

Plant Regeneration from Cell Suspension Protoplasts of Two Iranian Japonica Rice Cultivars and Ploidy Level of Regenerated Plants

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ABSTRACT

An efficient system has been developed for the reproducible regeneration of plants from cell suspension-derived protoplasts of the two commercial Iranian Japonica rice cultivars Tarom and Khazer. Friable embryogenic calli were used for cell suspension initiation and protoplasts were isolated from both varieties. When embedded in agarose, protoplasts failed to divide. However, sustained divisions were obtained by using nurse cells of *Lolium multiflorum*. Plant regeneration was 21.1% and 10.5% for Tarom and Khazer, respectively. Somaclonal variation was observed amongst regenerated plants, 16.2% of regenerants were tetraploid, and the rest were diploid.

Keywords: Cell suspension, Iranian Japonica rice, *Oryza sativa*, Plant regeneration, Ploidy, protoplast culture.

INTRODUCTION

Efficient and reproducible methods of plant regeneration from rice calli have been reported by several researchers (Ozawa and Komamine, 1989; Jain *et al.*, 1995). Such a development is largely due to improvements in cell suspension cultures and the enhancement of protoplast culture techniques. Plants regenerated through cell cultures are known to undergo various changes, especially in chromosome number and structure (Orton, 1980; Murata and Orton, 1983; D' Amato, 1965). Ploidy level is conducted by means of chromosome counting using the meristematic tissues of plants. Flow cytometry can also be used for rapid measurement of the DNA content of interphase nuclei or chromosomes in order to study ploidy levels (De Laat *et al.*, 1989).

Plants regenerated from somatic cells through tissue and protoplast cultures are not

genetically uniform and exhibit significant variability. This variability (somaclonal variation) can be utilized for the development of new varieties (Evans and Sharp, 1986). Some workers reported that there are somaclonal, protoclonal and molecular variations in the seed progeny of regenerated plants from callus and protoplasts of rice (Abdullah *et al.*, 1989; Zheng *et al.*, 1989; Saleh *et al.*, 1990; Müller *et al.*, 1990; Davey *et al.*, 1991). Commonly, variations observed in tissue or protoplast-derived rice plants included the number of tillers per plant, plant height, flag leaf length, flag leaf width, days to flowering, panicle length, number of branches per panicle, number of spikelets per panicle, number of grains per plant, grain length, grain width and the grain length/width ratio.

In this paper, an efficient procedure is reported that can enhance plant regeneration in the two commercially cultivated Iranian Ja-

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ponica rice plants cvs. Tarom and Khazer. Also the agronomic characteristics and ploidy levels of protoplast-derived plants were studied.

MATERIALS AND METHODS

Seeds of *Oryza sativa* L. cvs. Tarom and Khazer were supplied by Amol Rice Research Station, Amol, Mazandaran, Iran. For confirmation of varietal grouping, seeds were sent to the International Rice Research Institute (Philippines) for isosyme analysis. Both cultivars belong to the group VI-Japonica rice.

Establishment of Cell Suspension

Dehusked rice seeds were surface sterilized in 30% (v/v) 'Domestos' bleach (Lever Industrial Ltd., Runcorn, Cheshire, UK.) and inoculated onto the surface of LS medium (Linsmaier and Skoog, 1965) supplemented with 2.5 mg l⁻¹ 2,4-D and made semi-solid by the addition of 4 g l⁻¹ SeaKem agarose (FMC BioProducts, Rockland, ME, USA). Embryogenic calli which developed on the scutellar surface of mature seeds (2-3 months post initiation) were used for the initiation of cell suspension cultures. Approximately 1 g f. wt. of callus was transferred to a 25 ml conical flask containing 7 ml of liquid medium. R2 (Ohira *et al.*, 1973) and AA2 (Müller and Grafe, 1978) for the initiation of cell suspension cultures of cvs. Tarom and Khazer, respectively. Cultures were incubated on a rotary platform shaker at 120 rpm (2 cm throw) at 26 ± 1°C in the dark. During the first stage of the initiation of suspension cultures, 6 ml of the culture medium was replaced with the same volume of fresh medium at three to four day intervals. After three to four weeks, cultures were transferred to 100 ml Erlenmeyer flasks and all the culture medium was replaced with 14 ml of fresh medium. Two weeks later, the culture medium was replaced with 21 ml of fresh medium and the

suspension was subcultured at seven day intervals by transferring 1 ml packed cell volume (PCV). After another two weeks, the culture medium was again replaced with 26 ml of fresh medium. Established embryogenic cell suspension cultures (3-4 months old) were used for protoplast production.

Protoplast Isolation

Protoplasts were isolated enzymatically using the procedure given by Thompson *et al.*, (1986). Protoplasts were then resuspended in KPR (Kao and Michayluk, 1975) liquid medium at a density of 5 x 10⁵ ml⁻¹. Protoplasts were washed twice in CPW13M solution and cultured using two methods.

Protoplast Culture

Agarose embedding method: Isolated protoplasts were cultured in KPR liquid medium at a density of 5 x 10⁵ ml⁻¹ made semi-solidified with 1.2% (w/v) of Sea Plaque agarose (FMC Crop., Rockland, ME, USA). The medium was prepared by mixing equal volumes of double-strength KPR medium with molten 2.4% (w/v) Sea Plaque aqueous agarose solution to give a final concentration of 1.2% (w/v). Protoplasts were suspended again at a density of 5x10⁵ ml⁻¹ in a molten KPR medium containing 1.2% (w/v) Sea Plaque agarose. The protoplasts were cultured as a 1.5 ml layer in sealed 3.5 cm diameter Petri dishes (A/S Nunc, Roskilde, Denmark) in the dark at 26 ± 1°C.

Nurse culture method: To prepare the feeder plates, 5 ml packed cell volume (PCV) of *Lolium multiflorum* cells were suspended in 50 ml of KPR medium. Cells were harvested three to four days after subculture and nurse plates were set up one day prior to protoplast isolation. Cell suspension cultures of *L. multiflorum* were obtained from Dr. P. J. Dale, (IRAT-CIRAD, BIOTROP- GERDAT laboratory, BP 5035, 34032 Montpellier Cédex 01, France) and

were maintained by weekly subculture in N6 medium (Chu *et al.*, 1975) at Nottingham.

The nurse cells were mixed with double-strength KPR liquid medium containing molten 2.4% (w/v) Sea Plaque agarose (Jain *et al.*, 1995) and were dispensed into 3.5 cm Petri dishes, 5ml aliquots per dish. A Whatman cellulose nitrate filter membrane (47 mm dimension, 0.2 mm pore size; Whatman Paper Ltd., Kent, UK) was placed on the surface of this feeder culture. A 0.2 ml aliquot of protoplasts, at a density of $5 \times 10^5 \text{ ml}^{-1}$ was cultured on the surface of the filter membranes. Petri dishes were sealed with Nescofilm and incubated in the dark at $26 \pm 1^\circ\text{C}$. The protoplast plating efficiency was determined as the percentage of the total number of protoplast-derived colonies counted on the 14th and 28th day of culture divided by the protoplasts plated.

Plant Regeneration from Protoplasts

Protoplast-derived micro-calli (1-20 mm in diameter; 4-5 weeks old) were transferred onto agarose-solidified regeneration MS medium (Murashige and Skoog, 1962) supplemented with 2 mg l^{-1} kinetin, 0.5 mg l^{-1} NAA and 3% (w/v) sucrose; this was designated MSKN medium. After being transferred onto MSKN medium, protoplast-derived colonies continued growth and plantlets were regenerated from some colonies after two to four weeks.

ANALYSIS OF PLOIDY LEVEL

The ploidy level was assessed by flow cytometric analysis of protoplast-derived and seed-derived control plants (three samples of each cultivar). Leaf samples were collected from *in vitro* regenerated plants that had been subcultured for two to three weeks on Murashige and Skoog (1962) medium supplemented with 2 mg l^{-1} BAP, 0.4% agarose and 3% (w/v) sucrose at pH 5.8 (designated MSBP). Young leaves were excised from individual regenerants and the seedlings of

controls and 0.5 g wt. leaf tissues were chopped (0.5 mm^2) and placed in a 9 cm glass Petri dish containing 1.5 ml of a buffer solution (15 mM Tris, 2mM EDTA, 0.5 mM Spermine, 80 mM KCl, 20 mM NaCl, 0.1% (v/v) Triton X-100, 50 $\mu\text{g/ml}$ ethidium bromide, pH 7.5; Dolzel *et al.*, 1989). All reagents were obtained from Sigma Chemical Co. Ltd., UK.. Nuclei (and the rest of the homogenates, containing cell organelles, tissue and cellular debris) were passed through a $30 \mu\text{m}$ pore size nylon sieve. Isolated nuclei were collected in the 1.5 ml centrifuge tube, and were used for flow cytometric analysis. Fifty μl of a solution of fluorescently labeled 'Immuno-Check' polystyrene beads (Coulter Electronics Ltd., Luton, UK) were added to the filtrate as an internal fluorescence standard. Fluorescence measurements were performed using a Coulter EPICS 541 flow cytometer. The argonion laser was tuned to produce 100 mw output at a wavelength of 488 nm. Ten thousand particles were analyzed per sample. Green fluorescence histograms were collected and following transfer to a PC computer and the relative mean linear fluorescence values of the Go/G1 nuclei were determined.

Morphological Characteristics of Regenerated Plants

A population of 51 protoplast derived plants (34 of Tarom and 17 of Khazer) and 16 seed-derived plants (8 of Tarom and 8 of Khazer) were analyzed morphologically. Regenerated protoclonal, approximately 1.0-2.0 cm in height, were transferred to MSBP medium and grown under continuous light ($55 \mu\text{mol m}^{-2} \text{ s}^{-1}$, daylight fluorescent tubes) at $26 \pm 1^\circ\text{C}$ for micro propagation. After three weeks multiple shoots, developed on MSBP medium, were separated and transferred to 175 ml glass jars containing 30-40 ml of MSN1.5 [MS-based medium supplemented with 1.5 mg l^{-1} NAA and 3% (w/v) sucrose] and grown under the same conditions. Plants (10-12 cm in height) were removed from these jars, their roots washed

gently with tap water to remove agarose. Individual plants were placed in 10 cm plastic pots which contained a mixture of John Innes No. 3 (Joseph Bentley Ltd. South Humberside, UK.), Levington M3 soil-less composts (Fisons plc, Ipswich, Suffolk, UK.) and perlite (Silvaperl Ltd., Gainsborough, Lincolnshire, UK.) in the ratio of 6:6:1 (v/v). When plants showed sustained growth, the ventilation was progressively increased to a maximum after 1-2 weeks.

The recording of plant characteristic such as, plant height, total number of tillers, flag leaf length, flag leaf width, flag leaf length/width ratio, number of days to flowering, panicle length, number of primary branches per panicle, number of secondary branches per panicle, number of spikelets, grain length, grain width and grain length/

soft and yellow-to-brown colour, whilst embryogenic callus was identifiable by its compact, dry and globular appearance and pale yellow colour. Some calli of both cultivars were non-embryogenic with root primordia (Table 1). There was a significant difference ($p < 0.05$) in embryogenic calli production between the two varieties.

For the establishment of embryogenic cell suspension cultures, during the first 2-3 weeks, the majority of cells, or groups of cells were observed to become thick-walled and vacuolated. This phase was associated with a gradual acceleration of growth, and the development of densely cytoplasmic cells. The groups of cells with dense cytoplasm were actively dividing, and the population of these dividing groups of cells gradually increased with time. Weekly sub-

Table 1. Type of callus and callus induction frequencies from mature embryos of different varieties of *Oryza sativa*.

Cultivar	Callus type	% seeds producing callus	% callus with root primordia	% of embryogenic calli
Tarom	Friable	84 ± 1.88	2.67 ± 0.94	68.00 ± 4.33*
Khazer	Friable, mucilaginous	78 ± 2.50	5.33 ± 0.51	43.33 ± 3.40

Data represent the means ± standard error (SE) based on three replicate experiments; 60 mature seeds were used in each experiment. *: Significantly at $p < 0.05$.

width ratio were recorded for each plant including controls.

Statistical analyses were performed according to the programme InStat Graphic Software (1992). Means, standard error and variances were calculated and statistical significance between mean values was assessed using a Student *t*-test. A probability of $p < 0.05$ was considered significant.

RESULTS

After 2-3 weeks of protoplast culture following cell division, callus masses were produced. Initially, both embryogenic and non-embryogenic calli were formed (Table 1). Non-embryogenic callus consisted of rooty, elongated cells, sometimes with a wet,

culture led to the development of cultures containing densely cytoplasmic cell colonies. It was observed that AA2 medium was suitable for the initiation of cell suspension cultures of cv. Khazer. In the case of Tarom, when calli were transferred to AA2 medium, browning of calli occurred. However, R2 medium was subsequently used for the initiation of cell suspension cultures in this cultivar. After 3-4 months, the cell suspension cultures were suitable for protoplast isolation.

Protoplasts were isolated routinely from cell suspension cultures of both cultivars. The yield of protoplasts for both cultivars is shown in Table 2. The age of cell suspension cultures greatly influenced the yield of protoplasts (Figure 1). Efficient protoplast isolation could be obtained from 5-12 month-

Table 2. Protoplast plating efficiency under two culture methods and plant regeneration from protoplast-derived calli of rice cultivars.

Cultivar	Protoplast yield ($\times 10^6/g$ f.wt. cells)	Plating efficiency (%)				No. of plants re-generated	(% Plant regeneration frequency *)
		14d		28d			
		Embedding	Nurse method	Embedding	Nurse method		
Tarom	3.98 ± 0.51	0	2.7 ± 0.09	0	0.11 ± 0.01	143	14.3 ± 1.98
Khazer	10.75 ± 0.87	0	1.2 ± 0.11	0	0.07 ± 0.01	42	10.5 ± 1.64

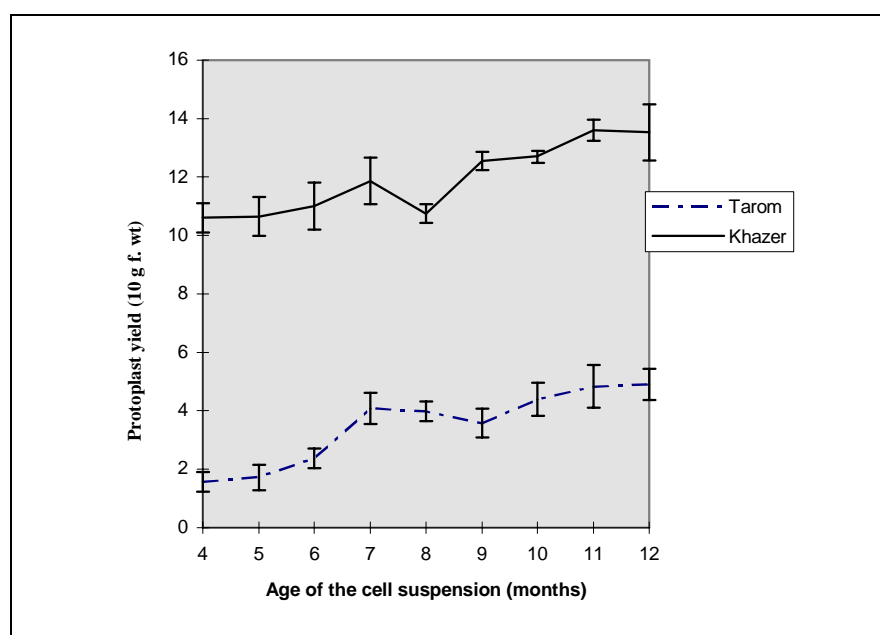
* Percentage of calli, which produced plants when, transferred to regeneration media. Data were collected from three replicates and represent the mean \pm standard error (SE).

old cell suspension cultures of both cultivars. Generally, the older the suspension cultures, the higher the protoplast yield in the eight month period after initiation.

Protoplasts embedded in agarose failed to divide. In order to obtain sustained protoplast division, protoplasts of cvs. Tarom and Khazer had to be cultured using the nurse culture method. The influence of culture method on callus induction from protoplasts is shown in Table 2.

Protoplasts cultured on membrane over feeder cells began to swell during the first week of culture and microcolonies had

formed after two weeks. It was difficult to observe the first divisions of protoplasts, although dividing protoplasts could be observed with a microscope seven days after protoplast culture. At this stage, the transfer of membranes containing microcolonies to fresh KPR medium was found to be essential for continuous growth. Statistical analysis showed that there was significant difference ($p < 0.01$) between plating efficiency of two cultivars. Plating efficiency was on average 2.7 and 1.2% (2 weeks) and 0.11% and 0.07% (4 weeks) for cvs. Tarom and Khazer, respectively (Table 2).

**Figure 1.** Effect of age of cell suspension cultures on protoplast yield of cvs. Tarom and Khazer.

The values represent the mean protoplast yields from 3 replications and the bars represent the standard error (SE).

Table 3. Comparison of the phenotypic characteristics of the protoplast regenerated plants with rice plants (control) of the same age under glasshouse conditions (cvs. Tarom and Khazer).

Trait	Tarom		Khazer	
	Control plants (mean ± SE)	Protoplasts re-generated plants (mean ± SE)	Control plants (mean ± SE)	Protoplasts re-generated plants (mean ± SE)
Plant height (cm)	137.90 ± 4.42	134 ± 1.18	94.47 ± 2.63*	83.31 ± 1.93
Number of tillers/plant	6.75 ± 0.92	8.75 ± 0.72	7.55 ± 0.34	8.50 ± 0.56
Flag leaf length (cm)	55.97 ± 3.81	56.65 ± 2.9	43.75 ± 0.93	46.01 ± 1.37
Flag leaf width (mm)	10.08 ± 0.4	10.25 ± 0.03	10.67 ± 0.02	10.59 ± 0.05
Flag leaf length/width ratio	55.52 ± 2.72	55.27 ± 2.2	41.11 ± 0.41	43.45 ± 0.85
Number of days to flowering	90.25 ± 3.01**	105.12 ± 0.84	113 ± 3.25*	124.12 ± 5.43
Panicle length (cm)	26.58 ± 1.4	25.98 ± 0.41	23.17 ± 0.48	24.75 ± 0.68
Primary branches/panicle	7.75 ± 0.60	8.00 ± 0.26	12.25 ± 0.60	12.00 ± 0.67
Secondary branches/panicle	24.25 ± 2.80	18.00 ± 0.83	26.00 ± 0.5	23.00 ± 1.54
Number of spikelets	131 ± 10.99**	75.75 ± 4.96	179 ± 8.19*	130.62 ± 11.14
Grain length (mm)	8.90 ± 0.14	8.24 ± 0.11	9.05 ± 0.05	8.32 ± 0.35
Grain width (mm)	2.37 ± 0.21	2.30 ± 0.06	2.10 ± 0.03*	2.00 ± 0.03
Grain length/width ratio	3.75 ± 0.04*	3.58 ± 0.03	4.29 ± 0.37	4.14 ± 0.27

* Significant at $p \leq 0.05$; ** Significant at $p \leq 0.01$

After placing protoplast-derived embryonic calli on MSKN regeneration medium, white embryo-like structures developed on the surface of the micro-calli, after seven to twelve days of culture. Later, after further incubation in the dark, organised and defined bipolar structures were formed, each consisting of a coleoptile and a radicle. In most cases, shoots and roots developed together. For the further development of regenerated plants, it was essential to transfer material to the MSN1.5 medium for rooting and to keep the plants in the light ($55 \mu \text{mol m}^{-2} \text{s}^{-1}$) (Table 2).

Flow cytometric studies of nuclei isolated from all samples (controlled growth room-grown diploid seedlings and *in vitro* grown plantlets) showed that there was variation in the DNA index among protoplast-derived plants as compared to the standard control plants (1.00) (Figure 2). There was variation in the DNA index among the protoplast-derived plants of Tarom (0.92-2.09) and Khazer (1.01-2.46). The ploidy level varied from tetraploid to diploid. From the flow cytometric studies carried out on 37 random samples of the total population of plants

(more than 185 plants for cvs. Tarom and Khazer), 6 were tetraploids (16.21%) and the rest were diploids (83.79%). The plants which were tetraploid, did not flower in the glasshouse.

A population of 51 protoplast-derived plants (34 of Tarom and 17 of Khazer) and 16 control plants (8 of Tarom and 8 of Khazer) were grown to maturity under glasshouse conditions. The results of mor-

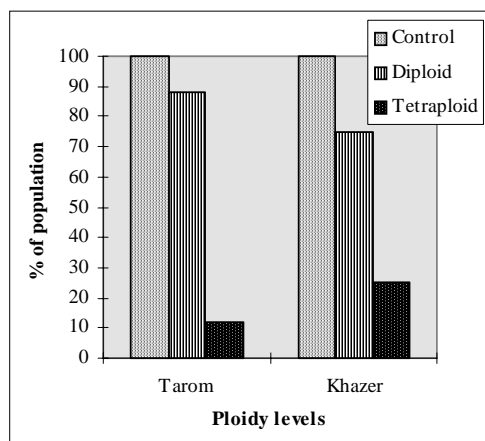


Figure 2. Ploidy level of protoplast-derived plants of cvs. Tarom and Khazer.

phological analysis are given in Table 3. The mean value between the two groups of plants (protoplasts and seed-derived control plants) was compared for thirteen phenotypic characters, for both cultivars. Analysis showed, for the majority of phenotypic characteristics examined under glasshouse conditions, that protoplasts of Tarom had significantly delayed flowering, had less spikelets and a lower grain length/width ratio. Protoplasts of Khazer had significantly decreased mean plant height, delayed flowering, a decreased number of spikelets and decreased grain width, cultures being optimum. Younger cultures gave spontaneous fusion bodies for both cultivars which are considered to be of no value in the context of somatic hybridisation.

DISCUSSION

Mature embryos have been found suitable for establishing suspension cultures with regeneration capability in *Japonica* and *Indica* rice (Lee *et al.*, 1989). In this study fertile plant regeneration was also possible from embryogenic cell suspension-derived calli of *Japonica* rice and cvs. Tarom and Khazer. Plant regeneration has not previously been reported for these varieties. In this study, the role of the feeder layer technique in obtaining sustainable division in protoplast culture was found to be essential (Table 2). Previously plant regeneration from rice protoplasts of *Indica* rice cultivars by using nurse cells has been reported (Lee *et al.*, 1989; Jain *et al.*, 1995). The ploidy studies involving flow cytometric analysis of 37 randomly selected regenerated plants of cvs. Tarom (25), Khazer (12) and six control plants showed the occurrence of diploid and tetraploid plants to be at frequency of 16.2% and 83.8%, respectively in this population of regenerated plants. However, the plants which exhibited tetraploid characteristics, did not flower under glasshouse conditions. Chromosomal changes in rice may have been induced by prolonged exposure of cells to growth regulators in the culture medium.

In this regard, a chromosomal study in one month - and six month- old calli of *Oryza sativa* L. by Fatima and Anwar (1993) revealed that cells with haploid and triploid chromosome numbers were observed in six month old calli.

Analysis showed that, for the majority of phenotypic characteristics examined under glasshouse conditions, protoplasts of Tarom had significantly delayed flowering, a lower number of spikelets and a lower grain length/width ratio. Protoplasts of Khazer had significantly decreased in mean plant height, delayed flowering, decreased number of spikelets and decreased grain width (Table 3). This reduction in fertility has also been described by other researchers in studies of *Japonica* and *Indica* rice strains (Hayashimoto *et al.*, 1990; Peng *et al.*, 1992). Changes in panicle morphology, fertility and days to flowering were observed by Abdullah *et al.* (1989) among the R1 generation protoplast-derived from *Japonica* rice plants, in field conditions. Protoplast variation can be used in rice breeding.

It is concluded that fertile plants can be regenerated from protoplasts of the Iranian *Japonica* rice cultivars, Tarom and Khazer. It should be possible to improve these cultivars through protoplast mediated transformation.

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باززایی گیاه از پروتوپلاستهای برنج ایرانی (ارقام طارم و خزر) و تعیین سطح پلوئیدی آنها

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چکیده

یک روش بسیار مؤثر جهت باززایی گیاه از پروتوپلاستهای جداشده از سوسپانسیون سلولی دو رقم برنج تجاری ایرانی (ارقام طارم و خزر) تشکیل شد. در این مطالعه برای تشکیل سوسپانسیون سلولی از کالوسهای جنین‌زا استفاده گردید. پس از تشکیل سوسپانسیون سلولی پروتوپلاستها از سوسپانسیون هر دو رقم جدا شد. پروتوپلاستهای کشت شده در آگارز شروع به رشد و تولید کالوس ننموده اند. اما وقتی که از روش کشت حمایتی استفاده شد پروتوپلاستها شروع به تقسیم و تولید کالوس نموده اند. نتایج نشان داد که فراوانی باززایی گیاه سبز از کالوسهای بدست آمده از پروتوپلاست بترتیب ۲۱/۱ و ۱۰/۵ در صد برای ارقام طارم و خزر بوده است. تنوع سوماکلونال در میان گیاهان باززایی شده مشاهده شده است. بطوری که ۱۶/۲ در صد از گیاهان باززایی شده تراپلوئید و بقیه دیپلوئید بوده‌اند.