British Biotechnology Journal
5(1): 1-12, 2015, Article no.BBJ.2015.001
ISSN: 2231–2927

SCIENCEDOMAIN *international*
www.sciencedomain.org

Somatic Embryogenesis and Plantlet Regeneration from Protoplast Culture of *Stevia rebaudiana*

Marisol Lopez-Arellano[1], Seema Dhir[2], Nilmarie Colón Albino[3], Anais Santiago[4], Terrica Morris[5] and Sarwan K. Dhir[6*]

[1]Department of Biological Sciences, Northern Arizona University, Flagstaff, Arizona 86011, USA.
[2]Department of Biology, 1005 State University Drive, Fort Valley State University, Fort Valley, Georgia 31030, USA.
[3]School of Science and Technology, Universidad Metropolitana, San Juan, Puerto Rico 00928, USA.
[4]Department of Sciences and Technology, Interamerican University, San Juan, Puerto Rico 00936, USA.
[5]Department of Biological and Physical Sciences, Saint Augustine University, Raleigh, North Carolina 27610, USA.
[6]Center for Biotechnology, Department of Plant Sciences, 1005 State University Drive, Fort Valley State University, Fort Valley, Georgia 31030, USA.

Authors' contributions

This work was carried out in collaboration between all authors. Author SD designed the study, wrote the protocol and the first draft of the manuscript. Author SKD managed the analyses of the study and literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BBJ/2015/13884
Editor(s):
(1) Melina A. Talano, Dept. of Molecular Biology, Universidad Nacional de Río Cuarto, Argentina.
(2) Anil Kumar, School of Biotechnology, Devi Ahilya University, Madhya Pradesh, India.
Reviewers:
(1) Anonymous, Lille University of Science and Technology, France.
(2) Anonymous, Institute of Biosciences and Technology, India.
(3) Paula Cristina da Silva Angelo, Embrapa Western Amazon, Manaus, Brazil.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=801&id=11&aid=6884

Original Research Article

Received 8th September 2014
Accepted 21st October 2014
Published 11th November 2014

ABSTRACT

In order to develop a high-efficiency and reproducible regeneration protocol for *Stevia* protoplasts, various factors such as type and concentration of enzymes, osmoticum, incubation time, plant material type and age were studied. Protoplasts were successfully isolated from leaves of four-week-old *in vitro* grown plants using an enzyme mixture comprising of 2% (w/v) Cellulase Onozuka R-10, 1.5% (w/v) Macerozyme Onozuka R-10, 0.2% (w/v) Driselase and 0.1%(w/v) Pectolyase Y-23 in 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2 (N-morpholino)-ethanesulfonic acid (MES)

**Corresponding author: Email: sarwandhir@gmail.com, dhirs0@fvsu.edu;*

at pH of 5.8. Approximately 8.4±0.40x10^6 protoplasts g^{-1}fresh weight with 98.8±1.39% viability was obtained after incubating in enzyme solution for 4 hours in dark. Viable protoplasts were collected by centrifugation in the presence of 16% sucrose solution. Protoplasts at density of 5x10^5 mL^{-1}were cultured on modified KM8P medium supplemented with 0.2 mg L^{-1} 2,4-dicholorophenoxyacetic acid (2,4-D), 1 mg L^{-1} α-naphthalene acetic acid (NAA), 0.5 mg L^{-1} zeatin, 0.15 M sucrose and 0.3 M mannitol by agarose-bead or thin layer liquid culture technique. The protoplasts regenerated cell walls within 24 hours. First cell division was observed after culturing for 2-3 days and micro-colonies were formed within 4 weeks. Gradually adding fresh medium of lower osmotic pressure into the medium for protoplast culture favored cell division. Compared to liquid culture, agarose bead culture improved division frequency almost 1.5 times effectively and showing a plating efficiency of 13% and 9.1% respectively with survival rate of 23.5% to 14.8%. Upon transfer to Murashige and Skoog's medium (MS) with 1 mg L^{-1}BA, alone or in combination with NAA or 2, 4-D at 0.1 mg L^{-1}, protoplast-derived calli produced complete plantlets through somatic embryogenesis in 8-weeks. The regenerated plants survived in soil and all were normal with respect to morphology and growth characters. This protocol might lead to the improvement of the *Stevia* through somatic hybridization, somaclonal variation and genetic engineering by using protoplast based regeneration system.

Keywords: Stevia rebaudiana; protoplast isolation; protoplast culture; somatic embryogenesis; plant regeneration.

1. INTRODUCTION

Stevia rebaudiana (*Bertoni* belong to Asteraceae family), herbaceous, perennial, commonly known as a natural sweetener, is a zero calorie value natural alternative source to traditional sugar (sucrose) obtained from sugarcane or sugarbeet [1-3]. It is native to Paraguay and Southern Brazil. The leaves are a significant source of diterpene *steviol* glycosides that are about 300 times sweeter than sucrose at their concentration of 4% (w/v). Hence, *Stevia* has been named as calorie free "Biosweetener" of high quality as it does not contain calcium cyclamate, saccharin and aspartame and causes no side effects [4-6]. The plant is propagated by seed or by cutting; however seeds are inefficient due to poor seed germination, low fertility and self-incompatibility of the flowers [7]. Recent studies indicate that *Stevia* displays a high degree of variability in sweetening level and composition due to the heterogeneous populations obtained through propagation by seed [8]. Vegetative propagation is also limited by the low number of individuals obtained simultaneously from a single plant. Classical breeding methods including radiation mutation or *in vitro* induction of polyploidy techniques have yielded little improvement for disease resistance and improved sweetener quality in *Stevia* [9-12].

To overcome propagation efficiency it is important to develop an alternate method for rapid production of transgenic homogenous a population of disease free *S. rebaudiana* plants in a short period of time and limited space with high and uniform yield of sweetener plants (glycoside production) [13]. Therefore, interest has been directed towards the use of *in vitro* techniques such as somatic hybridization or direct genetic transformation that can be exploited for plant improvement if a reliable and efficient regeneration system from isolated protoplast could be developed to create genetically diverse non-chimeric transgenic plants [14].

Over two decades, genetic manipulation of plants through protoplast transformation and fusion has been intensively reported [15,16]. The current plant regeneration procedures for *Stevia* are less amenable to gene transformation at the whole plant level. Using *Agrobacterium*-mediated or particle bombardment procedures, the transformation competence of regenerative tissue is low for most of medicinal crops. Furthermore, the use of multicellular explants for transformation often results in the formation of chimeric callus and plants [17]. Therefore, development of techniques for protoplast isolation, culture, fusion and regeneration of transgenic clones of single cell origin may lead to production of a homogenous population and new varieties and could increase the market of *Stevia* species in pharmaceutical industry [16].

Several reports are available on *Stevia* clonal multiplication [18-20] via organogenesis and embryogenesis using different explants [21-24]. Recently, *Agrobacterium*-mediated transgenic plant production through direct and indirect regeneration from leaves with their glycoside

profile has also been reported [25]. However, for the successful application of transformation technique at the single cell level, the availability of an efficient procedure for protoplast isolation and culture is prerequisite but, so far, the protoplast isolation and culture of this species has not been reported. Therefore, in this study for the first time, we attempted to establish efficient protoplast isolation and culture procedure of *Stevia* in order to produce high quality protoplasts.

2. MATERIALS AND METHODS

2.1 Plant Materials and Medium Preparation

Young *S. rebaudiana* plantlets were produced under aseptic conditions *via* node and or shoot tip culture as described in Singh et al. [26]. They were maintained and sub-cultured on half-strength Murashige and Skoog's (MS) medium [27]. The medium consists of MS salts and vitamins with 3% (w/v) sucrose and 0.3% (w/v) gelrite. The pH of the medium was adjusted to 5.8. The cultures were incubated in a 16/8h (light/dark) photoperiod at 25±1°C and sub-cultured onto the same fresh medium at 4 week intervals. Leaf, petiole, hypocotyl and root explants from 4-weeks-old *in vitro* grown plants were used for optimization of protoplasts isolation, culture and regeneration experiments.

2.2 Factors Affecting Protoplast Yield and Viability

2.2.1 Standard protocol for protoplast isolation

Approximately 1 g of fresh young leaves (transversely sliced into small pieces, effect of other explants described in section 2.4.4) excised from 4-week old *in vitro* grown plants were incubated in 10 m L of enzyme solution in dark on a gyratory shaker (55 rpm) at 25±1°C for 4 h. Standard concentration of digestive enzymes consisting of 2% (w/v) Cellulase Onozuka R-10, 1.5% (w/v) Macerozyme Onozuka R-10, 0.2% (w/v) Driselase and 0.1%(w/v) Pectolyase Y-23 in 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2 (N-morpholino)-ethanesulfonic acid (MES), pH 5.8, filter sterilized (0.22 µM, Millipore) was used for all protoplast isolation. The incubated enzyme-tissue mixture was then gently filtered through nylon meshes of 60 µm pore size to remove undigested cell clumps and debris. The filtrate was centrifuged for 10 min at 1000 rpm. The supernatant was discarded and the pellet was washed twice by centrifugation at 1000 rpm for 10 min in the Cell Protoplast Washing (CPW) 0.5 M mannitol (pH 5.8) washing solution. The pellet was suspended in 8 ml of 16% sucrose solution with 5 mM MES and overlaid with 2 ml of KM8P (Kao and Michayluk [28]) protoplast culture medium. After centrifugation at 1000 rpm for 10 min, purified protoplasts were localized in the interphase between two solutions. The collected protoplasts were washed and re-suspended in KM8P culture medium.

2.2.2 Effect of different enzymes on protoplast isolation

To optimize the suitable conditions of enzymes for high yield of viable protoplast isolation, different enzyme combinations and concentrations were used. The effect of applying a combination of both Cellulase and Macerozyme enzymes for protoplast isolation were studied (See Table 1). Seven different filter-sterilized digestion solutions tested contained different concentrations of Cellulase Onozuka-10, Macerozyme Onozuka-R, Driselase and Pectolyase Y-23; all in a cell protoplast wash (CPW) salt solution with 0.5M (w/v) mannitol, pH 5.8 as used in the protoplast isolation process. The CPW with 0.5M (w/v) mannitol, pH 5.8 solution without enzyme was used as the control.

2.2.3 Effect of different concentrations of mannitol

In this study, to identify the optimal concentration of mannitol as sole osmoticum that influences the yield and viability of protoplast, the standard digestive enzymes (optimized from above experiment) and the control protoplast isolation solution used was without Mannitol. The enzyme solution composing of 2% Cellulase Onozuka RS, 1.0% Macerozyme and 0.7% Pectolyase Y-23, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2 (N-morpholino)-ethane sulfonic acid (MES) and various mannitol concentrations (0.2, 0.3, 0.4, 0.5, 0.6 and 0.7 M) were tested for their effects on the yield and isolation of viability of *Stevia* protoplasts. After 4 hr. of incubation, protoplasts were collected to determine the yield and viability, as previously described.

2.2.4 Effect of incubation time on protoplast yield

Incubation time is the duration required to immerse the minced explant (leaf) samples in the protoplast isolation solution with enzyme. To determine the suitable duration required for

obtaining the highest yield of viable protoplasts, one gram of 4-week old *in vitro* grown leaves were incubated in 10 m L^{-1} of standard digestive enzyme containing 0.5 M mannitol as previous described. The digestion was performed on a gyratory shaker (55rpm) form 1 to 10 h in dark (see Table 3). Then the protoplasts were collected and purified as previously described. The viability and yield of protoplasts were recorded to determine the effect of incubation periods.

2.2.5 Effect of different type of explants on protoplast yield and viability

Protoplasts were isolated from four different organs: Leaves, petiole, hypocotyl and roots from 4-week old *in vitro* raised plants. Approximately 1g FW of tissue was placed in a petri-dish with 10 ml of enzyme solution in CPW 0.5 M mannitol solutions (pH 5.8), cut into small pieces and then incubated for 4 h. in dark at 25±1°C, purification and protoplast yield was determined by counting the number of viable protoplasts using a hemocytometer chamber and viability was tested by FDA staining assay.

2.2.6 Effect of age of leaves on protoplast yield and viability

Comparing the effect of different explants as described above, leave explants was found the best for maximum yield of viable protoplast. Further, the effect of age of plant material on protoplasts yield and viability was examined using leaf tissue excised from 1 to 7 weeks old *in vitro* grown plants. One gram of leaves was subjected to enzyme mixture- 2% Cellulase Onozuka R-10, 0.2% Pectolyase Y-23, 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM MES, and incubated in dark on a gyratory shaker (40 rpm). The protoplasts were incubated for 4 h in dark at 25±1ºC. Protoplasts were harvested and purified prior to measurement of protoplast yield and viability as described above.

2.3 Determination of the Protoplasts Yield and Viability

After purification, protoplast yield was determined by using a Hemocytometer chamber. The protoplasts were viewed at 100 x magnification and number of protoplasts' observed was recorded. Total protoplasts yield was calculated using the formula:
Protoplast Yield:

$$\frac{\text{Total protoplasts counted}}{4} \times \frac{\text{total Volume}}{\text{Weight of Fresh Tissue (g)}}$$

The working density of protoplasts was adjusted to $5x10^5$ protoplasts mL^{-1}. The suspended protoplasts (500µl) were mixed with 12 µl Fluorescein diacetate (FDA**)** stock solutions (5mg/mlacetone), then20 µl of suspended protoplasts were placed on a Hemocytometer chamber [29]. After 5min, protoplasts were counted under white light, results were expressed as protoplast yield g^{-1}FW, and all yield assessments were repeated atleast five times. The viability of protoplasts was examined by FDA staining assay under B-2A illumination using an Epifluorescence microscope (Olympus). Viability expressed as percentage is determined as the number of protoplasts that fluoresced yellow-greenunder UV lightout of the total number of isolated protoplasts observed in the same microscopic field under normal light. Removal of cell wall was confirmed by Calcofluor White ST staining [30]. Protoplasts were observed under bright field light using an Olympus BX51 microscope with a 40 x objective and quantified by a Hemocytometer. All experiments were carried out in triplicate and repeated.

2.4 Protoplast Culture Technique

Purified protoplasts at a density of $5x10^5$ protoplasts mL^{-1} were cultured using the liquid thin layer and or agarose bead method. For the liquid thin layer method, 1.5 mL of protoplast suspension in 10 mL liquid KM8P medium was poured onto the bottom of a 9 cm Petri dish. For agarose bead method, equal volume of the protoplast suspension was gently mixed with KM 8 P culture medium containing 0.2 mg L^{-1} 2,4-D, 1 mg L^{-1} NAA and 0.5 mg L^{-1} zeatin with 1.2% (w/v) agarose (SeaPrep®, FMC BioProducts, USA). The protoplast suspension was pipetted and dropped into a 9 cm Petri dish. After gelling of agarose, the layer was covered with 3 m L^{-1} of modified liquid KM 8 P medium. Petri dishes were sealed with micro pore tape and incubated at 25±1ºC in dark for 7 days, dim light for 7 days and then in light for 30 days. The osmotic pressure of the liquid medium was gradually reduced at 7 days intervals: In the agarose bead culture, 0.5 ml of spent medium was replaced with a similar volume of 1:1 0.1 (v/v) mixture of KM 8 P and KM 8 medium containing 1.5 mg L^{-1} of 2, 4-D and 0.5 mg L^{-1} BA; for liquid culture 0.5 ml of above protoplast culture medium mixture was added to each dish. Cell wall regeneration was observed with 0.01% (w/v) Calcofluor white staining under a fluorescence microscope. The protoplast survival rate and plating efficiency

(colony formation) were determined after 7, 14, 21 and 28 days of culture. The division frequency (number of dividing protoplasts/total protoplast x 100) and the plating efficiency (percentage of the plated protoplasts forming cell colonies after 21 days) were expressed as mean ± standard error of three independent replicates which were repeated three times. After 28 days of culture, the cell colonies from the liquid culture or agarose beads were transferred on to agarose-solidified KM 8 (0.3%) medium for proliferation and maturation into embryogenic callus. Protoplast derived small calli, 3-5 mm in diameter were isolated and cultured on MS medium as described for somatic embryos induction (below) and sub- cultured every 21 days. All cultures were maintained at 25±1°C under 16/8 h. (light/dark) photo-period with cool-white fluorescent light supplied at intensity of 30 $\mu molm^{-2}$ per second.

2.5 Somatic Embryo Induction and Plant Regeneration

For inducing somatic embryogenesis and plantlet regeneration, the protoplast-derived micro-calli were transferred onto MS medium containing 2, 4-D (0.5-2.0 mgL^{-1}), BA mgL^{-1} with 500 mgL^{-1}CH, 3% sucrose (w/v) and 0.3% (w/v) Gelrite. After 4 weeks of culture, data on percentage and number of calli producing somatic embryos and plantlet regeneration were recorded. Each treatment was replicated three times and 24 pieces of calli were used each time. Cultures with embryos were transferred onto a MS medium supplemented with 1.0 mg L^{-1}NAA for rooting. All cultures were maintained at 25±1ºC under 16/8 hr. (light/dark) photo period with cool-white fluorescent light supplied at intensity of 30 $\mu molm^{-2}$ per second.

3. RESULTS AND DISCUSSION

3.1 Effect of Concentration and Enzyme Combinations on Protoplast Yield and Viability

The availability of a large number of protoplasts with high viability is the first step for successful protoplast culture. Protoplast could be easily released using 3 weeks old *in vitro* raised plant leaves. Spherical protoplasts were released from the leaf tissues after 4–5 h of incubation in the enzyme mixture (Fig. 1A). In leaf protoplasts, numerous green chloroplasts randomly distributed in the cytosol were visible and showed no fluorescence when stained with calcofluor white (data not shown). Some leaf protoplasts were pink probably due to the presence of anthocyanins in a vacuole. In order to obtain large quantities of viable protoplasts, we tested seven different mixtures of enzymes to find the optimum isolation conditions (Table 1). The results indicated that the kind and concentration of enzymes significantly affected protoplast yield and quality. It can be seen from Table 1 that culture in group E7, which contained 2% (w/v) Cellulase R-10, 1.5% (w/v) Macerozyme Onozuka R-10, 0.2% (w/v) Driselase and 0.1%(w/v) Pectolyase Y-23, resulted in the highest yield 8.4±0.40 x 10^6 protoplasts g^{-1} FW and viability 98.8±1.39size on average 30-35 µm in diameter of *Stevia* mesophyll protoplasts. When the concentration of Cellulose R-10 was decreased to 1.0% or increased to 3%, the yield (group E1, E3) and viability of the protoplast significantly decreased (81.8±3.49 to 79.9±3.12). However, viability of protoplast obtained from E3 (81.8±3.49 x10^6 protoplasts g^{-1} FW) was not significantly different from that of E2 (82.1±2.51 x16^5 protoplasts g^{-1} FW). Absence of Pectolyase Y-23 (E1-E4) from the enzyme mixture also resulted in lower viability of the protoplast. Cellulase R-10 was more effective for protoplast isolation than Cellulase R-S (data not shown). Pectolyase Y-23 was more effective and has been used successfully for isolating mesophyll protoplasts in many species such as *Crytocoryne wendtii* [31]. Nagata and Ishii [32] indicated that Pectolyase Y-23 has about 50 times stronger endo-polygalacturonase activity than Macerozyme R-10.

3.2 Effect of Osmotic Potential of the Enzyme Solution

The number of viable protoplasts isolated was strongly affected by the osmotic potential condition of mannitol used in protoplast isolation and purification process given the fresh weight of leaf tissue was fixed at 1.0 g fresh weight. Intact protoplasts were observed in most of the mannitol concentrations (Table 2) suggesting that *Stevia* protoplasts were capable of withstanding a wide range of osmotic potential.

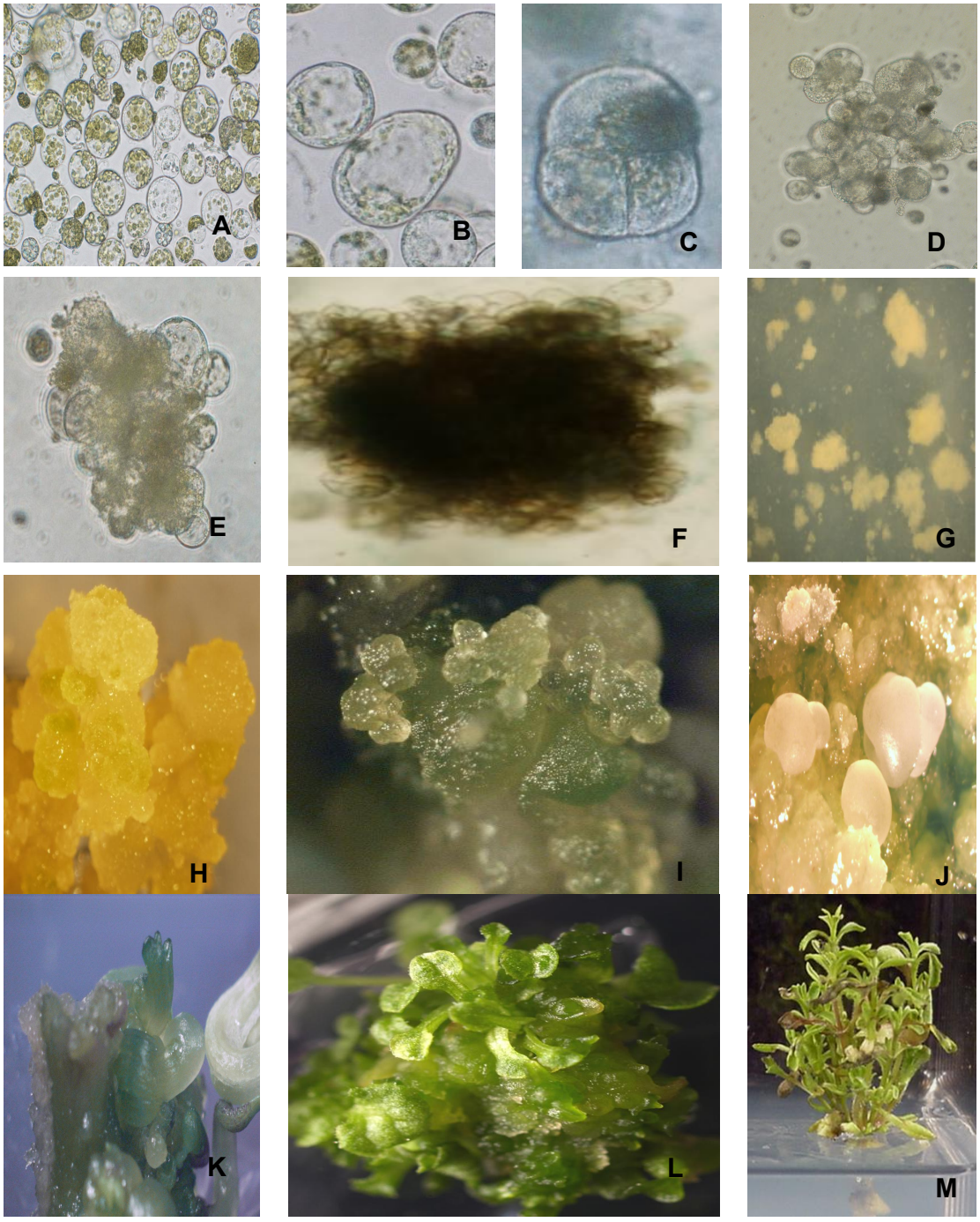

Fig. 1A-M. Plant regeneration from mesophyll protoplast-derived embryogenic calli of Stevia *rebaudiana: (A) Freshly isolated mesophyll protoplast, (B) Enlarged leaf-derived protoplasts with visible cytoplasmic strand before mitotic division, (C-E) First symmetric and asymmetric division; and cell colony formation, respectively, (F-G) Protoplast-derived microcalli on agarose-solidified KM8P medium after 8 weeks of culture, (H-J) Protoplast-derived globular and heart-shaped somatic embryos on solid MS medium, (K) Somatic embryos in cotyledonary stage formed on MS solid medium, (L) Adventitious shoots produced from protoplast-derived embryogenic callus on MS medium, (M) Plantlets from protoplast via somatic embryogenesis*

However, for following experiments, mannitol concentration in the enzyme solution was kept at 0.5 M corresponding to an osmotic potential of -2.25 MPa, as this gave the highest yield (8.66±0.32 x10^6 protoplasts g^{-1} FW) and viability (98.0±1.25%) (Table 2). When using mannitol at 0.2 or 0.7 M concentration, both protoplast yield and viability were lower than the optimal concentration (0.5 M Mannitol). Generally, protoplasts burst in hypotonic solution and collapse in hypertonic solution [33]. It was concluded that 0.5 M has suitable osmotic pressure for mesophyll protoplast isolation of *S. rebaudiana*. This concentration was different from that used in aquatic plant *Cryptocoryne wendtii* De Wit [31,34].

3.3 Effect of Enzyme Incubation Period on Protoplast Yield and Viability

The effect of duration for which leaf segments and protoplast digestive enzymes (3-10 h) are incubated was found to have a significant influence on yield and viability of protoplasts. As shown in Table 3, protoplast yield and viability increased when the duration of enzyme digestion was increased from 3 to 5 hrs. Optimal time of incubation was 5 h, which gave the highest yield (8.21±1.08 x 10^6 protoplast g^{-1} FW) and viability (94.9±1.79%) of protoplasts. However, when the incubation time was longer than 5 h, the viability of protoplasts decreased. Prolonged incubation of leaves in enzyme solution could potentially lead to protoplast mass breaking and turning brown. Damage could be minimized by modifying the enzyme solution (lower concentration of enzymes) or duration (shortening) of enzymatic treatment [35].

3.4 Effect of Different Explants on Protoplast Yield and Viability

The effect of different explants on protoplast yield and viability was studied using *in vitro* raised plants. Direct observation under inverted microscope revealed that maximum digestion of cell walls was found in the leaf tissue (approximately 6.98±0.97 x10^6 protoplasts g^{-1} FW with 98.0±1.25% converted protoplasts being viable). In comparison to the leaf explants, the conversion of cells into protoplasts for the petiole was 2.03±0.96; hypocotyl, 0.76±0.28% and root 1.22±0.19 x 10^6 protoplasts g^{-1} FW (Table 4). Since the protoplasts obtained from leaf explants were found rich in cytoplasm and had regularly arranged chloroplasts (Fig. 1A), leaf explants were used to study the effect of age of explants on protoplast yield and viability and for the rest of the experimental treatments.

Table 1. Effect of enzyme concentration and combinations on protoplast yield and viability

Enzyme solution	Cellulase onozuka R-10 (%)	Macerozyme onozuka-R-10 (%)	Driselase (%)	Pectolyase Y-23 (%)	Protoplast yield[a] (x 10^6 rotoplasts g^{-1} FW)	Protoplast viability[a] (%)
E1	1.0	1.0	0.2	0	6.4±0.76	79.9±3.12
E2	2.0	1.0	0.2	0	5.4±0.76	82.1±2.51
E3	3.0	1.0	0.2	0	5.6±1.10	81.8±3.49
E4	1.5	1.0	0.2	0	6.7±0.72	79.8±3.97
E5	1.5	1.0	0.5	0.1	4.9±0.38	88.2±1.93
E6	2.0	1.0	0.2	0.1	6.1±0.41	87.0±2.00
E7	2.0	1.5	0.2	0.1	8.4±0.40	98.8±1.39

.[a]Data represent mean ± standard error of three independent replicates

Table 2. Effect of osmotic potential on protoplast yield and viability

Mannitol concentration (M)	Protoplast yield[a] (x 10^6 protoplasts g^{-1} FW)	Protoplast viability[a] (%)
0.0	0.93±0.31	28.9±5.67
0.2	0.93±0.31	30.2±4.85
0.3	4.14±0.53	75.9±3.73
0.4	7.38±0.44	94.9±1.79
0.5	8.66±0.32	98.0±1.25
0.6	6.00±0.34	73.3±2.91
0.7	2.01±0.41	60.0±4.39

[a]Data represent mean ± standard error of three independent experiments

3.5 Effect of Plant Material Age on Protoplast Yield and Viability

In vitro grownyoung leaves of one to seven-week-old *Stevia* plants were used for protoplast isolation. It was found that the age of leaves influenced the viability and yield of protoplasts. Four week old leaves provided the highest yields (6.89±1.10 x10^6 protoplasts g^{-1} FW) and viability (98.1±1.20%) of protoplasts. older leaves (five, six and seven-week-old) or younger leaves (one and two week-old) resulted in a lower protoplast yield and viability (Table 5). Similar results indicating that age of leaves could influence the number of isolated protoplasts were shown in plants such as sweet potato [36], soybean [37] *Muscari* [38] and carrot [39]. It was difficult to isolate protoplasts from older leaves because more lignin substances accumulate in cell walls of old cells. Therefore, a four-week-old leaf was the appropriate age for protoplast isolation.

3.6 Protoplast Culture Method

To assess protoplast viability and plating efficiency, leaf protoplasts were cultured in KM 8 P media. Significant differences in plating efficiencies and protoplast survival rates were found in the two different culture methods tested. Culturing protoplasts using agarose-bead compared to thin liquid layer culture method resulted in high cell division and plating efficiencies (Table 6). Cell wall synthesis occurred within 24 h and could be observed by Calcofluor white staining. In the first day of culture the proportion of viable protoplasts was high (81.5%), in the next 5 days it decreased to 75% and started decreasing afterwards even after 7 days of culture (Fig. 1B).

Table 3. Effect of enzyme incubation period on protoplast yield and viability of *Stevia*

Incubation time (h)	Protoplast yield[a] (x 10^6 protoplasts g^{-1} FW)	Protoplast viability[a] (%)
1	1.57±0.42	60.0±4.14
2	2.06±0.17	58.4±3.34
3	7.14±0.77	75.9±3.73
4	6.89±1.10	98.0±1.25
5	8.21±1.08	94.9±1.79
6	5.82±0.41	90.2±1.81
8	3.74±0.34	73.3±3.08
10	2.10±3.73	60.0±3.05

[a]*Data represent mean ± standard error of three independent experiments.*

Table 4. Isolation efficiency of protoplast yield and viability from different *Stevia* explants

Explant type	Protoplast yield[a] (x10^6 protoplasts g^{-1} FW)	Protoplast viability[a] (%)
Leaf	6.98±0.97	98.0±1.25
Petiole	2.03±0.96	57.0±6.22
Root	1.22±0.19	41.3±5.59
Hypocotyls	0.76±0.28	22.3±1.42

[a]*Data represent mean ± standard error of three replicates.*

Table 5. Effect of plant material age on protoplast yield and viability

Age of leaf tissue	Protoplast yield[a] (10^6 protoplasts g^{-1} FW)	Protoplast viability[a] (%)
1 week	2.00±0.52	61.4±5.96
2 weeks	1.94±1.01	71.4±6.41
3 weeks	4.11±0.85	89.4±2.31
4 weeks	6.89±1.10	98.1±1.20
5 weeks	2.22±0.69	89.7±2.45
6 weeks	1.13±0.12	78.9±4.41
7 weeks	1.09±0.14	60.0±3.50

. [a]*Data represent mean ± standard error of three replicates.*

Table 6. Effect of culture methods on survival rate and plating efficiency of protoplasts

Days on culture medium	Culture method	Survival rate % of individual protoplast/colony	Plating efficiency %
1 day	Liquid layer culture	66.1±2.47	27.5±2.27
	Agarose bead	81.5±4.21	33.6±2.00
7 days	Liquid layer culture	61.7±2.26	21.3±1.90
	Agarose bead	75.9±2.47	29.4±2.58
14 days	Liquid layer culture	36.7±2.20	12.7±1.74
	Agarose bead	61.6±2.22	20.1±1.20
21 days	Liquid layer culture	20.0±1.41	11.3±1.25
	Agarose bead	31.3±1.41	14.1±1.10
28 days	Liquid layer culture	14.7±1.63	9.1±1.54
	Agarose bead	23.4±1.43	12.7±1.75

[a]*Data represent mean ± standard error of three replicates*

Three days after culture most of the protoplasts enlarged and started to change their shape from spherical to oval indicating a reconstruction of the cell wall (Fig. 1C). Two kinds of first cell division, i.e. symmetrical as well as asymmetrical, were observed. After 7 days of culture, when division frequency was measured, the protoplasts in the liquid media had increased in size, but still had not divided. In 7-days-old agarose bead cultures two-, four-, and multi-cell colonies were formed (Fig. 1D). At that time about 30% of plated protoplasts underwent first cell division while 14 days later the frequency of cell colony formation was almost 65%. Microscopic observations showed that cells in the colonies were tightly packed and had dense cytoplasm, suggesting that they might be embryogenic (Fig. 1E, F and G). Due to lack of synchrony in development, the size of the colonies varied considerably. Beneficial influence of embedding protoplasts in agarose or calcium alginate was proven in numerous studies [40]. Those types of cultures keep protoplasts from rupturing or aggregating and also stabilize membranes by inhibiting lipid peroxidation and by preventing leakage of cell wall precursors and other metabolites [41]. The level of ethylene in protoplast culture in medium solidified with calcium alginate can be even ten times less compared to liquid medium [36, 42]. In a study on *Gentiana crassicaulis*, plating efficiency with agarose medium was 14% higher than with liquid culture [43-45]. Our study also shows that solidifying the culture medium with Sea Plaque agarose stimulates the initial stages of protoplast division.

3.7 Somatic Embryogenesis and Plant Regeneration

Protoplast-derived micro-calli was transferred onto MS medium containing 2, 4-D (0.5-2.0 mg L^{-1}NAA) and BA 1.0 mg $L^{-1}$with 500 mg/l CH, 3% sucrose. After 4 weeks of culture, yellowish, granular callus was observed on the surface of micro-calli in the agarose beads. Transferring of agarose beads to an agar MS medium containing 2, 4-D (0.5-2.0 mg L^{-1}NAA) and BA 1.0 mg L^{-1} with 500 mg/l CH, 3% sucrose resulted in embryogenic callus. All the stages of somatic embryogenesis such as globular, heart-shaped and bipolar embryos were observed (Figs. 1g and H). Somatic embryo development in the present case was asynchronous and various stages of globular, heart-shaped and torpedo like embryos could be observed simultaneously in the same embryogenic calli, as previously described for some members of *Liliaceae* and *Iridaceae* [44]. A few days later, it was possible to isolate bipolar embryos that subsequently germinated on MS medium supplemented with 1.0 mg L^{-1}NAA for rooting.

Conversion of somatic embryos to plantlets was obtained on the same medium after 1 month of culture (Fig. 1I). The data reported here demonstrated plantlet regeneration from protoplasts derived embryogenic calli of *Stevia* for the first time. This effective approach offers the possibility to mass multiply material that has been improved by genetic manipulation experiments.

4. CONCLUSION

We developed an efficient method for isolation, culture and regeneration of protoplast of *Stevia*. Protoplasts were successfully isolated from leaves of four-week old *in vitro* grown plants using an enzyme mixture comprising of 2% (w/v) Cellulase Onozuka R-10, 1.5% (w/v) Macerozyme Onozuka R-10, 0.2% (w/v) Driselase and 0.1%(w/v) Pectolyase Y-23 in 0.5 M mannitol, 2.5 mM $CaCl_2.2H_2O$ and 5 mM 2 (N-

morpholino)-ethanesulfonic acid (MES) at pH of 5.8. Approximately 8.4±0.40 x 10^6 protoplasts g^{-1} FW) fresh weights with 98.8±1.39% viable protoplasts were obtained after 4 hrs of incubation in the dark. First cell division was observed after culturing protoplasts for 2-3 days and micro- colonies were formed within 4 weeks. Plant regeneration was achieved through somatic embryogenesis. This protocol might lead to the improvement of the *Stevia* through somatic hybridization, somaclonal variation and genetic engineering by using protoplast based regeneration system.

ACKNOWLEDGEMENTS

This work was supported by the National Science Foundation grants (Division of Biological Infrastructure: Directorate for Biological Sciences, REU-Site #1004764 and Division of Human Resource Development: Directorate for Education & Human Resources HBCU-UP #0625289). Author Alicia Williams supported the work by providing material support.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

REFERENCES

1. Hanson JR, De Oliveira BH. *Stevioside* and related sweet diterpenoid glycosides. Natural Product Reports. 1993;10:301–309.
2. Lewis WH. Early uses of *Stevia rebaudiana* (*Asteraceae)* leaves as a sweetener in Paraguay. Economic Botany. 1992;46:336–337.
3. Kinghorn AD, Soejarto DD. Discovery of terpenoid and phenolic sweeteners from plants. Pure and Applied Chemistry. 2002;74:1169–1179.
4. Skaria BP, Joseph R, Mathew G, Malhew S, Joy PP. *Stevia:* A sweet herb. Indian Journal of Arecanut Spices and Medicinal Plants. 2004;6:24–27.
5. Chatsudthipong V, Muanprasat C. Stevioside and related compounds: Therapeutic benefits beyond sweetness. Pharmacology & Therapeutics. 2009*;*121: 41–54.
6. Lemus-Mondaca R, Vega-Galvez A, Zura-Bravo L, Ah-Hen Kong. Stevia rebaudiana Bertoni, source of a high-potency natural sweetener: A comprehensive review on the biochemical, nutritional and functional aspects. Food Chem. 2012;132:1121–1132.
7. Mishra PK, Singh R, Kumar U, Prakash V. *Stevia rebaudiana* a magical sweetener. Global J Biotechnol Biochem. 2011;5:62–74.
8. Yadav AK, Singh S, Dhyani D, Ahuja PS. A review on the improvement of stevia [*Stevia rebaudiana* (Bertoni)]. Canadian Journal of Plant Science. 2011;91:1–27.
9. Kalpana M, Anbazhagan M, Natarajan V, Dhanavel D. Improved micropropagation method for the enhancement of biomass in *Stevia rebaudiana Bertoni.* Recent Research on Science and Technology. 2010;2:8–13.
10. Madan S, Ahmad S, Singh GN, Kohli K, Kumar Y, Singh R, Garg M. *Stevia rebaudiana* (Bert.) *Bertoni*—a review. India J Nat Prod Resource. 2010;1:267–286.
11. Hassanen SA, Khalil RMA. Biotechnological studies for improving of Stevia (*Stevia rebaudiana* Bertoni) *in vitro* plantlets. Middle-East Journal of Scientific Research. 2013;14:93–106.
12. Javad S, Naz S, Ilyas S, Mateen B. Establishment of the honey crop (*Stevia rebaudiana*) in hot semi-arid climate. The Journal of Animal and Plant Sciences. 2013;23:108–113.
13. Dey A, Kundu S, Bandyopadhyay A, Bhattacharjee A. Efficient micropropagation and chlorocholine chloride induced *stevioside* production of *Stevia rebaudiana bertoni.* CR Biologies. 2013;336:17–28.
14. Grosser J, Calovic M, Louzada E. Protoplast fusion technology–somatic hybridization and cybridization. Plant cell culture: Essential methods. John & Wiley Sons; 2010.
15. Davey M, Anthony P, Patel D, Power J. Plant Protoplasts: Isolation, Culture and Plant Regeneration. Plant cell culture: Essential methods. John Wiley & Sons; 2010.
16. Eeckhaut T, Shankar Lakshmanan P, Deryckere D, Van Bockstaele E, Van Huylenbroeck J. Progress in plant protoplast research. Planta. 2013;238:991-1003.
17. Rirera AL, Gomez-Lim M, Fernandez F, Loske AM. Physical Methods for Genetic Plant transformation. Physics of Life Review. 2012;9:308-345.

18. Giridhar P, Sowmya SK, Ramakrishna A, Ravishankar GA. Rapid clonal propagation and stevioside profiles of *Stevia rebaudiana* Bertoni. Int J Dev Biol. 2010;4:47–52.
19. Janarthanam B, Gopalakrishnan M, Lakshmi Sai G, Sekar T. Plant regeneration from leaf derived callus of *Stevia rebaudiana Bertoni*. Plant Tissue Culture and Biotechnology. 2009;19:133–141.
20. Kalpana M, Anbazhagan M, Natarajan V, Dhanavel D. Improved micropropagation method for the enhancement of biomass in *Stevia rebaudiana bertoni*. Recent Research on Science and Technology. 2010; 2: 8–13.
21. Mehta J, Sain M, Sharma DR, Gehlot P, Sharma P, Dhaker JK. Micropropagation of an anti-diabetic plant—*Stevia rebaudiana bertoni,* (Natural Sweetener) in Hadoti region of south-east Rajasthan, India. ISCA Journal of Biological Science. 2012;1:37–42.
22. Lata H, Chandra S, Wang YH, Raman V, Khan IA. TDZ-induced high frequency plant regeneration through direct shoot organogenesis in *Stevia rebaudiana bertoni*: An important medicinal plant and a natural sweetener. American Journal of Plant Sciences. 2013;4:117–128.
23. El-Motaleb MA, El-Hady MAS, El-Kholy MA, Badr A. *In vitro* propagation of *Stevia rebaudiana bertoni* in Egypt. Journal of Applied Science Research. 2013;9:4597–4605.
24. Pande SS, Gupta P. Plant tissue culture of *Stevia rebaudiana* (*Bertoni*): A review. Journal of Pharmacognosy Phytotherapy. 2013;5:26–33.
25. Khan SA, Rahman LU, Shanker K, Singh M. *Agrobacterium tumefaciens*-mediated transgenic plant and somaclone production through direct and indirect regeneration from leaves in *Steviai rebaudiana* with their glycoside profile. Protoplasma. 2014;251: 661-670.
26. Singh HP, Dhir S, Dhir SK. *Stevia*: In the Transgenic and Molecular Tailoring in Plants, Transgenic Series, Pulses, Sugar and Tuber Crops, Blackwell Publishing, (eds.), Chitta R. Kole, Ralph Scorza and Timothy C. Hall. 2008;3:97-115.
27. Murashige T, Skoog F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiology Plant. 1962;15:473–497.
28. Kao KN, Michayluk MR. Nutritional requirements for growth of *Viciahajastana* cells and protoplasts at a very low population density in liquid media. Planta. 1975;126:105–110.
29. Widholm JM. The use of fluorescein diacetate and phenosafranine for determining viability of cultured plant cells. Stain Techno. 1972;47:189–194.
30. Nagata T, Takebe I. Cell wall regeneration and cell division in isolated tobacco mesophyll protoplasts. Planta. 1970;92:301–308.
31. Pongchawee K, Na-Nakorn U, Lamseejan S, Poompuang S, Phansiri S. Protoplast isolation and culture of aquatic plant *Crytocoryne wendtii* De Wit. Kasetsart J. 2007;41:300-310.
32. Nagata T, Ishii S. A rapid method for isolation of mesophyll protoplast. Can. J. Bot. 1979;57:1820-1823.
33. Davey MR, Anthony P, Power JB, Lowe KC. Plant protoplasts: Status and biotechnological perspectives. Biotechnol Adv. 2005;23:131–171.
34. Pongchawee K, Na-Nakorn U, Lamseejan S, Poompuang S, Phansiri S. Factors affecting protoplast isolation and culture of *Anubias nana* Engler. International Journal of Botany. 2006;2(2):193-200.
35. Navratilova B, Rokytova L, Lebeda A. Isolation of mesophyll protoplasts of *Cucumis* spp. And *Cudurbita* spp. Acta Hortic. 2000;510:425-431.
36. Dhir SK, Oglesby J, Bhagsari AS. Plant regeneration via somatic embryogenesis and transient gene expression in sweet potato protoplasts. Plant Cell Rep.1998;17:665-669.
37. Dhir SK, Dhir S, Widholm JM. Plantlet regeneration from immature cotyledon protoplasts of Soybean (Glycine max (L) Merr.). Plant Cell Rep. 1991;10:39-43.
38. Karamian R, Ranjbar M. Somatic embryogenesis and plantlet regeneration from protoplast culture of *Muscari neglectum* Guss. African Journal of Biotechnology. 2010;10:4602- 4607.
39. Ewa G, Marek S, Rafal B. An improved protocol for plant regeneration from leaf and hypocotyl-derived protoplasts of carrot. Plant Cell Tiss Organ Cult. 2012;109:101–109.
40. Conde P, Santos C. An efficient protocol for *Ulmus minor* Mill. Protoplast isolation

and culture in agarose droplets. Plant Cell Tissue Organ Cult. 2006;86:359–366.
41. Fiuk A, Rybczynski J. The effect of several factors on somatic embryogenesis and plant regeneration in protoplast cultures of *Gentiana kurroo* (Royle). Plant Cell Tiss Org. 2007;91:263-271.
42. Wang J, Jiang J, Wang Y. Protoplast fusion for crop improvement and breeding in China. Plant Cell Tiss Org. 2013;112:131-142.
43. Deryckere D, Eeckhaut T, Van Huylenbroeck J, Van Bockstaele E. Low melting point agarose beads as a standard method for plantlet regeneration from protoplasts within the *Cichorium* genus. Plant Cell Rep. 2012;31:2261-2269.
44. Suzuki S, Nakano M. Organogenesis and somatic embryogenesis from callus cultures in *Muscari armeniacum* Leichtl. Ex Bak. *In vitro* Cell. Dev. Biol. Plant. 2001;37:382-387.
45. Karamian R. Somatic embryogenesis and plant regeneration from protoplast culture of *Crocus pallasii* subsp. haussknechtii. Pak. J. Biol. Sci. 2007;10(4):659-663.

Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=801&id=11&aid=6884