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IMPROVED ISOLATION AND CULTURE OF PROTOPLASTS FROM *S. chacoense* AND POTATO: MORPHOLOGICAL AND CYTOLOGICAL EVALUATION OF PROTOPLAST-DERIVED REGENERANTS OF POTATO CV. DESIREE

Bushra Sadia*

Pakistan Centre for Advanced Studies in Food Security/ Agriculture (PCAS-FSA) University of Agriculture, Faisalabad. Pakistan.
***Corresponding author's e-mail: bushra.sadia@uaf.edu.pk**

The study reports the establishment of an improved and repeatable protoplast-to-plant protocol using axenic cultures of wild diploid species *S. chacoense* Bitt. and potato cv. Desiree and cell suspensions alone of cv. Desiree, as a pre-requisite to somatic hybridization. The protoplast isolation was influenced by type and age of explants, composition of enzyme and wash solutions, and duration of explant incubation in enzymes. Five different enzyme mixtures having varied combinations of Cellulase R10, Macerozyme, Hemicellulase, Cellulase RS, Pectolyase Y23 and Xylanase were used. The Xylanase solution produced the highest yield of viable protoplasts in both the species. Leaf explants were taken from 10, 20 and 30 days old axenic cultures, with 20 days old cultures being the highest protoplast yielders (7.61±0.31 and 9.74±0.50 10^6 protoplasts/ g fresh weight for *S. chacoense* and cv. Desiree, respectively). A decline in the yield and viability of protoplasts was also observed with the cell suspension age. The suspensions three days after subculture produced the highest yield (10.20±1.13 10^6 protoplasts/ g f. wt.) of viable protoplasts (96.66±0.72%). Out of four plating densities tested (0.5, 1.0, 1.5 and 2.0 x 10^5 protoplasts ml^{-1}), culture of protoplasts at 1 x 10^5 protoplasts ml^{-1} proved efficient for enhanced mitotic division of protoplasts. Mesophyll protoplasts of *S. chacoense* being non-totipotent, failed to regenerate. Whereas, the protoplasts of cv. Desiree regenerated plants with cytological and morphological variations. Cell suspension protoplast-derived regenerants had higher somatic chromosome numbers and a wide range of aneuploidy (2n=4x=48+2-to-8). However, a much higher percentage of leaf protoplast-derived regenerants had 2n=4x=48 chromosomes and variants had close to normal chromosomal complement (2n=4x=48+2). The protoplast-to-plant regeneration of potato under the same culture conditions where leaf protoplasts of *S. chacoense* remained non-totipotent, may be exploited as a unilateral selection strategy for possible somatic hybrids of *S. chacoense* and potato.

Keywords: protoplast culture, somatic hybridization, regeneration, *Solanum chacoense*, protoclonal variations.

Abbreviations: Gram fresh weight (g f. Wt.), Days (d), Minutes (mins.), Endosperm balance number (EBN), Murashige and Skoog medium with no growth regulators (MS0), Uchimiya and Skoog medium (UM), LS (Linsmaier and Skoog), Diameter (dia), Initial plating efficiency (IPE), Final plating efficiency (FPE), Steroidal glycoalkaloids (SGAs), Cell Protoplast Wash Solution containing 9% (w/v) mannitol (CPW9M), fluorescein diacetate (FDA), Settled Cell Volume (SCV).

INTRODUCTION

Cultivated tetraploid potatoes (*Solanum tuberosum* L.) are no fun to dissect at molecular level owing to their heterozygous nature, low seediness, vegetative propagation and Endosperm Balance Number (EBN) incompatibility barriers. Despite having wide adaptability to varied environmental conditions, the crop is threatened by different insect pests and diseases. Genus *Solanum* is comprised of more than 900 species, out of which 228 are wild diploids (Hawkes, 1990). These wilds are valued as rich genetic sources for improving cultivated potatoes (Moweeta *et al*., 2012).

Solanum chacoense Bitt. (a tuber-bearing wild diploid relative of potato) is of particular importance having fungal, viral, bacterial resistance and tolerance to abiotic stresses like drought and heat (Rakosy-Tican *et al*., 2011). It produces novel steroidal glycoalkaloids (SGAs) (leptines and leptinines) in some accessions. These SGAs act as defence compounds. The species synthesizes SGAs only in aerial parts with no traces in tubers. Hence, it can be safely exploited to improve potato. However, ploidy differences, post- and pre-zygotic barriers do not let breeders exploit its full potential. Plant biotechnology has emerged as an efficient alternative to these problems. Somatic hybridization via protoplast fusion offers significant opportunities for whole genome manipulation by bridging these sexual incompatibilities. Somatic hybrids of potato have been successfully created having wild *Solanum* species as donor

parent (Chen *et al*., 2008, Tiwari *et al.*, 2010; Sarkar *et al.*, 2011).

Establishment of *in vitro*- and protoplast-to-plant regeneration protocol is a pre-requisite to somatic hybridization. While, frequent record exists for cultivated potato varieties; protoplast isolation, culture and regeneration methods are available only for few wild *Solanum* species e.g., *S. acaule*, *S. brevidens, S. pinnatisectum*, *S. cardiophyllum*, *S. dulcamara, S. nigrum*, *S. bulbocastanum* and *S. chacoense* (Chen *et al*., 2008; Iovene *et al*., 2012). For efficient exploitation, these protocols still need improvement.

The study explores and optimizes factors affecting protoplast isolation, culture and regeneration in *S. chacoense* and Desiree. Ploidy evaluation and morphological characteristics of Desiree protoclones are also reported. These optimized protocols provided the baseline for successful somatic hybridization of Desiree with *S. chacoense* (Sadia, 2002).

MATERIALS AND METHODS

***Plant material for protoplast isolation*:** Leaf explants of *S. chacoense* and cv. Desiree and cell suspensions of cv. Desiree alone were used for protoplast isolation. Axenic cultures of *S. chacoense* maintained in RMB5 medium (Menzel *et al*., 1981) and those of cv. Desiree in MS medium (Murashige and Skoog, 1962) without growth regulators (MS0 medium; Kumar, 1994) were used 2-3 weeks post subculture for leaf-derived protoplast isolation. Cell suspension cultures of cv. Desiree maintained in liquid UM medium (Uchimiya and Murashige, 1974). (3 months post- initiation) were also used as protoplast source (Plate 1).

***Enzyme mixtures*:** Five different enzyme mixtures named as Enzyme solution 1, 2, 3, 4 and 5 (E1, E2, E3, E4 and E5) (Table 1) were prepared in a range of wash solutions viz; Solution S, Cell Protoplast Wash Solution salts containing 9% (w/v) mannitol (CPW9M), WSS (Sidorov *et al*., 1984), Wash solution 1 and 2 (W1 and W2) (Table 2). Enzyme mixtures were filter sterilized and kept frozen in aliquots of 10ml each before use. The glassware, Pasteur pipettes, nylon sieves and cotton wool syringes etc were autoclaved at 121°C for 20 min at 10.3 KN/m^2 prior to protoplast isolation.

***Protoplast isolation from leaves*:** Fully expanded leaves (4^{th} to 7^{th} leaf as measured from apex) excised from *in vitro*-grown plants of Desiree and *S. chacoense* were transversely cut into 1mm^2 with a straight-edged scalpel blade. Aliquots of 1.0 g fresh weight (g f. wt.) were transferred to 9.0 cm diameter (dia) Petri dishes and incubated in 10 ml each of filter sterilized enzyme solutions (E1, E2 or E3). Whereas, for a separate protocol, 0.25 g f. wt. of leaves were used with 10 ml of sterilized enzyme solutions E4 and E5 (following Butenko and Kuchko, 1980) (Table 1). Petri dishes were sealed with Nescofilm and digestion preceded on a horizontal rotary shaker (40 revolutions per minute, 2 cm throw) in the dark at 25±2°C for 3-7 hrs. Digested tissues were passed through a sterilized 100 µm nylon sieve and then through a layer of hydrophilic cotton wool contained in a syringe barrel, to remove the undigested tissues. Protoplast suspensions were transferred to 12 ml screw capped tubes and centrifuged (50-70 *xg*, 15 minutes). The protoplast pellet was washed thrice by re-suspension and centrifugation in a range of wash solutions prior to counting and viability assessments.

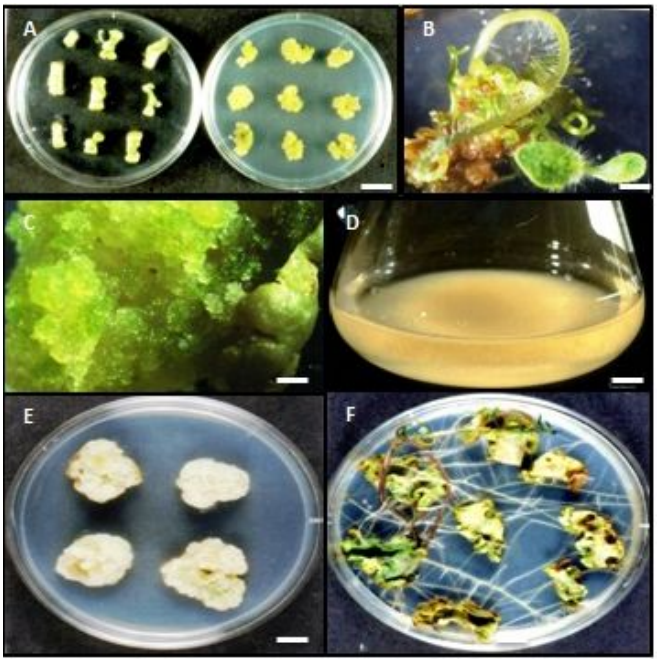

Plate 1. ***In vitro* culture of *S. chacoense* and cv. Desiree: A.** Stem and leaf explants of cv. Desiree (Left-right) 2 weeks after culture on LSR1 medium (Bar = 1.5 cm); **B.** Plant regeneration from stem-derived callus of cv. Desiree 6 weeks after culture on W2 medium (Bar = 0.12 cm); **C.** Leaf-derived callus of cv. Desiree 4 weeks after culture on W1 medium (Bar = 0.21 cm); **D.** Cell suspension culture (6 d old) of cv. Desiree in UM medium (Bar = 0.72cm); **E.** Leaf-derived compact callus from glasshouse-grown *S. chacoense* after 5 weeks culture on MSP1 medium (Bar = 1.05 cm); **F.** Direct plant regeneration from leaves of *S. chacoense* 6 weeks after culture on VR medium (Bar = 1.1 cm).

***Protoplast isolation from cell suspension cultures*:** Protoplasts were isolated from cell suspension cultures of Desiree 1-7 days post-subculture. A 2ml Settled Cell Volume (SCV) of cell suspension was incubated overnight in 10 ml each of enzyme solutions (E1, E2, E3, E4 and E5) on a rocker platform (40 rpm) in dark at 25±2°C.

Table 1. Enzyme solutions for protoplast isolation

Components	Composition in enzyme solution (g/L)				
	E1	**E2**	**E3**	**E4**	**E5**
Cellulase R10	-	15.00	10.00	-	-
Macerozyme	-	5.00	-	-	-
Hemicellulase	10.00	10.00	-	-	-
Cellulase RS	4.00	-	-	-	-
Pectolyase Y23	1.00	-	0.50	-	-
Xylanase	-	-	-	15.00	35.00
MES	1.10	1.10	0.50	-	-
Wash solution	WSS	S	CPW9M	W1	W2
pH	5.8	5.8	5.8	6.1	6.1

All solutions were filter sterilized (0.2 µm pore size).

Table 2. Wash solutions for protoplast isolation

Components	Composition of Wash solution (mg/L)				
	S	**CPW9M**	**WSS**	**W1**	**W2**
KCl	2,490.0	-	-	-	-
$CaCl_2.2H_2O$	2,058.0	1,480.0	-	880.0	880.0
MES	1010.0	1010.0	-	-	-
KNO_3	-	101.0	-	-	-
$MgSO_4.7H_2O$	-	246.0	-	-	-
KH_2PO_4	-	27.2	-	-	-
KI	-	0.16	-	-	-
$CuSO_4.5H_2O$	-	0.025	-		-
D-Mannitol	-	90,000.0	-	54,950.0	73,268.0
L-Glutamine	-	-	500.0	-	-
Yeast extract	-	-	100.0	-	-
Xylose	-	-	250.0	-	-
Sodium succinate	-	-	111,80.0	-	-
Thiamine HCl	-	-	10.0	-	-
Pyridoxine HCl	-	-	1.0	-	-
Nicotinic acid	-	-	1.0	-	-
NAA	-	-	2.0	-	-
BAP	-	-	0.5	-	-
2,4-D	-	-	0.2	-	-
Myo-inositol	-	-	100.0	-	-
Sucrose	-	-	167.72	-	-
pH	5.6	5.8	5.8	-	-

All media were autoclaved (121°C), for 20 min at 10.3 KN/m^2.

Suspension-derived protoplasts were harvested as described for leaf source.

***Determination of protoplast yield and viability*:** Leaf- and cell suspension-derived protoplasts were re-suspended in 10 ml of respective wash solution and an aliquot was transferred to a haemocytometer (Weber Scientific International Ltd., Teddington, Middlesex, UK). The number of protoplsts in 5 tripple-lined squares was counted. Total yield was determined using the following formula and expressed as the number of protoplasts per g f. wt. of input material.

Total yield (per g f.wt.) = (n x 5 x 10^3 x volume of protoplast suspension) / (Input weight of incubated cells)

n = mean number of protoplasts in one triple-lined square.

Protoplast viability was measured using a modification of fluorescein diacetate (FDA) staining method (Widholm, 1972). 10 µl of FDA stock solution (3 mg ml^{-1} in acetone) was added to 10 ml of respective wash solution). A 1.0 ml aliquot of this solution was mixed with equal volume of protoplast suspension (in their respective wash solution). The protoplasts were washed 2-3 times to remove excess FDA and examined (after 5 min) at room temperature under UV light (Nikon 'Diaphot TMD inverted microscope with high pressure mercury vapour lamp HBO 100 W/2, fitted with a 350 nm exciter filter and a GG4+2E barrier filter). Viable protoplasts labelled with FDA were assessed and percentage protoplast viability was calculated by counting total number of protoplasts in a field of view (under bright field illumination) and then measuring the counterpart

number of protoplasts which fluoresced yellow/ green in the same field of view under UV light. Viabilities were determined by counting at least 200 protoplasts and calculated as percent.

***Protoplast culture*:** Protoplasts of Desiree and *S. chacoense* were cultured in agarose droplets of three different culture media; V8PCL medium (Foulger and Jones, 1986), MSC medium (Cheng *et al*., 1995) and B medium (Engler and Grogan, 1983). Culture media were prepared double strength and semi solidified with 1.0% (w/v) Sea-Plaque agarose (FMC Bio-Products, Rockland, USA). Protoplasts were suspended in molten (40ºC) media at different plating densities (0.5, 1, 1.5 and 2.0 x 10^5 protoplasts ml^{-1}). Seven to ten droplets (100µl) of semi-solidified protoplast suspension were placed on the base of each 5.5 cm dia. Petri dish to settle for 30 mins. Cultured protoplasts were incubated at 25±2°C in dark for 2 weeks and then in the light for callus proliferation and plant regeneration.

***Assessment of protoplast and protoplast-derived micro colony viabilities and plating efficiencies*:** Cultured protoplasts were assessed after 15 days for initial plating efficiency (IPE) and 30 d for final plating efficiency (FPE) and for dead or non-dividing protoplasts. Two hundred protoplasts were counted for each assessment. Protoplast-derived colonies were scored by superimposing a $1cm^2$ grid on Petri dish lids and recording number of colonies in each square.

***Shoot regeneration from cultured protoplasts and multiplication of plantlets*:** Protoplast-derived calli (2-6 mm^2) were carefully removed from callus proliferation medium and placed on a range of regeneration media (Table 9). Five callus pieces per each 9 cm dia. Petri dish were assessed in ten replications (n=50). Incubation conditions were as described earlier. Shoot regeneration efficiency was recorded as the percentage of individual protoplast-derived calli giving one or more shoots. Individual shoots (2cm in height) were detached from calli and transferred to semi solidified MS30 medium for rooting. Fully developed plants (8-10 cm high) were transferred to pots in glasshouse for hardening.

***Cytological studies*:** Somatic chromosome complements of regenerated plants were assessed following Andras *et al*. (1999) using root tips excised from *in vitro*-grown plants.

***Morphological characterization of regenerants*:** Data regarding morphological and tuber traits were collected from randomly selected tuber-derived and protoplast-derived plants of both the *Solanum* species, 8 weeks and 4 months post-transfer to glasshouse, respectively.

***Statistical analyses*:** Statistical analyses of protoplast yield, viability, IPE, FPE and plant regeneration data were performed using Standard analysis of variance (ANOVA). The means and standard error of means were computed and Turkey's test was employed to find out statistical significance between means. A probability of $P < 0.05$ considered significant and $P < 0.01$ denoted highly significant values.

RESULTS

***Protoplast isolation from leaves*:** Both yield and viability of protoplasts were genotype-dependent. Among five different enzyme solutions, E4 gave the highest yields and viabilities for leaf-derived protoplasts of Desiree (2.73±0.41 protoplasts per g f. wt. and 78.33±3.60% viability values) and *S. chacoense* (3.00±0.05 protoplasts per g f. wt. with 81±1.7% viability) (Table 3). So, E4 was used throughout the study. E3 did not yield any protoplasts from any source. When assessed on varying incubation times (3, 5 and 7 hrs), the yield and viability of leaf protoplasts of Desiree and *S. chacoense* were the highest at 5 hrs incubation in E4 (Figs. 1 & 2). Leaf explants of both the species were taken from 10, 20 and 30 day old axenic cultures to monitor the effect of

Table 3. Effect of enzymes on yield and viability of protoplasts of cv. Desiree and *S. chacoense*

Protoplast source	Enzyme solution				
	E1	**E2**	**E3**	**E4**	**E5**
- *S. chacoense* (Leaf)					
Yield (10^6 g f.wt.)	1.21 ± 0.13	0.24 ± 0.02	-	3.00 ±0.05	0.10 ±0.04
Viability (%)	53.33 ± 3.57	28.66 ±3.66	-	81 ±1.7**	6.66 ±1.36
- *Desiree (Leaf)*					
Yield (10^6 g f.wt.)	2.70 ±0.21	2.00 ±0.10	-	2.73 ±0.41	0.86 ±0.02
Viability (%)	63.30 ± 2.72	29.33 ±2.00	-	78.33 ±3.60	15.33 ±1.18
- *Desiree (Cell Suspension)*					
Yield (10^6 g f.wt.)	-	-	-	0.46 ± 0.02	7.36 ±0.43**
Viability (%)	-	-	-	24.33 ± 2.84	96.66 ± 0.72**

Protoplast viability was assessed by FDA. Values are mean ± Standard Error of Means (SEM) of 3 replications with at least 200 protoplasts counted per replication. Protoplast yields were assessed for 1 g f. wt. of leaves/ 10 ml of each for E1,E2,E3; 0.25 g f wt. was assessed for 10 ml of E4 and E5. Figures followed by * differ at $P < 0.05$ and by ** differ at $P < 0.01$ level of significance.

Table 4. Effect of leaf explant age on protoplast yield and viability

Explant age	*S. chacoense*		cv. Desiree	
(d)	Yield (10^6 /g f.wt.)	Viability (%)	Yield (10^6 / g f.wt.)	Viability (%)
10	3.03 ± 0.12	72.66 ± 2.66	2.68 ± 0.27	61.66 ±1.66
20	7.61 ± 0.31*	81.00 ±2.10	9.74 ±0.50*	82.66 ±2.66*
30	4.00 ± 0.10	40.3 ± 3.00	4.20 ± 0.20	56.66 ± 3.52

Protoplast viability was assessed by FDA. Values are mean ± SEM of three replicates with at least 200 protoplasts counted per replication. Figures followed by * differ at P < 0.05 level of significance.

explant age on protoplast yield and viability. Leaf explants taken from 20 day old cultures gave significantly higher (P<0.05) yield and viability values of Desiree protoplasts, significantly higher values for yield and higher values for viability of *S. chacoense* protoplasts (Table 4). The wash solution W1 was the most appropriate for handling protoplasts of *S. chacoense* and W2 for leaf and suspension-derived protoplasts of Desiree.

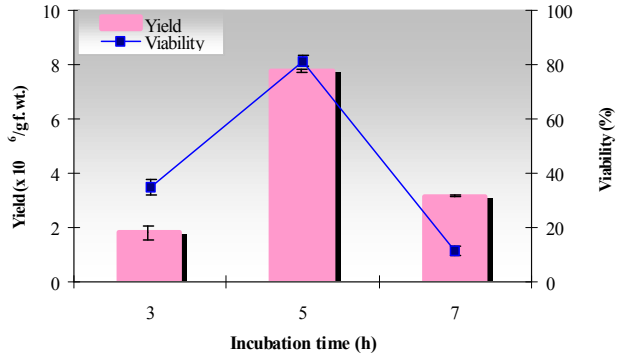

Figure 1. Effect of incubation time on yield and viability of leaf- derived protoplasts of *S.chacoense*

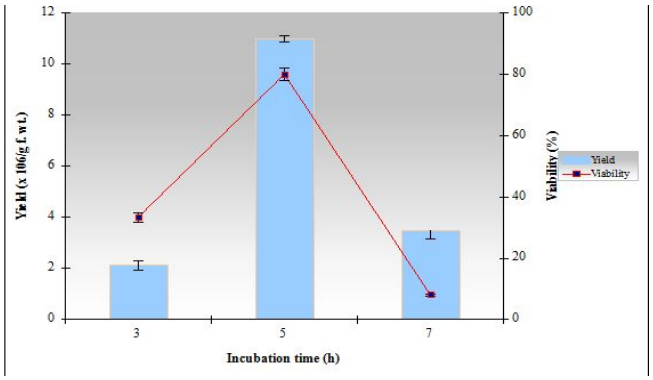

Figure 2. Effect of incubation time on yield and viability of leaf- derived protoplasts of cv. Desiree. Protoplast yield calculated as x 10^6 /g f. wt. and viability by FDA fluorescence. Values are means ± SEM of 3 replicates with at least 200 protoplasts counted per replicate. Bars indicate SEM.

***Protoplast isolation from cell suspension cultures of Desiree*:** Enzyme solutions E1, E2 and E3 failed to release any protoplasts. Whereas, E5 gave highly significant (P<0.01) values for protoplast yield (7.36±0.43 protoplasts per g f. wt.) and viability (96.66 ± 0.72%) compared to E4 (0.46±0.02 protoplasts per g f. wt. and 24.33±2.84%) (Table 3). Suspension cultures, 1-7 d after subculture were assessed for suspension age effects on protoplast yield and viability. A decline in the yield and viability of protoplasts was observed with the explant age, suspensions three days after subculture being the most suitable for higher yield of viable protoplasts (Table 5).

***Culture of protoplasts*:** Four different plating densities were evaluated (0.5, 1.0, 1.5 and 2.0 x 10^5 protoplasts ml^{-1}). A protoplast plating density 1.0 x $10^5 ml^{-1}$ gave the highest IPE and viability values 15 d after culture for *S. chacoense* and cv. Desiree protoplasts. The proportion of dividing protoplasts was 29.20±3.11, 33.40±1.64 and 52.10±2.45% for leaf protoplasts of *S. chacoense* and cv. Desiree and suspension-derived protoplasts of cv. Desiree, respectively. The viable protoplasts ranged in the cultures of all these three sources as 50.00±7.60, 46.00±4.17 and 35.40±1.00%, respectively (Table 6).

Table 5. Influence of suspension age on yield and viability of protoplasts

Cell suspension (day after subculture)	Yield (10^6/ g f.wt.)	Viability (%)
1	0.48 ± 0.07	84.66 ± 2.12
2	0.77 ± 0.02	88.30 ± 0.27
3	10.20 ± 1.13**	96.66 ± 0.72
4	4.00 ± 0.43	84.33 ± 2.62
5	2.30 ± 0.22	81.30 ± 1.51
6	0.85 ± 0.06	81.00 ± 0.47
7	0.20 ± 0.47	80.00 ± 1.00

Protoplast viability was assessed by FDA. Values are mean ± SEM of 3 replicates with at least 200 protoplasts counted per replication. Figures followed by * differ at P < 0.05 and by ** differ at P < 0.01 level of significance.

***Agarose droplet culture of protoplasts*:** Protoplasts divided in all three culture media evaluated (Table 7). First indication of regeneration was 1-3 d after initial plating of cultures involving a rapid enlargement, cell wall formation, degeneration of chloroplasts and formation of extensive cytoplasmic strands. Initial division was clearly evident 3-5 d post-culture (Plate 2). In many cases protoplasts divided

Table 6. Effect of plating density on growth and division of protoplasts cultured on MSC medium

Plating density (ml^{-1})	Proportion of dividing protoplasts (%)	Proportion of viable protoplasts (%)	Proportion of dead protoplasts (%)
Leaf-derived protoplasts of *S. chacoense*			
0.5 x 10^5	9.00 ± 0.31	36.80 ± 2.74	54.20 ± 2.74
1.0 x 10^5	29.20 ± 3.11*	50.00 ± 7.60	20.80 ± 7.6
1.5 x 10^5	17.70 ± 2.04	50.00 ± 2.00	32.3 ± 1.83
2.0 x 10^5	14.70 ± 0.73	10.30 ± 1.01	75.00 ± 1.01
Leaf-derived protoplasts of cv. Desiree			
0.5 x 10^5	9.30 ± 1.04	31.70 ± 0.77	59.00 ± 0.77
1.0 x 10^5	33.40* ± 1.64	46.00 ± 4.17	20.60 ± 4.17
1.5 x 10^5	14.30 ± 0.70	26.40 ± 3.32	59.30 ± 3.32
2.0 x 10^5	5.30 ± 1.00	7.10 ± 2.00	87.60 ± 2.00
Cell suspension-derived protoplasts of cv. Desiree			
0.5 x 10^5	37.20 ± 2.06	32.00 ± 1.01	30.80 ± 1.01
1.0 x 10^5	52.10 ± 2.45*	35.40 ± 1.00	12.5 ± 1.00
1.5 x 10^5	29.60 ± 1.73	38.40 ± 4.00	32.00 ± 2.00
2.0 x 10^5	15.80 ± 2.00	12.50 ± 3.20	71.7 ± 3.20

Values are mean ± SEM of 3 replications with at least 200 protoplasts counted per replication, Figures followed by * differ at $P < 0.05$ and by ** differ at $P < 0.01$ level of significance.

more rapidly on the periphery of the agarose lenses. Leaf-derived protoplasts of *S. chacoense* and suspension protoplasts of Desiree showed significantly higher ($P < 0.05$) IPE values on MSC medium, whereas V8PCL medium showed significantly higher IPEs for Desiree leaf protoplasts (Table 7). Protoplast-derived micro colonies were clearly visible 40-50 d after culture. Mean FPE values recorded for leaf derived protoplasts of *S. chacoense* were very low, rapidly became necrotic and failed to proliferate. None of the culture media favored subsequent development and growth of protoplast-derived micro colonies of *S. chacoense*, hence, these could not be transferred to callus proliferation media (Table 8). Leaf and suspension protoplasts of cv. Desiree on the other hand sustained division as reflected by their FPE values (Table 8).

Table 7. IPE values of protoplasts cultured in agarose droplets

Medium	IPE (%)± SEM		
	Leaf-derived protoplasts (*S. chacoense*)	Leaf-derived protoplasts (Desiree)	Suspension-derived protoplasts (Desiree)
V8PCL	13.50 ± 0.45	37.11 ± 5.10*	24.70 ± 1.90
MSC	31.21 ± 0.66*	19.63 ± 0.58	54.42 ± 1.53
B	16.82 ± 2.40	13.67 ± 1.31	44.04 ± 1.95

IPE was assessed 15d after culture. Values are mean ± SEM of 3 replicates with at least 200 protoplasts counted per replication, Figures followed by * differ at $P < 0.05$.

Table 8. FPE values of protoplasts cultured in agarose droplets

Medium	FPE (%)± SEM		
	Leaf-derived protoplasts (*S. chacoense*)	Leaf-derived protoplasts (Desiree)	Suspension-derived protoplasts (Desiree)
V8PCL	0.05 ± 0.004	0.10 ± 0.003	0.10 ± 0.007
MSC	0.05 ± 0.008	0.07 ± 0.007	0.30 ± 0.002
B	0.03 ± 0.002	0.03 ± 0.001	0.18 ± 0.010

FPE calculated 50d after culture. Values are mean ± SEM of 3 replicates with at least 200 protoplasts counted per replication, Figures followed by * differ at $P < 0.05$.

***Measurement of growth of protoplast-derived micro colonies*:** Micro colonies of leaf- and cell suspension-derived protoplasts of cv. Desiree were transferred to four different callus proliferation media MSP1 medium (MS with BAP and NAA), UM, CG medium (Shepard, 1980) and C medium (Cheng *et al*., 1995). All micro colonies growing on CG medium were dark green and became nodular (Plate 2).Colonies growing on UM medium on the other hand, were friable and creamy yellow in appearance. These colonies showed progressive mitotic activity and gained 1-1.5 mm in dia. only after two subcultures (subcultured every two weeks) on the same fresh medium. Both MSP1 and C medium produced light green nodular calli. After 2-4 subcultures on the respective fresh media, all these calli (3-5 mm in dia.) were transferred to regeneration medium. Mesophyll protoplasts of *S. chacoense*, being non-totipotent, could not be transferred to proliferation/regeneration medium.

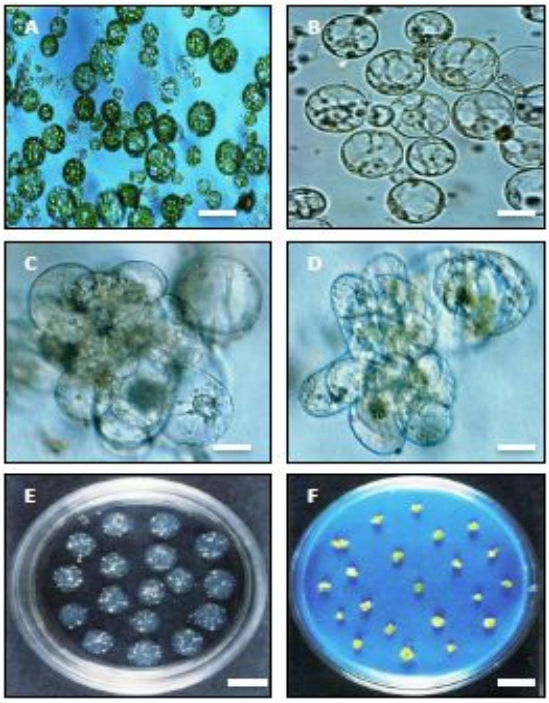

Plate 2. Protoplast isolation and culture in *S. chacoense* and cv. Desiree: A. Freshly isolated mesophyll-derived protoplasts of *S. chacoense* (Bar = 56 µm); **B.** Freshly isolated cell suspension- derived protoplasts of cv. Desiree (Bar = 30 µm); **C.** Leaf protoplast- derived colony of cv. Desiree 2 weeks after culture on V8PCL liquid medium (Bar = 30 µm); **D.** Micro colony of cell suspension-derived protoplasts of cv. Desiree 3 weeks post initiation in agarose droplets of MS C medium (Bar = 30 µm); **E.** Cell suspension-derived protoplasts of cv. Desiree 4 weeks after culture in agarose droplets of B medium (Bar = 0.71 cm); **F.** Leaf protoplast-derived calli of cv. Desiree 2 weeks after culture on CG medium (Bar = 1.1 cm). Protoplasts were cultured at a plating density of 1.0 x $10^5 ml^{-1}$ throughout.

***Shoot regeneration from protoplast-derived tissues and multiplication of plantlets*:** The regeneration response of protoplast-derived potato calli was measured on four regeneration media. Protoplast-derived calli regenerated shoots only on MSZ and LSR2 media (Kumar, 1994) (Table 9). Multiple shoots were initiated 8 weeks post culture on regeneration media, from leaf protoplast-derived calli of potato, whereas, cell suspension protoplast-derived calli regenerated shoots 10 weeks post culture on regeneration medium.

Based upon these results, CG and LSR2 media were used for routine proliferation and regeneration of protoplast-derived calli, respectively. Shoots were multiplied in LSR2 medium, induced to root (MS30 medium) and then transferred to the glasshouse as described earlier). Plants (20 each from leaf- and cell suspension-derived protoplasts of cv. Desiree) were maintained in the glasshouse till tuber production.

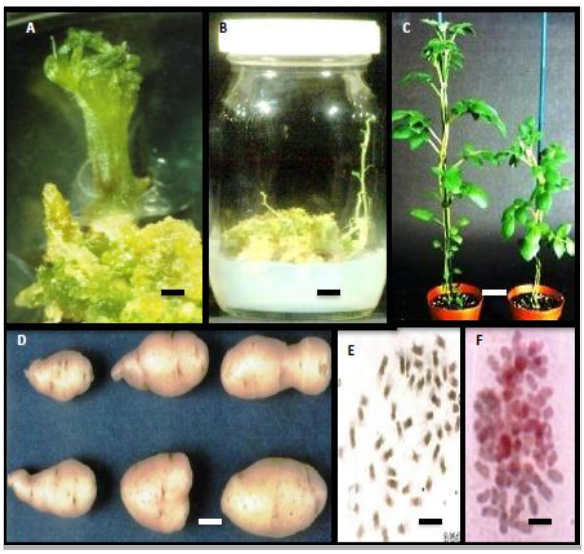

Plate 3. Morphological and cytological evaluation of protoplast-derived plants of cv. Desiree: A. Shoot bud initiation from leaf protoplast-derived colonies of cv. Desiree 8 weeks after culture on LSR2 medium (Bar = 0.18 cm); **B.** Shoot formation response (10 weeks post culture on LSR2 medium) of cell suspension-derived protoplasts of cv. Desiree (Bar = 1.8 cm); **C.** Tuber- derived (control) and protoplast- derived plants of cv. Desiree (Left-right), 45 d after transfer to the glasshouse (Bar = 6.3 cm); **D.** Tubers of protoplast- derived plants of cv. Desiree 4 months after transfer to the glasshouse (Bar = 0.82 cm); **E-F.** Aneuploid chromosomes ($2n=4x=47$ and $2n=4x=54$) of protoplast- derived plants of cv. Desiree, respectively (Bar = 5 µm).

Table 9. Regeneration response of leaf- and cell suspension protoplast-derived calli of cv. Desiree

Medium code	Percent surviving calli	Percent regenerating calli
Calli from leaf-derived protoplasts of cv. Desiree		
SH	60.0 ± 9.0	0.0 ± 0.0
D	50.0 ± 6.0	0.0 ± 0.0
MSZ	40.0 ± 7.5	16.0 ± 5.4
LSR2	66.0 ± 6.0	33.0 ± 8.0*
Calli from suspension-derived protoplasts of cv. Desiree		
SH	63.0 ± 8.0	0.0 ± 0.0
D	28.0 ± 7.1	0.0 ± 0.0
MSZ	51.0 ± 6.0	17.0 ± 5.2
LSR2	80.0 ± 5.2	42.0 ± 6.1*

Values are mean ± SEM of 50 observations of individual protoplast-derived calli. Figures followed by * differ at $P < 0.05$ level of significance.

***Cytological and morphological assessment of protoplast-derived plantlets of potato*:** Ploidy variations were exhibited by protoplast-derived regenerants of potato (randomly selected 20 each from cell suspension- and leaf-derived protoplasts) (Plate 3). Plants regenerated from cell suspension–derived protoplasts had higher than normal (2*n*=4*x*=48) chromosome complement, 50% were normal tetraploids (2*n*=4*x*=48), 35% had 2*n*=4*x*=48+2-to-8 and 15% had 2*n*=4*x*=48-2 chromosomes. In the case of leaf protoplast-derived plants of cv. Desiree, 70% plants were normal tetraploids, 5% had 2*n*=4*x*=48-4 chromosomes, 15% had 2*n*=4*x*=48-2 chromosomes and the chromosome complement of 10% was 2*n*=4*x*=48+2. Regarding morphological assessment, (Table 10) protoplast-derived regenerants of cv. Desiree showed significantly higher (P<0.05) number of nodes per plant as compared to control Desiree plants.

Table 10. Comparison of morphological characters of control and protoplast- derived plants of cv. Desiree

Character	Control plants	Protoplast-derived plants
No. of primary leaflets per leaf	5.53 ± 0.20	3.60 ±0.20
No. of secondary leaflets per leaf	5.62 ± 0.35*	3.00 ±0.15
Leaf index (cm)	1.41 ± 0.20	1.41 ±0.10
Stem length (cm)	108.10 ± 3.60**	75.00 ±2.60
No. of nodes per plant	16.56 ± 0.77	24.22 ±1.60*
Days to flowering	100.00 ± 1.87	112.00 ±2.70
No. of tubers per plant	5.00 ± 0.33	4.00 ±0.43
Tuber wt. per plant (g)	40.55 ± 2.37*	23.11 ±2.40

Values are mean ± SEM of 10 randomly selected tuber-derived (Control) and protoplast-derived plants of cv. Desiree. Figures followed by * differ at P < 0.05 and by ** differ at P < 0.01 level of significance.

However, number of secondary leaflets per leaf and total tuber wt. of control plants was significantly higher (P<0.05) and stem length was highly significant (P<0.01) than protoplast regenerants. No significant differences were observed for the rest of morphological characters between parental and protoplast-derived potato plants.

DISCUSSION

Optimization of a plant growth regime in relation to the choice of genotype, culture vessel and culture medium is a pre-requisite to protoplast isolation on a routine basis from such shoot cultures. The most widely used and the best donor sources of protoplasts are young leaves and cell suspensions. Present study reports the establishment of efficient protocol for higher yields of highly viable protoplasts of cell suspension cultures of cv. Desiree and leaves of both the *Solanum* species. The highest yields of intact protoplasts were from cell suspension cultures in their exponential growth phase, containing mainly small spherical aggregates. When protoplast isolation was attempted from older, highly vacuolated cells (stationary phase) cell walls were resistant to enzyme degradation.

The composition of enzyme and wash solutions were of particular importance among other exogenous factors influencing the efficiency of protoplasts. Xylanase was found to be the most efficient enzyme for both species, using the protoplast isolation protocol of Butenko and Kuchko (1980) with some modifications {reducing the concentration of Xylanase from 2% (w/v) to 1.5% (w/v) in the present study}. The epidermis and its cuticle limit the ability of digestive enzymes to penetrate into the leaf. However, removal of lower epidermis of leaves with fine forceps or dissection of leaves into thin strips facilitates wall digestion and maximises protoplast release (Davey *et al*., 2010). Since *Solanum* epidermis cannot be readily stripped off, slicing the leaves of *S. chacoense* and cv. Desiree, in the present study, was found to be the quickest and most efficient method of mechanical pretreatment before enzyme incubation. These findings are in agreement with Espejo *et al*. (2012) working with *Solanum papita* and Ehsanpour and Jones (2001) working with Delaware.

Protoplasts in the present research were cultured in agarose droplets. A general finding of the study was the improved mitotic division of protoplasts in this culture system. This is in accordance with earlier postulations on the positive effects of agarose embedding owing to easy handling of protoplast cultures, replacement of the liquid bathing medium in the cultures without the risk of disturbance of developing protoplast-derived microcalli and prevention of microbial contamination (Rakosy-Tican *et al*., 2007). Moreover, the microcalli frequently underwent mitosis at the periphery of the agarose droplets and transfer of microcalli to proliferation medium was easy. Unlike liquid culture, embedding technique prevented protoplast aggregation and browning (Lian *et al*., 2011). This culture technique has been found to be beneficial for improved growth and development of protoplasts in other crops as well, like *Cichorium* (Deryckere *et al*., 2012), *Nicotiana tabacum* and *Lotus corniculatus* (Pati *et al*., 2005), carrot (Grzebelus *et al*., 2012) and aroid (Lakshmanan, 2013).

The density at which protoplasts are plated in the medium is crucial in sustaining mitotic division and cell colony formation. Generally, the optimum plating density is 5 x 10^2 to 1.0 x 10^6 protoplasts ml^{-1}. For potato, however, it can range between 10^4-10^5 ml^{-1} (Espejo *et al*., 2012). Out of four plating densities tested in the present study, culture of protoplasts at 1 x 10^5 protoplasts ml^{-1} gave higher values for IPE and FPE of protoplasts of both species. Anjum (1998) found the same plating density to be efficient for protoplast-to-plant-regeneration from mesophyll- and cell suspension-

derived protoplasts of cv. Desiree and Maris Piper. Protoplasts of both cv. Desiree and *S. chacoense* failed to grow when plated at lower plating density (0.5 x 10^5ml^{-1}). It is known that dividing cells stimulate division of neighbouring cells, probably as a result of release of growth factors, particularly amino acids into the surrounding medium. Presumably it is less effective below the minimum cell density.

Mesophyll protoplasts of *S. chacoense* utilized in this study did not regenerate to plants, whereas, under the same culture conditions and regeneration media, leaf- and suspension-derived protoplasts of potato were totipotent. This may provide the basis of a half (unilateral) selection strategy for possible somatic hybrid production involving *S. chacoense* and potato. Other researchers also support the effectiveness of half selection tool to eradicate nonfused protoplast regeneration of recalcitrant parent (Lakshmanan, 2013; Patel *et al*., 2011; Wang *et al*., 2012). Non regenerative mesophyll cells are routinely used as one of the fusion partners in citrus somatic hybridizations (Grosser *et al*., 2010).

Chen *et al*. (2008) also reported lack of regeneration potential in *S. pinnatisectum* protoplasts on the media where other *Solanum* protoplasts regenerated plants. Confirming the fact that potato protoplast isolation and culture is highly genotype-dependent.

Totipotency of a protoplast system is reported to be influenced by protoplast structural diversity. Tylicki *et al*. (2002) distinguished four types of protoplasts after isolation viz., mononuclear, polynuclear, anuclear and homogeneous protoplasts from cell suspension culture of *Solanum lycopersicoides* Dun. They observed cell division only in mononuclear protoplasts. Non-totipotency of *S. chacoense* protoplasts in the present study might also be linked to the absence of mononuclear protoplasts in the protoplast mixture. Potato protoplasts in this study regeneratedplants on culture medium containing zeatin and GA_3. These two growth hormones gave enhanced regeneration from protoplast-derived calli in other reports as well (Ehsanpour and Jones, 2001; Sharma *et al*., 2011).

Present results also show differences in the time required for shoot regeneration from leaf- and cell suspension-derived protoplasts of potato, with leaf protoplast-derived calli regenerating shoots 2 weeks earlier than suspension protoplast-derived calli. Probably because cell suspensions were maintained in UM medium containing 2,4-D (2 mgl^{-1}), which affected the morphogenic ability of cells resulting in delayed differentiation. These results are consistent with those of Anjum (1998) who also noticed initiation of regeneration earlier in leaf protoplast-derived calli of cv. Desiree and Maris Piper, than their cell suspension-derived calli.

Cytological analysis of potato protoplast-derived plants in the present study revealed variations in chromosome number. Cell suspension protoplast-derived regenerants were characterized by higher somatic chromosome numbers and a wide range of aneuploidy (2*n*=4*x*=48+2-to-8). Whereas, a much higher percentage of leaf protoplast-derived regenerants had 2*n*=4*x*=48 chromosomes and variants were within a more limited aneuploid range (2*n*=4*x*=48+2). Further reports also confirmed that plants regenerated from protoplasts of mesophyll tissues maintained the normal ploidy status of the donor plants (Jones *et al*., 1989). Whereas, contradictory evidence exists regarding ploidy instability in suspension protoplast-derived regenerants (Xu and Xue, 2000).

Scientists have described different factors like genotype of donor plants, type of donor tissue, method of protoplast culture and protoplast structural variations being responsible for ploidy changes. Occurrence of cytological variations in the protoplast-derived potato plants is well-documented; these may be both numerical (Sree Ramulu *et al*., 1985) and structural changes (Creissen and Karp, 1985) in the chromosomes. These chromosomal changes may be one of the causes of protoclonal variations. In the present study, however, only numerical changes have been observed in the protoplast-derived regenerants of potato. Two aneuploid classes recognized in the present case probably arise in different ways; the 48 ± plants through spindle errors (Creissen and Karp, 1985). Whereas, in the origin of the 48 + + plants, a doubling step must have preceded chromosome loss (Karp *et al*., 1982). The establishment of efficient protocols for potato and *S. chacoense* protoplast isolation and culture and regeneration of Desiree protoplasts alone, described here, served as a baseline for the creation of potato and *S. chacoense* somatic hybrids.

***Conclusion*:** Scarce record exists on protoplast and *in vitro* culture of *S. chacoense*. Here, the efficient protoplast isolation systems for both *S. chacoense* and cv.Desiree have been optimized. Plant regeneration from leaf and cell suspension of Desiree protoplasts is reported. The study identified an efficient selection criterion for somatic hybrid material as non-totipotency of *S. chacoense* protoplasts on the same medium where Desiree protoplasts were totipotent. The efficient protocols reported here were exploited for successful creation of potato + *S. chacoense* somatic hybrids (Sadia, 2002).

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