo explants (Figure 1B). Development of somatic embryos completed in 5-6 weeks, while, maturation continued until 8-weeks of culture initiation (Figure 1C and 1D). The process of somatic embryogenesis in guava was observed and analysed under the six different parameters viz. (i) frequency of embryogenesis (FE), (ii) intensity of embryogenesis (IE) [represented as average number of embryos produced per explants per culture (ANEPC)], (iii) frequency of elongated torpedo-stage somatic embryos (ET), (iv) frequency of short torpedo-stage somatic embryos (ST), (v) frequency of lower- (cotyledonary, heart, and globular) stages somatic embryos (CHG), and (vi) efficiency of embryogenesis (EE) calculated as relative numbers according to the formula described in [6,7,8,9,10].

3.1.1. Interaction of 2,4-D Concentrations and Treatment Durations

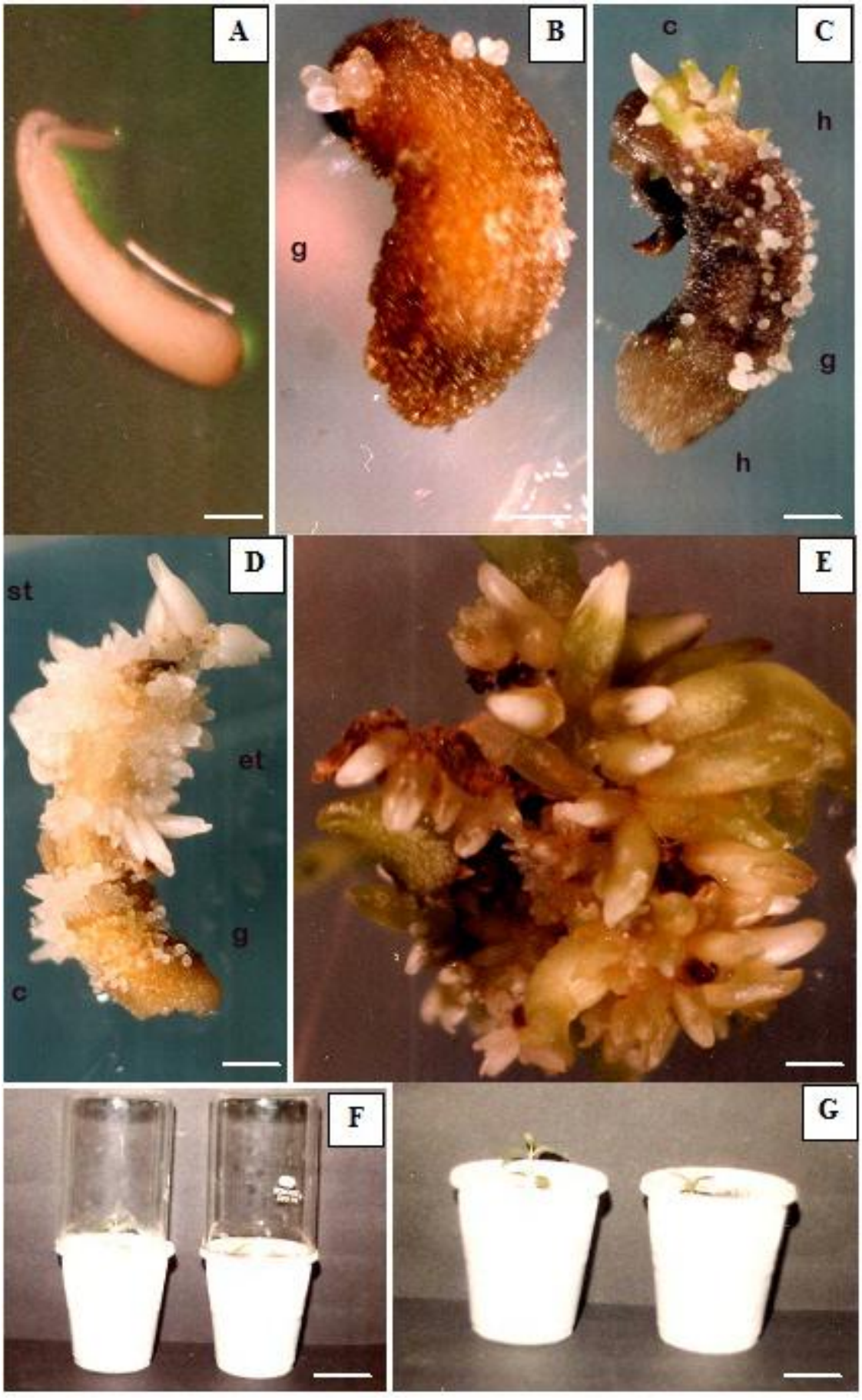
The interactive effects of various durations of pulse treatment and 2,4-D concentrations on the six embryogenesis parameters presented in Figure 2. The result of multivariate analyses of variance showed a significant ($P \leq 0.05$, Table 2) interactions and indicated that 2,4-D exerted a concentration dependent temporal effects on all the six somatic embryogenesis parameters. There was a significantly gradual shift ($P \leq 0.05$, Table 2) in maximum response from 0.01 mgl^{-1} (continuous and 38-days) to 0.05 mgl^{-1} (28- and 18-days) then to 0.1 mgl^{-1} (14- and 12-days) and finally to 0.5 mgl^{-1} (10- and 8-days) 2,4-D (Figure 2). Approximately 30% enhancement in the frequency, almost 6-fold increase in intensity and about 8-fold improved efficiency of embryogenesis was observed in 8-days treatment with 0.5 mgl^{-1} 2,4-D (Table 1) in the present study as compared to continuous treatment of zygotic embryo of guava [7].

Somatic embryo appeared as 5-6 distinctive stages (Figure 1D) on one and the same zygotic embryo explants indicating asynchronous development. The frequency of convertible elongated- and short-torpedo stages somatic embryos were inversely proportional whereas, frequency of lower stages (non-convertible) somatic embryos showed a positive correlation with 2,4-D concentrations and treatment periods (Figure 2D-2F & Table 1). A large values of *F* for one way ANOVA indicated that concentration of 2,4-D exerted a highly significant ($P \leq 0.05$, Table 1) effects on the six embryogenesis parameter in 8-days treatment of zygotic embryo explants with 0.5 mgl^{-1} 2,4-D compared to the continuous treatment [7]. These results clearly indicated a concentration dependent temporal regulation of somatic embryogenesis in guava (*P. guajava* L.) cv. Allahabad safeda.

3.1.2. Polyamines and Somatic Embryogenesis

The endogenous levels of total, free, conjugated, and bound forms of different polyamines were analyzed after various durations of zygotic embryo explants treatments during induction and development phases in order to understand the correlation between the polyamines metabolism and somatic embryo development. The

gradual increase in various forms of different polyamines during induction phase in the presence of 2,4-D (until 8-days), followed by a sudden burst and subsequent decline in their concentrations during development and maturation of somatic embryos in the absence of PGR clearly indicated that metabolism of cellular polyamines might be involved in modulation of regulatory signals of 2,4-D for the process of somatic embryogenesis (Figure 3).



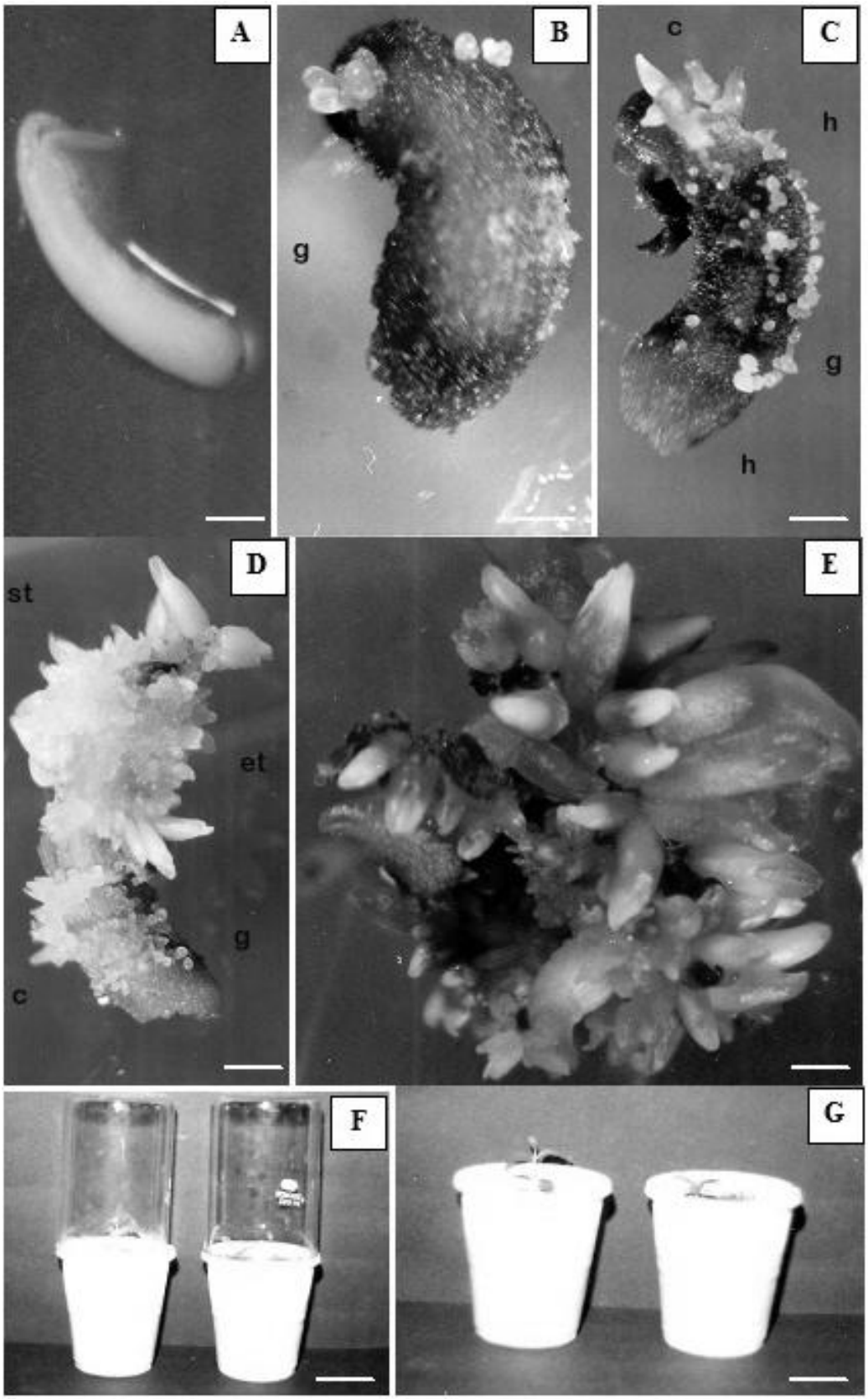


Figure 1. Induction of somatic embryogenesis from immature zygotic embryo of guava (*Psidium guajava* L.) 'Allahabad sfeda'.A, a zygotic embryo (10 weeks post-anthesis) used as an explants (Scale bar = 1.0 mm). B, a zygotic embryo explant after 20 days of sub-culture on 3% (w/v) sucrose containing full-strength MS development medium following an initial 8- days treatment with 0.5 mg l^{-1} 2,4-D showing the globular stages somatic embryos (Scale bar = 0.35 mm). C, zygotic embryo 5-weeks after culture initiation showing development of lower-stages somatic embryos (Scale bar = 0.5 mm). D, development of different-stages somatic embryos from entire hypocotyle region of a zygotic embryo explants 6-weeks after culture initiation (Scale bar = 1.0 mm). E, germination of different stages of somatic embryos in first week of sub-culture to half-strength agar-solidified MS basal medium with 3% (w/v) sucrose (Scale bar = 0.5 mm). F, somatic plantlets in the process of acclimatisation (Scale bar = 32 mm). G, somatic plantlets after 2-weeks of acclimatization (Scale bar = 23.5 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.

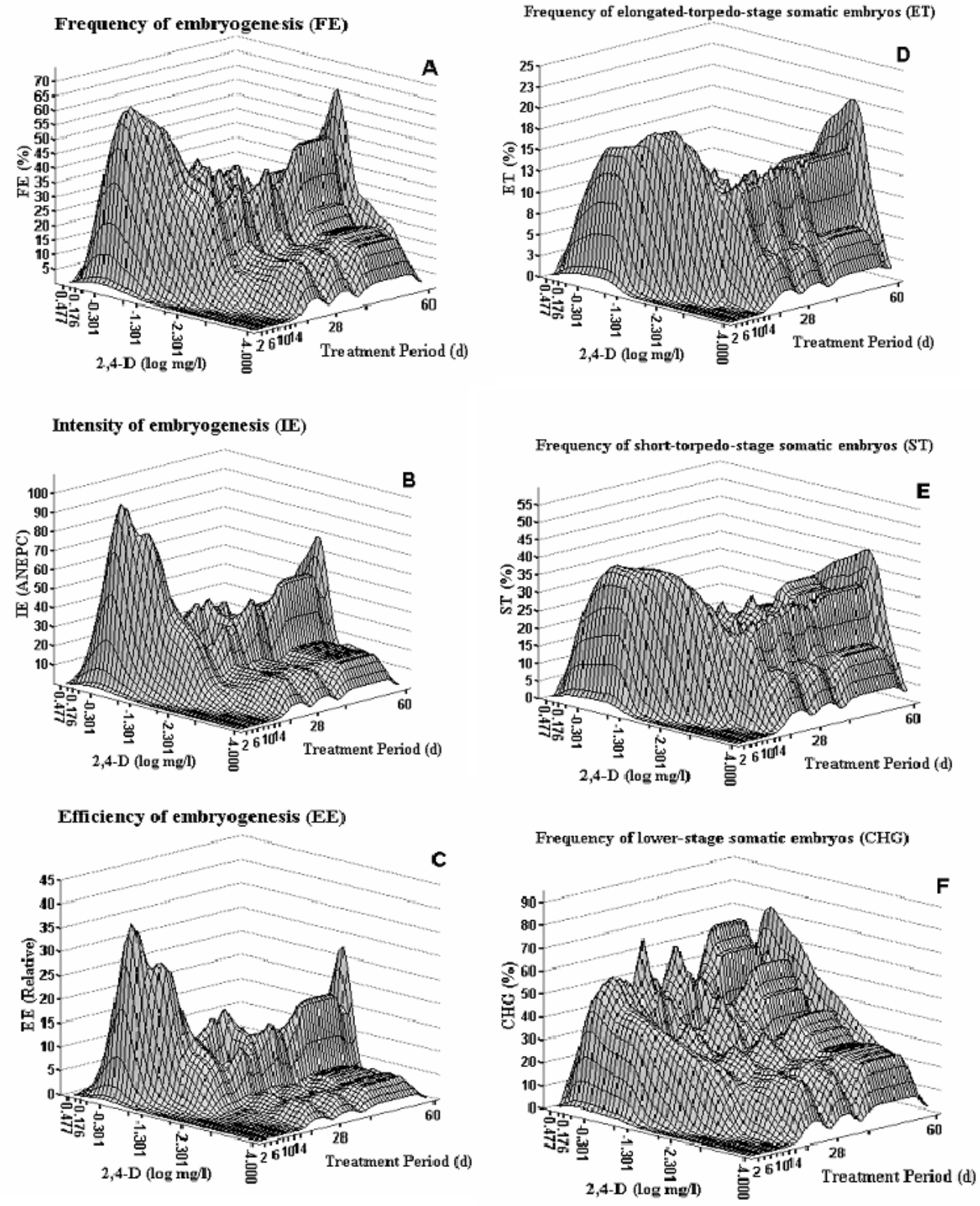


Figure 2. Interactive effect of 2,4-D concentrations and treatment durations on six different somatic embryogenesis parameters. A, frequency of embryogenesis (FE) represented as percentage of response. B, intensity of embryogenesis (IE) [represented as average number of embryos produced per culture per treatment (ANEPC)]. C, frequency of elongated torpedo-stage somatic embryos (ET) represented as percentage of total somatic embryos produced. D, frequency of short torpedo-stage somatic embryos (ST) represented as percentage of total somatic embryos produced. E, frequency of lower- (cotyledonary, heart, and globular) stages somatic embryos (CHG) represented as percentage of total somatic embryos produced. F, efficiency of embryogenesis (EE) represented as relative numbers and calculated as described by Akhtar (2010). Zygotic embryo explants (10-weeks post-anthesis) of guava (*Psidium guajava* L.) cv. Allahabad safeda were treated for various durations with different concentrations of 2,4-D, followed by sub-culture on 3% (w/v) sucrose-containing growth regulator-free full-strength MS agar medium for development and maturation of somatic embryos. Mean values for the entire data set were plotted and presented as 3-D interactive graphs using software SPSS for Windows Version 10. Axis- X represented log to the base 10 transformed values of 2,4-D concentrations (see materials and methods). Axis- Y represented treatment duration in days after culture initiation. The Z- axis represented the six somatic embryogenesis parameter described in panel A, B, C, D, E, and F above. Statistical analysis for the significance of the data is shown in Table 2.

Table 2. Full factorial multivariate analysis of variance (ANOVA) showing the interactions of 2,4-D concentrations and treatment durations (d) on six somatic embryogenesis parameters in guava (*Psidium guajava* L.) cv. Allahabad sfeda

Source	Dependent Variable	Type I Sum of Squares	df	Mean Square	F	Sig.
Corrected Model	FE (%)	196940.288	120	1641.169	92.975	.000
	IE (ANEPC)	296195.252	120	2468.294	106.874	.000
	ET (%)	26882.401	120	224.020	104.061	.000
	ST (%)	124411.013	120	1036.758	104.347	.000
	CHG (%)	335309.630	120	2794.247	194.424	.000
	EE (Relative)	40836.842	120	340.307	81.287	.000
Intercept	FE (%)	123375.408	1	123375.408	6989.461	.000
	IE (ANEPC)	132731.041	1	132731.041	5747.111	.000
	ET (%)	15153.868	1	15153.868	7039.254	.000
	ST (%)	101017.417	1	101017.417	10167.122	.000
	CHG (%)	269950.366	1	269950.366	18783.198	.000
	EE (Relative)	9315.128	1	9315.128	2225.046	.000
2,4-D (mg/l)	FE (%)	75408.575	10	7540.858	427.204	.000
	IE (ANEPC)	102334.475	10	10233.447	443.097	.000
	ET (%)	7772.164	10	777.216	361.031	.000
	ST (%)	39283.162	10	3928.316	395.374	.000
	CHG (%)	143003.701	10	14300.370	995.022	.000
	EE (Relative)	10879.729	10	1087.973	259.877	.000
Treatment period (d)	FE (%)	32936.136	10	3293.614	186.590	.000
	IE (ANEPC)	42112.023	10	4211.202	182.341	.000
	ET (%)	3667.890	10	366.789	170.380	.000
	ST (%)	24677.639	10	2467.764	248.374	.000
	CHG (%)	73315.492	10	7331.549	510.131	.000
	EE (Relative)	3625.142	10	362.514	86.592	.000
2,4-D (mg/l) * Treatment period (d)	FE (%)	88595.577	100	885.956	50.191	.000
	IE (ANEPC)	151748.755	100	1517.488	65.706	.000
	ET (%)	15442.347	100	154.423	71.733	.000
	ST (%)	60450.212	100	604.502	60.841	.000
	CHG (%)	118990.436	100	1189.904	82.794	.000
	EE (Relative)	26331.970	100	263.320	62.898	.000
Error	FE (%)	4271.696	242	17.652		
	IE (ANEPC)	5589.054	242	23.095		
	ET (%)	520.969	242	2.153		
	ST (%)	2404.438	242	9.936		
	CHG (%)	3478.001	242	14.372		
	EE (Relative)	1013.130	242	4.186		
Total	FE (%)	324587.392	363			
	IE (ANEPC)	434515.348	363			
	ET (%)	42557.239	363			
	ST (%)	227832.868	363			
	CHG (%)	608737.997	363			
	EE (Relative)	51165.100	363			
Corrected Total	FE (%)	201211.984	362			
	IE (ANEPC)	301784.306	362			
	ET (%)	27403.371	362			
	ST (%)	126815.451	362			
	CHG (%)	338787.631	362			
	EE (Relative)	41849.972	362			

a R Squared = .979 (Adjusted R Squared = .968); b R Squared = .981 (Adjusted R Squared = .972); c R Squared = .990 (Adjusted R Squared = .985) d R Squared = .976 (Adjusted R Squared = .964)

The exogenous application of various polyamines (Figure 4) showed variable response in frequency, intensity and efficiency of somatic embryogenesis until 1.0mM concentration and higher concentrations were inhibitory to the process. The frequencies of different categories of somatic embryos were affected significant. Most normal and convertible somatic embryos were produced in 64.72, 61.58 and 56.86 relative efficiencies in the presence of 1.0mM concentrations of putrescine, spermidine and spermine, as against the 61.10, 58.77 and 51.97, respectively, in control cultures without any polyamines.

3.2. Maturation and Germination of Somatic Embryos

Development and maturation of somatic embryos were completed within the 5-6 weeks (Figure 1C, Figure ID) of culture initiation and followed more normal trajectory in pulse treatment compared to continuous treatment of

zygotic embryo explants [7]. However, somatic embryos were allowed to develop and mature up to the 8-10 weeks of culture initiation in the presence of 2,4-D (0.005-0.5 mgl^{-1}) or on PGR-free development medium after 8-18 days of inductive treatments for normal germination (Figure 1E) and plantlet formation. About 95-99% of elongated torpedo and 77-89% of short torpedo category of somatic embryos from various 2,4-D concentrations in different days of pulse treatments were germinated and converted into normal plantlets when sub-cultured to 3% (w/v) sucrose containing half strength agar-solidified MS germination medium. Lower stages somatic embryos turned light green to green in colour indicating germination but never converted into plantlets.

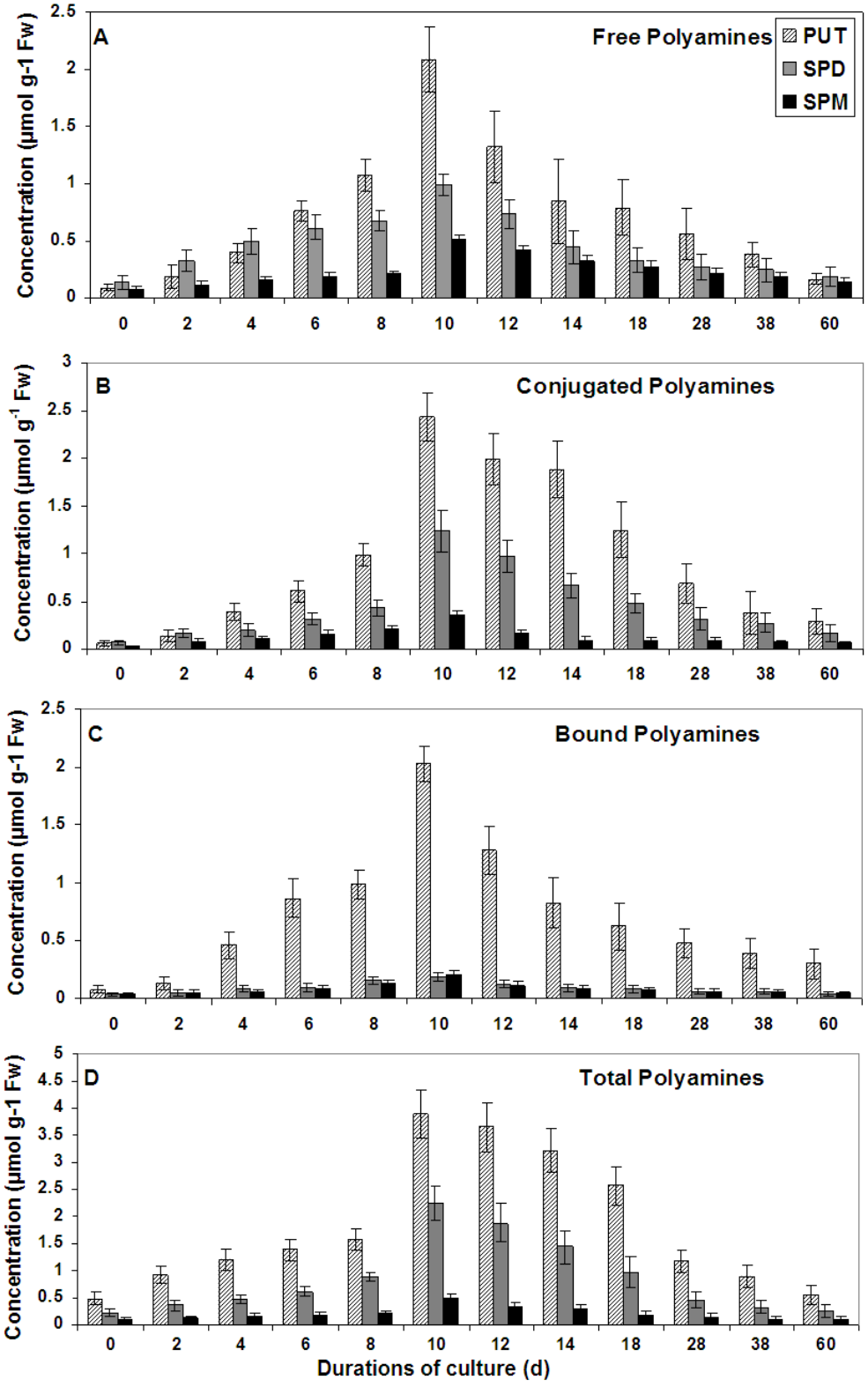


Figure 3. Temporal changes in endogenous profile of various cellular polyamines during induction phase in the presence of 2,4-D (till 8 days) and developmental phase in the absence of 2,4-D (> 8 days) of somatic embryogenesis on 3% (w/v) sucrose containing full-strength MS agar medium. A, showing changes in free-polyamines concentrations. B, showing changes in conjugated-polyamines concentrations. C, showing changes in bound-polyamines concentrations. D, showing changes in total-polyamines concentrations. The bars in figure represented mean value of 3-independent experiments performed in three consecutive years at the similar flowering seasons. The error bar on each chart represented mean ±standard deviation

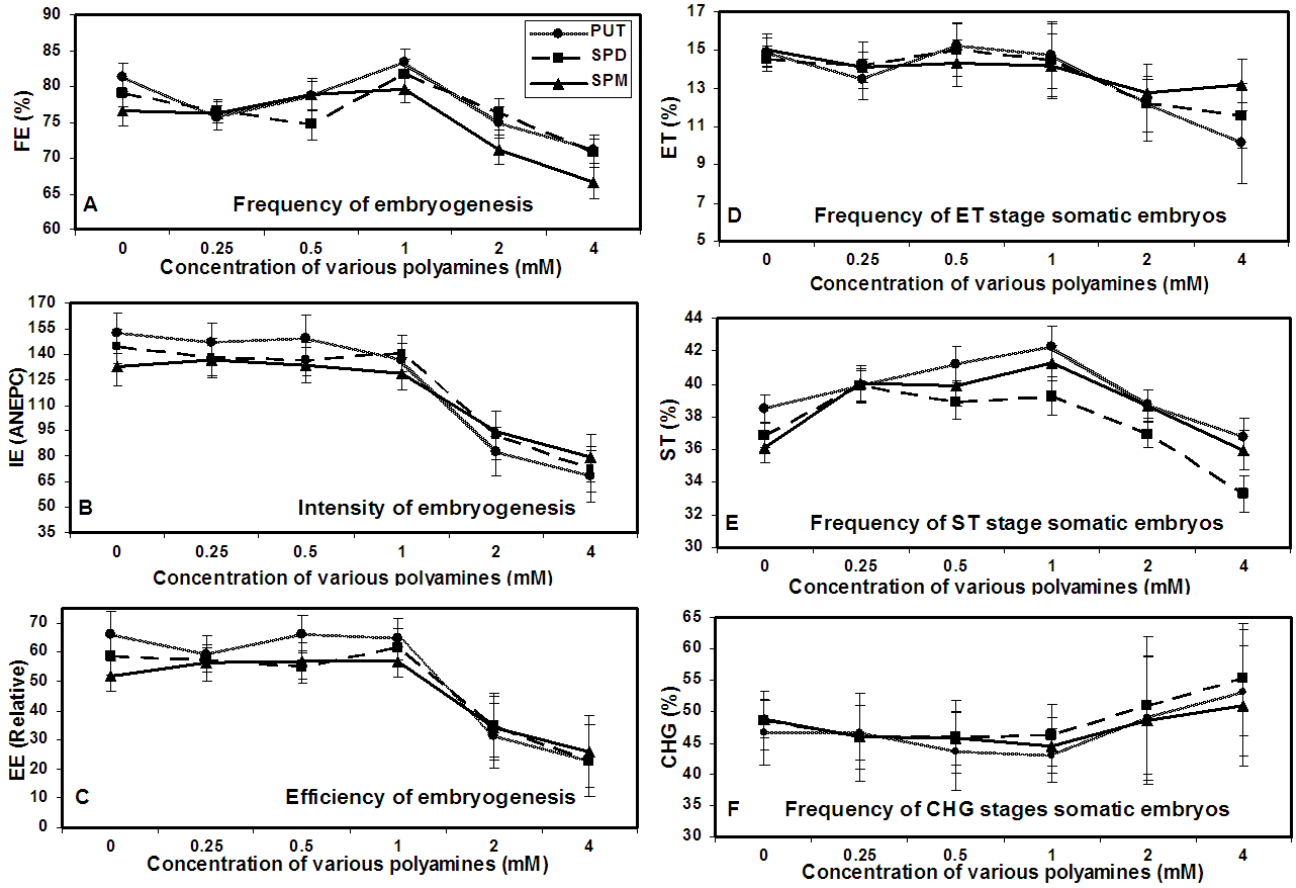


Figure 4. Effect of exogenous application of various polyamines, putrescine (PUT), spermidine (SPD), or spermine (SPM) on the induction of somatic embryogenesis in guava (*Psidium guajava* L.) 'Allahabad safeda'. Zygotic embryo explants (10-weeks post-anthesis) were treated with 0.5 mg l^{-1} 2,4-D for 8 days followed by sub-culture to PGR-free 3% (w/v) sucrose-containing MS agar medium. Both induction and development medium were added with different concentrations of putrescine, spermidine, or spermine. A, frequency of embryogenesis (FE) expressed as percentage on y-axis. B, intensity of embryogenesis (IE) expressed on y-axis as average number of embryos produced per explants per culture (ANEPC). C, efficiency of embryogenesis (EE) expressed as relative number on y-axis; D, frequency of elongated torpedo (ET) stage somatic embryos expressed as percentage on y-axis. E, Frequency of short torpedo (ST) stage somatic embryos expressed as percentage on y-axis. F, frequency cotyledonary, heart, and globular (CHG) stage somatic embryos expressed as percentage on y-axis. The error bar on each chart represented mean ±standard deviation.

3.3. Growth and Performance of Plants

Somatic plantlets were grown for an extended period of 2-weeks in full strength MS liquid growth medium supplemented with 3% (w/v) sucrose prior to soil transfer and acclimatization. Well grown plantlets from growth medium were processed for soil transfer and hardening (Figure 1F, Figure 1G) as in [6,7,8,9,10]. Hardened plants were transferred to the grower's field, performed well and fruited in less than 1½-years of time.

4. Discussions

A reliable and systematic regeneration of guava plants was reported recently through the process of somatic embryogenesis by continuous treatment of zygotic embryo explants with 2,4-D [7]. The periodic sub-culture of the explants onto PGR-free development medium following different durations of inductive treatments with 2,4-D showed significant effects on all the parameters with an overall increase in the efficiency of somatic embryogenesis in the present study. In several recalcitrant fruits and tree species induction of somatic embryogenesis was achieved using zygotic embryos as the starting explants material [14,33]. Exogenous application of auxins, mainly 2,4-D, and was demonstrated as one of the inductive factors associated with cell division, polar transport and production of organized cell clusters [16]. In *Norway* spruce the initiation rate of somatic embryogenesis was higher when immature zygotic embryos were used, and transfer of the embryogenic tissue from proliferation onto maturation medium leads to the embryo development [15]. Similarly, in the present study the process was improved with the gradual shift from 0.01 mgl^{-1} (continuous and 38-days) to 0.05 mgl^{-1} (28- and 18-days) then to 0.1 mgl^{-1} (14- and 12-days) and finally to 0.5 mgl^{-1} (10- and 8-days) 2,4-D indicating a temporal regulation of somatic embryogenesis by 2,4-D from zygotic embryo culture of guava cv. Allahabad safeda. Development of somatic embryos in the complete absence of PGRs following an initial inductive treatment with a particular growth regulators were also reported in *Carya illinoinensis* [34], *Feijoa sellowiana* [12,35], myrtle [13], *Picea abies* [17], *Populus* spp. [36], *Theobroma cacao* [37]. Consequently, it would now be accepted conceptually that sensitivity of the explanted tissue to the exogenously supplied PGRs might be regulated in time and space in higher plants for induction of somatic embryogenesis [2,17,38].

Further, unusually low frequency of anomalous and secondary embryo formation in the present study

compared to continuous treatment [7,10] indicated a highly regulated developmental response as a function of 2,4-D concentrations and treatment durations. In Paluma cultivar of guava high levels of anomalies and secondary embryo development was observed in the combined presence of auxin and cytokinin in the medium [39]. Long term and continuous presence of auxins during the later phase of somatic embryogenesis were always been associated with high frequency of anomalies in other species including *Feijoa sellowiana* [12,35], pecan [34], mango [40], *Theobroma cacao* [37]. This further supports the concept that embryonic development in plants might require a temporal and spatial regulation [2,17,38].

Additionally, gradual increase in all the forms of various cellular polyamines in response to the 2,4-D in the induction medium and their sudden burst within 2-days of 2,4-D removal in the present study further support that cellular metabolism do follow a temporal response leading to the somatic embryo development. Cellular polyamines found critical, as double mutation on two spermidine-synthase encoded gene were lethal to embryogenesis in *Arabidopsis* [26]. Similarly, spatial endogenous polyamine levels were associated with differential embryogenic ability of explanted tissue [41,42]. Further, exogenous supply of the putrescine increased cellular concentrations of putrescine but not the spermidine and spermine [43] a prerequisite for cell division [44]. The lower concentrations of spermidine and spermine compared to putrescine indicating the metabolic involvement of these polyamines in regulation of somatic embryogenesis in the present study similar to the other species [27,45]. The results of exogenous application of different polyamines suggested that in guava polyamines might regulated the process probably by modulating the cellular concentration as a function of 2,4-D during induction phase and being rapidly metabolized in the production of developmentally normal and mature somatic embryos in the absence of growth regulators.

Santa-Catarina *et al.* [46] suggested that putrescine was related to the formation of new somatic embryos, whereas, spemidine and spermine stimulated somatic embryo development and maturation in *Ocotea catharinensis.* Similarly, polyamines were critically involved in regulation of somatic embryogenesis in the temporal manner in eggplants [43]. Previously, Rey *et al.* [47] showed that accumulation of putrescine in plant tissue induced senescence, however, the spermine was identified as an anti-senescence-inducing factor [48] and must be absent during the maturation phase of the process. On the other hand, the senescence hormone abcisic acid (ABA) was involved in the maturation of somatic embryos in mango [40] and other species [17,49]. These observations further supported the findings that increased cellular polyamine and overall efficiency of the process as a function of 2,4-D might be highly regulated (temporally), and modulated by the fast metabolism and utilization of different polyamines during somatic embryo development in the present study. But a more detailed biochemical and molecular studies required in understanding the crucial role of polyamines and other metabolites involved in acquisition of embryogenic competence for the normal development and maturation of somatic embryos.

In conclusion, somatic embryogenesis in guava (*P. guajava* L.) cv. Allahabad safeda was regulated in a temporal manner as a function of 2,4-D concentrations. Polyamine modulate the regulatory signals of 2,4-D for the enhanced production of most normal and convertible somatic embryos. The regulatory signals of 2,4-D modulated the cellular pools of polyamines in temporal manner for the production of most normal and convertible somatic embryos. These findings in guava might be helpful in development of regeneration protocol through induction of somatic embryogenesis in other recalcitrant plant species but further investigations required to identify the interacting role of polyamines with other molecules involved in regulation of the process.

Acknowledgement

Financial support for the Laboratory of Morphogenesis, Centre of Advanced Study in Botany, Banaras Hindu University, Varanasi, was provided by the University Grant Commission, the Council of Scientific and Industrial Research, the Central Silk Board, the Department of Biotechnology, and the Department of Science and Technology, of the Government of India is gratefully acknowledged. I thank the Jain Institute of Vocational and Advanced Studies, Bangalore and the Post Graduate and Research Department of Microbiology and Biotechnology, Al-Ameen Arts Science and Commerce College, Bangalore for access to laboratory facilities.

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