World Journal of Agricultural Sciences 5 (4): 415-421, 2009
ISSN 1817-3047

Long-Term Maintenance of Callus Cultures from Immature Embryo of *Sorghum bicolor*

[1]Sudhakar Pola, [2]N. Sarada Mani and [1]T. Ramana

[1]Department of Biotechnology,
[2]Department of Botany andhra University, Visakhapatnam, India 530 003

Abstract: A protocol was developed for long-term maintenance of callus cultures, induced from immature embryo of *Sorghum bicolor* (L.) Moench. Maintenance of callus cultures for prolonged duration in a regenerable state is one of the most important requirements for using *in vitro* techniques for plant improvements. In the present study calli were maintained satisfactorily on MS medium over 57 weeks and plantlets regenerated from them on transfer to light. Immature embryo was used as source material for callus initiation. The callus cultures were cultured on MS media with 1.5 mg/L of 2,4-D (2,4-dichlorophenoxy acetic acid), 10mg/L silver nitrate ($AgNo_3$), 400mg/L casein hydrolysate (CH), 200mg/L L-proline and L-asparagine. After callus initiation cultures were maintained in dark conditions and subcultured for every 3 week intervals. Greening of callus and regeneration of shoot-buds from callus occurred on transfer to MS media with 2mg/L BAP + TDZ (Thidiazuron). These shoots grew further in media with both, BAP and TDZ, Rooting of shoots occurred on media with NAA, the rooted plantlets could be transferred to autoclaved soil. Genetically uniform plantlets regenerated continuously from established callus upto 57 weeks old; thereby helping in multiplication and to facilitate the year round availability of explants and/or somatic embryos for *Sorghum* tissue culture and transformation.

Key words: *Sorghum bicolor* · Immature embryo · Silver nitrate · Long-term · Casein hydrolysate

INTRODUCTION

Sorghum [*Sorghum bicolor* (L.) Moench] is an economically important cereal plant belonging to the family of Poaceae, it is one of the most significant crop in Asia, Africa and Latin America. *Sorghum is* the fifth most important cereal crop after wheat, rice, maize and barley [1,2]. The crop is tolerant of many biotic and abiotic stresses and is often grown in more marginal cropping areas. In developing countries it tends to be a staple food and forage of the poor. In developed countries it is used primarily as an animal feed. *Sorghum* is often preferentially grown in both situations as it is better adapted to water limited environments than other cereal crops.

Plant tissue culture represents the simplest of the biotechnologies available to plant scientists today for crop improvement. Certain *in vitro* conditions could induce heritable changes in the genomes of plant cells, plant tissue culture opened an avenue for the selection of various desirable traits from *in vitro* cultures [3]. Long-term maintenance of callus cultures is very useful in plant tissue culture to facilitate the year round availability of somatic embryos in a regenerable state. On the other hand, *Sorghum* along with other cereals, shares the problem of loss of regeneration ability within a few subcultures and the regeneration frequency of Embryogenic calli decreased with increasing age [4-7].

The development of suitable protocol for long-term maintenance of callus cultures is still inadequate and difficult process [8,9]. Suspension cultures which maintained embryogenic potency for more than 18 months were established [10] from excised immature embryos of rice. Brisibe *et al.,* [6] was worked on regulation of somatic embryogenesis in long-term callus cultures of sugarcane. In their study Calli maintained their embryogenic competence for up to 16 months using casein hydrolysate.

Consequently, these reports imply that, it is necessary to develop an effective and reproducible protocol for long–term maintenance of callus cultures in *Sorghum*. The main objective of the present study is to develop an efficient method of regeneration system from long-term callus cultures of *Sorghum* to facilitate the year round availability of somatic embryos in a regenerable stage.

Corresponding Author: Dr. Sudhakar Pola, Department of Biotechnology andhra University, Visakhapatnam, India 530 003

MATERIALS AND METHODS

Plant Material: Seeds of *Sorghum bicolor* (L.) Moench var. IS 3566, SPV 475, CSV 13, CSV 15, CSV 112 and IS 348 were obtained from National Research Centre for *Sorghum*, Hyderabad, India and were raised in the experimental fields of Andhra university. Field grown immature seeds (florets) of *Sorghum* containing immature embryos were harvested from main spikes of the caryopses of 14-17 days after anthesis. Healthy immature embryos were used as the source materials (explants) for callus initiation.

Surface Sterilization of the Explant: The harvested immature seeds were first washed thoroughly under running tap water for 20 min, followed by treating with 70% alcohol for 5 minutes and subsequently washed three times with sterile double distilled water. Approximately 50 immature embryos (0.5-2 mm size) were aseptically excised from the spikelets, using a dissecting microscope and washed with distilled water. Then the surface sterilization was done with 70 % (v/v) ethanol for one minute and 15 min in a 2.5 % (m/v) sodium hypochlorite solution and followed by rinsing with 0.1% $HgCl_2$ for one minute [11].

Callus Induction Medium: The callus induction medium consisted of MS[12] salts and vitamins, 30 g/ l sucrose, 0.2 -3.0 mg/L of 2,4-D and KN D with 200mg/L L-asparagine, L-proline, medium was solidified with 0.8% (w/v) Agar Agar and autoclaved for 20 min. All cultures were performed in Petri dishes (90x16 mm). Unless otherwise stated, all chemicals used were provided by Hi-media. Explants inoculated with their axes in contact with the callus induction medium and their scutella positioned upwards on 25-30 ml of solid agar MS medium.

Callus Maintenance Medium: Callus maintenance medium was also prepared as callus induction medium and it contains 3% sucrose, 2 mg/L 2,4-D, 0.5 mg/L Kn with 200mg/L L-asparagine, L-proline, 1-12 mg/L $AgNo_3$, 100-600mg/L Casein hydrolysate were used. Cultures were incubated under dark conditions at 25±2°C.

Regeneration Stage: For regeneration of plants or germination of somatic embryos, the MS medium was supplemented with 2mg/L BAP + TDZ in combination with 10 mg/L $AgNo_3$, 400 mg/L Casein hydrolysate, L-asparagine and L-proline were used. After somatic embryos transferred onto the regeneration or germination medium, the cultures were incubated in continuous cool white fluorescent light at an intensity of 25 µ mol m^{-2} S^{-1} at 25±2°C [13]. The cultures for shooting and rooting were carried out in glass vessels with plastic caps.

Rooting Stage: Regenerated Shoots after attaining 3 cm length were separated from the cultures individually and used for root induction. The media used for root induction was half strength MS media supplemented with 1.0 mg/L NAA.

Acclimatization: The regenerated plantlets were washed gently with double distill water for removing all traces of medium from the roots and then transferred to small plastic cups containing sterile sand. The plastic cups were covered with sealed plastic vinyl bags to keep full humidity at 25±2?C in light conditions (photon flux density at 25 ì mol m–2 s–1, 16 h). As the plants grew vigorous, the bags were poked with chopsticks to allow air into the bags until the plants self-supported. The polythene bags were removed after fifteen to twenty days. The plantlets were later transferred to larger pots containing sterile sand and soil (1:1 ratio) and kept under shade in the green house for another two weeks before transferring to field. Fully established regenerants were later established in the field for further growth.

RESULTS

The explant produced a range of responses within 10 -16 days in MS medium with the different types of growth regulators. White, compact, embryogenic calli were produced from cut ends of explants within 12 days in media with mg/L 2,4-D. The highest callusing was seen in 2,4-D and KN combination at concentrations of 2 mg/L + 0.5 mg/L, respectively, although the presence of 2,4-D alone was adequate to initiate embryogenic callusing (Table 1). Three week old embryogenic calli were separated from the non embryogenic portions of the explants and subcultured onto the same medium for the embryogenic callus maintenance. The cultures were subcultured for every three week intervals.

Following more than a few subcultures on callus maintenance medium, we have observed the embryogenic callus either degenerated or converted into non embryogenic calli and development of brown colour soft calli was observed in the cultures. Gradual loss of regeneration ability in the embryogenic callus cultures was observed from the 6^{th} week onwards.

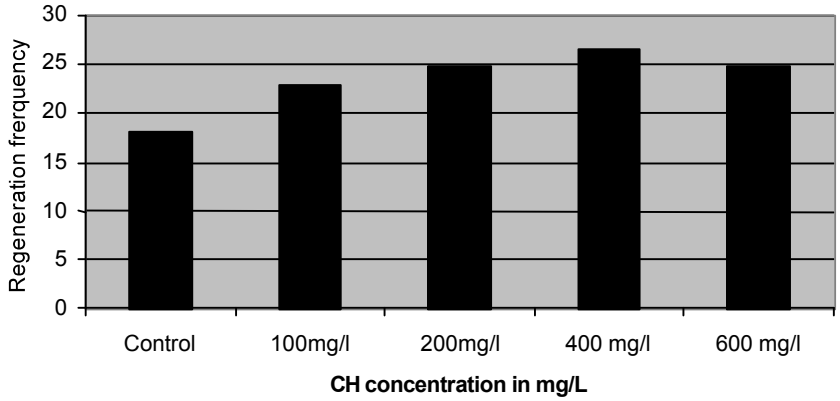

Fig. 1: Effect of casein hydrolysate concentration on regeneration efficiency of long-term maintained calli

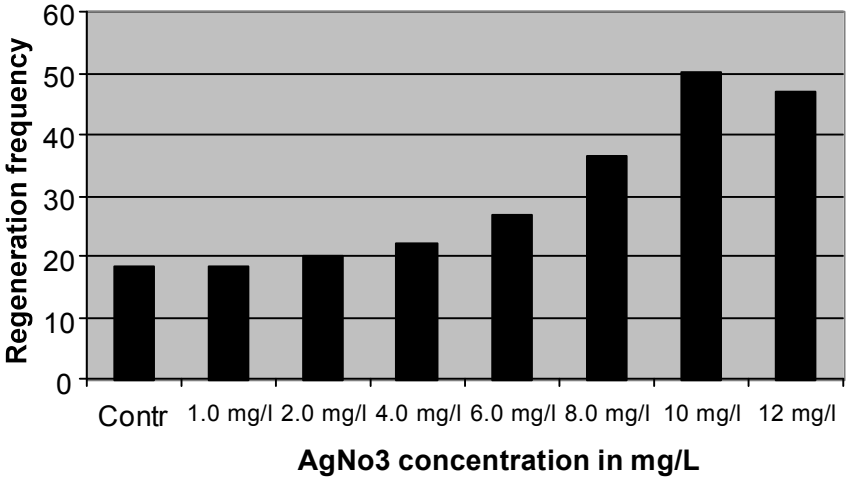

Fig. 2: Effect of silver nitrate concentration on regeneration efficiency of long-term maintained calli

We have conducted experiments to restoration for the plant regeneration from the embryogenic callus by the addition of $AgNo_3$ and Casein hydrolysate in the callus maintenance medium as well as in the regeneration medium.

Regeneration Response in the Long-Term Maintained Calli: The regeneration response of embryogenic callus in different varieties at different week intervals was summarized in the Table 2. (Prior to the addition of $AgNo_3$ and Casein hydrolysate in the regeneration medium). Shoot regeneration from long-term maintained calli was obtained after transferring the embryogenic calli onto BAP + TDZ medium (2 mg/L), BAP & TDZ could initiate shoot buds from the calli / somatic embryos. The calli became green colour after transfer to light (Fig. 3. A-D), shoot buds were produced within 9-12 days (Fig. 3. E-G). Shoots were proliferated from shoot buds after 2 weeks of culture (Fig. 3. H&I). Shoots after attaining 3 cm length they were transferred onto rooting medium (Fig. 3.J).

The results indicated that, the regeneration response was highest in fresh cultures i.e., three week old cultures. After three weeks the regeneration response was gradually decreased with increasing the number of subcultures or embryogenic callus age. Regeneration response in the long-term callus cultures was observed upto 21 weeks in all the varieties. In IS 3566 and SVP 475 regeneration response was observed upto 27 weeks, despite the fact that regeneration response was observed, but the response was very low i.e., 6% in IS 3566 and 2% in SPV 475 (Table 2). After 27 weeks no regeneration response was observed and the cultures underwent depletion and acquired brown colour.

Restoration of Plant Regeneration in Long-term Callus Cultures: After addition of 400 mg/L Casein hydrolysate (Fig.1) and 10mg/L $AgNo_3$ (Fig.2) in the culture medium, the regeneration response was sustained upto 33 weeks in CSV 15, CSV 112 and IS 348. Regeneration was observed till 45 weeks in SPV 475 whereas in IS 3566

Fig. 3: Plantlet regeneration from long-term maintained embryogenic calli of Sorghum bicolor

Legends of Figure 3.

A-D. Long-term maintained Somatic embryo (57 weeks old) maturation and developmental stages on 2 mg/L BAP + TDZ medium.

E. Shoot bud formation from somatic embryos in presence of light

F. Green colour Shoot development on BAP + TDZ medium in presence of light

G. Shoot proliferation on BAP + TDZ medium

H& I. Shoot developmental stages on BAP + TDZ medium after six weeks

J. well developed shoots on rooting medium, ½ MS + 1 mg/L l NAA

K. Regenerated plantlets acclimatized to the green house.

Table 1: Effect of different concentration of 2,4-D and KN on fresh weight of callus after three weeks**

2,4-D	KN	2,4-D + KN	Callusing ability
1.0			+
1.5			++
2.0			+++
2.5			++
3.0			+
	0.2		+
	0.5		+++
	1.0		++
	1.5		---
	2.0		---
		1.0 + 0.1	++
		1.5 + 0.2	+++
		2.0 + 0.5	++++
		2.5 + 1.0	++
		3.0 + 1.5	---

++++ Extraordinarily high, +++ High, ++ Medium, + Low, --- None.

* 25 explants per treatment, each treatment repeated thrice.

**Concentration of 2,4-D and KN are in mg/L

Table 2: Regeneration response in the embryogenic callus cultures, prior to addition of $AgNo_3$ and Casein hydrolysate in the medium

Age of callus	Genotype	No. of embryos transferred	No. of embryos responded	No of shoots	No of Roots
3rd Week	IS 3566	100	100	100	100
	SPV 475	100	100	100	100
	CSV 13	100	100	100	100
	CSV 15	100	100	96	98
	CSV 112	100	100	98	96
	IS 348	100	100	84	80
9th Week	IS 3566	100	100	100	100
	SPV 475	100	100	100	100
	CSV 13	100	100	100	100
	CSV 15	100	100	96	94
	CSV 112	100	100	94	92
	IS 348	100	100	78	80
15th Week	IS 3566	100	36	46	94
	SPV 475	100	28	32	67
	CSV 13	100	22	26	54
	CSV 15	100	18	20	41
	CSV 112	100	16	18	38
	IS 348	100	12	12	26
21st Week	IS 3566	100	24	30	64
	SPV 475	100	18	22	48
	CSV 13	100	14	14	29
	CSV 15	100	9	9	21
	CSV 112	100	6	6	14
	IS 348	100	4	4	9
27th Week	IS 3566	100	6	6	13
	SPV 475	100	2	2	5
	CSV 13	100	-	-	-
	CSV 15	100	-	-	-
	CSV 112	100	-	-	-
	IS 348	100	-	-	-

Table 3: Regeneration response in Long-term maintained callus cultures on MS + 10 mg/L $AgNo_3$ and 400 mg/L Casein hydrolysate medium

Age of Callus	Genotype	No. of embryos transferred	No. of embryos responded	No of shoots	No of Roots
33rd Week	IS 3566	100	64	67	141
	SPV 475	100	54	56	120
	CSV 13	100	59	62	130
	CSV 15	100	38	41	83
	CSV 112	100	26	29	61
	IS 348	100	18	18	39
39th Week	IS 3566	100	46	48	110
	SPV 475	100	38	40	82
	CSV 13	100	40	46	108
	CSV 15	100	-	-	-
	CSV 112	100	-	-	-
	IS 348	100	-	-	-
45th Week	IS 3566	100	46	48	110
	SPV 475	100	38	40	82
	CSV 13	100	40	46	108
	CSV 15	100	-	-	-
	CSV 112	100	-	-	-
	IS 348	100	-	-	-
51st Week	IS 3566	100	28	34	69
	SPV 475	100	-	-	-
	CSV 13	100	18	22	46
	CSV 15	100	-	-	-
	CSV 112	100	-	-	-
	IS 348	100	-	-	-
57th Week	IS 3566	100	16	16	36
	SPV 475	100	-	-	-
	CSV 13	100	9	9	20
	CSV 15	100	-	-	-
	CSV 112	100	-	-	-
	IS 348	100	-	-	-

and CSV 13 the regeneration potential was observed upto 57 weeks. After 57 weeks no regeneration response was observed in all the varieties even though 10mg/L $AgNo_3$ and 400 mg/L Casein hydrolysate were added to the medium.

In the 3rd week, in IS 3566, SPV 475 and CSV 13 the regeneration potential was 90, 84 and 88 shoots respectively from 100 somatic embryos (Table 3). The gradual decrease in regeneration response was observed on 27 weeks onwards in all the varieties, from 39 weeks onwards the regeneration potential was completely absent in CSV 15, CSV 112 and IS 348. Though regeneration was observed in IS 3566 and CSV 13 upto 57 weeks the shoot number was insufficient i.e., 16% in IS 3566 and 9% in CSV 13.

DISCUSSION

Vasil *et al.,* [14] studied long-term maintenance of callus cultures in Zea *mays* using Casein hydrolysate and 2% sucrose in the culture maintenance medium. Long-term regeneration of sugarcane callus cultures was reported [15] using 0.5 mg/L picloram and 2,4-D, they maintained callus cultures upto 19 months. Xu *et al.,* [9] reported that the callus cultures after repeated subcultures were losing their embryogenic potentials. They studied the effect of abscisic acid on growth and plant regeneration from long-term maintained callus, initiated from mature seed cultures of rice.

Silver nitrate ($AgNo_3$), an ethylene inhibitor has shown to increase embryo production in cereals. Pius *et al.,* [5] reported that inhibitors of ethylene, such as $AgNo_3$, made it possible to sustain regeneration in cultures that lose their ability to regenerate. Oldach *et al.,* [16] reported a significant increase in the regeneration rates of Pearl millet cultures when medium supplementing with 5mg/L $AgNo_3$. Vikrant and Rashid [17] reported somatic embryogenesis from immature and mature embryos of minor millet on MS medium supplemented with 59 µM $AgNo_3$. In the present investigation 10mg/L $AgNo_3$ was effectively used to maintain the long-term maintained callus in a regenerable stage, in combination with 400mg/L casein hydrolysate and 2mg/L 2, 4-D. Bajaj and Rajam [18] reported that the loss of morphogenesis in long-term callus cultures in rice is due to the accumulation of polyamines. They reduced the polyamine content with the addition of spermidine. Visarda *et al.,* [19] observed a sharp decline in the regeneration capacity starting from 30 days after callus induction from shoot apices derived embryogenic callus. In contrast, they observed regeneration ability was sustained in calli derived from immature inflorescence and maintained upto 90 days. In our study we observed that, immature embryo exhibited better long-term maintenance capacity (upto 57 weeks).

In the present study 400 mg/L concentration of casein hydrolysate were found to be effective for long-term maintenance of callus cultures. Artunduga *et al.,* [20] also reported that casein hydrolysate was improved the tissue culture response in long-term cultures of Bermuda grass using a combination of 13.6 µM 2,4-D and 200 mg/L casein hydrolysate in culture medium. Li and Qu [21] maintained undifferentiated callus for a long period (14 months) during subculture in media supplemented with an elevated BAP conc. at 2.2 µ M. Hasler *et al.,* [22] made detailed study on long-term callus cultures in sugar beat; they reported that the long-term *in vitro* cultured plant cells show typical neoplastic features at the cytological level.

In conclusion, we have succeeded in maintaining the embryogenic callus cultures derived from immature embryo, upto 57 weeks in a regenerable stage by using MS medium containing 2 mg/L 2,4-D, 0.5 mg/L KN,10mg/L $AgNo_3$, 400mg/L Casein hydrolysate, 200mg/L L-asparagine and L-proline. Long-term maintenance of callus cultures is very useful in plant tissue culture to facilitate the year round availability of somatic embryos in a regenerable state. So that, we can regenerate plants at any time with out any seasonal limits. Thus, Loss of regeneration efficiency was prevented by the addition of $AgNo_3$, Casein hydrolysate, L-asparagine and L-proline in the callus culture medium.

REFERENCES

1. Nguyen, V., T. Thu., M. Claeys and G. Angenon, 2007. Agrobacterium-mediated transformation of *sorghum* (*Sorghum bicolor* (L.) Moench) using an improved in vitro regeneration system. Plant Cell Tiss Organ Cult, 91: 155-164.
2. FAO (Food & Agricultural Organization). 2008. FAOSTAT. http://faostat.fao.org/faostat.
3. Richard, M., S. Mulwa. and M.M. Lydia, 2006. Biotechnology approaches to developing herbicide tolerance/selectivity in crops. African Journal of Biotechnology, 5(5): 396-404.
4. Cai, T. and L. Butler, 1990. Plant regeneration from embryogenic callus initiated from immature inflorescence of several high tannins *Sorghum*s. Plant cell tiss. Org. Cult, 20: 101-110.
5. Pius, J., L. George, S. Eapen and P.S. Rao, 1993. Enhanced plant registration in Pearl millet (*Pennisetum americanum*), by ethylene inhibitors and Cefotaxime. Plant cell tiss. Org. Cult, 32: 91-96.
6. Brisibe, E.A., H. Miyake., T. Taniguchi and E. Maeda, 1994. Regulation of somatic embryo genesis in long-term callus cultures of sugarcane (*Saccharum officinarum* L.). New Phytol, 126: 301-307.
7. Lambe, P., H.S.N. Mutambel., R. Deltour and M. Dinant, 1998. Somaticembryogenesis in pearl millet (*Pennisetum americanum*): strategies to reduce genotype limitation and to maintain long-term totipotency. Plant cell tiss. Org. cult, 55: 23-29.
8. Asano, Y., Y. Ito, M. Ohara, K. Suguira and Fujiie, A. 1994. Improved plant regeneration response of creepingbent grass and japonica rice by maltose and lactose. Plant Cell Tiss. Org. Cult, 39: 101-103.

9. Xu, Z.J., K. Fujino, C. Furuya and Y. Kikuta, 1995. Plantlet regeneration and Novel protein synthesis in a long-term cultured callus of rice in response to Abscisic acid. Japanese Journal of Crop Science, 64(1): 109-114.
10. Ozawa, K. and A.C. Komamine, 1989. Establishment of a system of high frequency embryogenesis from long-term cell suspension cultures of rice (*Oryza sativa* L.). Theor. Appl. Genet, 77: 205-211.
11. Sudhakar Pola., T. Ramana and N. Saradamani, 2008. Plant tissue culture studies in *Sorghum bicolor*: immature embryo explants as the source material. International Journal of Plant Production, 2(1): 1-14 January.
12. Murashige, T. and F. Skoog, 1962. A revised medium for rapid growth and bioassayswith tobacco cultures. Physiologia Plantarum, 15: 473-497.
13. Pola -Sudhakar, R and N. SaradaMani, 2006. Somatic embryogenesis and plantlet regeneration in *Sorghum bicolor* (L.) Moench, from leaf segments. Journal of Cell and Molecular Biology, 5(2): 99-107.
14. Vasil, V., I.K. Vasil and C. Lu, 1984. Somatic embryogenesis in long-term callus cultures of *Zea mays* L. (Gramineae). American Journal of Botany, 71: 158-161.
15. Maureen, M., M. Fitch and H.M. Paul,. 1990. Comparison of 2,4-D and Picloram for selection of long-term totipotent green callus cultures of sugarcane, 20(3): 157-163.
16. Oldach, K., A. Morgenstern., S. Rother., M. Girgi., M. O'Kennedy and H. Lorz, 2001. Efficient *in vitro* plant millet [*Pennisetum glaucum* (L.) R.Br.] and *Sorghum bicolor*(L.) Moench. Plant cell reports, 20: 416-421.
17. Vikrant, A. and A. Rashid, 2001. Direct as well as indirect somatic embryogenesis from immature (unemerged) inflorescence of a minor millet *Paspalum scrobiculatum* L. Euphytica, 120: 167-173.
18. Bajaj, S. and M.V. Rajam. 1996. Polyamine accumulation and near loss of Morphogenesis in long-term callus cultures of rice. Plant physiol, 112: 1343-1348.
19. Visarada, K.B.R.S., N. Sai kishore, D. Balakrishna and S.V. Rao, 2003. Transient gus expression studies in *Sorghum* to develop a simple protocol for *Agrobacterium* mediated genetic transformation J. Genet and Breed, 7: 147-154.
20. Artunduga, R., C.M. Taliaferro and B.L. Johnson, 1989. Induction and growth of Callus from immature inflorescence of zebra Bermuda grass as affected by casein hydrolysate and 2, 4-d concentrations. *In vitro* Cell Dev. Biol., 25: 753-756.
21. LI, L. and R. QU, 2004. Development of highly regenerable callus lines and Biolistic transformation of turf. Type common Bermuda grass [*Cynodan dactylon* (l) pers. Plant Cell Reports, 22: 403-407.
22. Hasler, J., G.Thomas and C. Michèle, 2003. Long-term *in vitro* cultured plant cells show typical neoplastic features at the cytological level. Biology of the Cell, 95: 357-364.