*Pak. J. Bot.*, 39(7): 2431-2441, 2007.

# ESTABLISHMENT OF CALLUS AND SUSPENSION CULTURE IN *JATROPHA CURCAS*

**RASHIDA SOOMRO AND RABIA ASMA MEMON***

*Department of Botany, Shah Abdul Latif University, Khairpur, Pakistan.*
**Institute of Botany, University of Sindh, Jamshoro, Sindh, Pakistan*

**Abstract**

Callus cultures were initiated from leaf and hypocotyl explants isolated from 4 days old seedling of *Jatropha curcas* L., on Murashige & Skoog (1962) basal medium supplemented with different growth regulator formulations including 2,4-D, BA, GA3, and coconut milk. Excellent growth of callus was obtained in medium supplemented with 0.5mg/L 2, 4-D alone and with 2% v/v coconut milk in hypocotyl explants, Callus produced from hypocotyl explants grew faster during 7 to 30 days of culture then stabilized at a low growth rate. Calli cultured on this medium showed 8 fold increase in fresh weight by the fourth week of incubation. Callus was soft, friable, globular, lush green in color. Hypocotyl explant and 0.5mg/L 2, 4-D proved to be most effective in inducing of callus on a large scale in short period of time. The friable green callus was then used for establishment of homogeneous and chlorophyllous suspension culture. Maximum growth of suspension culture was achieved in medium supplemented with 0.5mg/L 2, 4-D, with initial inoculum cell density of 1%. The growth rates of cells were initially slow but as the cultures proceeded, the growth increased significantly and accumulated a great amount of fresh weight (5fold) over a period of 21 days then the growth of cells was stable for 30 days. The fresh weight was balanced in terms of dry weight which almost corresponded to fresh weight. Total chlorophyll content in cell culture varied between 50.7 to 75.7ug/g FW with in growth cycle of these cultures.

## Introduction

*Jatropha curcas* L., a soft wood perennial plant belongs to family Euphorbiaceae (Nasir, 1986), commonly known as Jamalghota, Physic nut, Ratanjot or Purgative nut. *Jatropha curcas* is one of the most valuable crude drugs of primitive times and is still widely used in modern medicine. It has natural distribution covering the Neo tropic from Mexico to Brazil including the Caribbean Island (Grim, 1996). It is now distributed throughout the entire tropics of Africa and Asia as well (Grim, 1996). In recent years this plant has received extensive attention of many scientists in view of its great economic importance, medicinal significant and for its seed oil as commercial source of fuel (Datta & Pandy, 1993). The superior quality oil can be extracted from the seeds. The oil can be used as a mixed fuel for diesel/gasoline engines (Yoshifumi, 1982). The oil is not edible due to the presence of toxic substance"Curcascine" (Gandhi *et al.,* 1995). It is conventionally used in making soaps, candles, paints, lubricants and medicinally as a purgative (Dastur, 1951; Sujatha & Mukta, 1996). It is also recommended as a drought resistant plant suitable for erosion control and is not palateable to grazing animals due to the toxicity (Munch & Kiefer1989). Plant cell cultures are generally more desirable than a solid medium because of higher growth rates resulting from a high medium to tissue contact. Plant cell and tissue cultures provide an alternative approach to the plants which are difficult to cultivate, or has a long cultivation period, or has a low yield, product yield by cell culture may be significantly produce a higher yield that obtained from the parents (Hippolyte *et al.,* 1992; Zhong *et al.,* 1994). The advantage of plant cell culture have for

enhancing shoot proliferation and growth is reported in several species (Ilan *et al.,* 1995; Escalona *et al.,* 1999). Further studies were also carried out on the effect of environmental condition on culture induction, maintenance, somatic embryogenesis, trans formation and plant regeneration (Kim *et al.,* 2003, 2004, 2005; Rao *et al.,* 2006, Nakamura & Ishikawa, 2006). However despite their economic importance, drought resistance characteristic and medicinal value only a limited number of *In vitro* culture studies have been reported, including formation of callus and plant regeneration through callus (Sujatha & Mukta, 1996). The objective of the present studies was to test different growth regulator formulations to establish callus and cell suspension cultures and to develop a long term maintenance on large scale. The evidence showed that there was no work done on establishment of such cultures and it is the first time establishment of callus and cell suspension cultures on a large scale through tissue culture techniques in *Jatropha curcas* L., is reported.

## Materials and Methods

**Plant material:** Identified seeds of *Jatropha curcas* L., were collected from Ministry of Sciences and Technology, Government of Pakistan, Islamabad. The seeds were washed with commercial detergent (Zip) followed by running tap water. The seeds were then soaked in autoclaved distilled water for 4 hours to soften the hard seed coat. The seeds were then surface sterilized with 0.1% Mercuric chloride solution for 3 minutes followed by 4 rinses in sterile distilled water under aseptic conditions. After removing the seed coat, the seeds were transferred aseptically into test tubes containing 0.9% (w/v) plain agar medium for germination. The seeds were incubated in dark at 28°C, and when hypcotyl had enlongated (day 2) the seedlings were transferred to light (2000 Lux 16/8 hr photo period) and maintained at 27°C ± 28°C in growth room. After 4 days then leaf and hypocotyl were aseptically separated and cultured on solid medium (MS macro and micro salts (Murashig & Skoog, 1962) supplemented with different growth regulator formulations including 2, 4-D GA3, BA and coconut milk (2% v/v) for induction of callus. Fast growing calli were dispersed in sterile water and examined under stereo microscope for the characterization of different stages.

**Initiation of callus and maintenance:** Leaf and hypocotyl explants were excised aseptically from 4 days old seedlings of *Jatropha curcas* and cultured on solid media MS macro and micro salts (Murashige & Skoog, 1962) supplemented with different growth regulator formulations including 2,4-D, GA3, BA and coconut milk (2% v/v) for initiation of callus. Agar 0.9% was added for the solidification of the medium. The pH of the medium was adjusted to 5.8 before autoclaving the medium at 120Cº for 20 minutes each treatment consisted of one explant per flask with three replicates. Cultured flaks were maintained in growth room at 25°C ± 2ºC under controlled light intensity of 2000 Lux provided by a light assembly consisting of a series of cool and florescent lamps, photoperiod of 18/6 hrs L/D cycle was adjusted with the help of an electric timer fitted with the light assembly. After 2 weeks, the cellular clumps consisting of small green very friable callus grew at the margins of the leaf and hypocotyl explants (Yu *et al.,* 2005).The callus was then maintained under the same conditions as above with subcultures at 2 weeks interval in fresh medium.

**Cell suspension, initiation and maintenance:** Cell Suspension cultures of *Jatropha curcas* were initiated from callus tissue developed from hypocotyl explants. One gram of friable green callus was excised in Petri dish containing Whatman No.1 filter paper. Callus was slightly mashed and carefully transferred with sterilized forceps to each of 250ml Erlenmeyer flasks containing 50ml liquid MS medium, supplemented with 0.5ml/L of 2, 4-D alone with 2% v/v coconut milk. The flasks were covered with loose cotton plugs and aluminum foil. The pH of medium was adjusted to 5.8 before autoclaving the medium at 120Cº for 20 minutes. The flasks were agitated at 120rpm on a gyratory shakers and incubated at 25Cº under continuous low light (Padgett & Leonard 1994, Fang *et al.,* 2005). After 15 days of cultivation the cellular clumps were initially green and then most of them turned brown. At this time the medium needed to be replaced by fresh medium. The procedure was repeated for 6 to 8 weeks during which time calluses began to dissociate into single cells and small cell clumps.

**Fresh weight and total chlorophyll:** Fresh weight of cultures was measured by filtering 12ml of culture through oven dried pre-weighed, Whatman No.1 filter paper. The growth of culture was monitored with sets of flasks harvested at 3-day intervals from the day of subculture (Day 0) up to 21 days readings were taken from three flasks for each parameter. Dry weight measurements were made after the filter paper with the cells was dried to constant temperature at 80°C (Aguado-Santacruz *et al.,* 2001). The Suspension cultures were maintained routinely by transferring 5ml of culture to 250ml flaks containing 50ml fresh medium at 2 week intervals. After weighing three of samples were used for determination of chlorophyll contents at each sampling data. Total chlorophyll contents (a & b) was determined from absorbance measurements at 663 and 645 nm for cells ground in 80% alcohol. Total chlorophyll contents were calculated according to the procedure of Arnon (1949).

## Result and Discussion

**Initiation of callus and maintenance:** Hypocotyl and leaf explants of *Jatropha curcas* were cultured on MS basal medium supplemented with different growth regulator formulation including 0.5mg/L 2,4-D alone and with coconut milk (2% v/v), 0.1mg/L 2,4-D, 0.5mg/L GA3, and coconut milk (2% v/v), 0.5mg/L 2,4-D, 0.1mg/L GA3 and 0.5mg/L BA,0.5mg/L BA, 0.1mg/L 2,4-D and coconut milk (2% v/v) and 0.5mug 2,4-D and 0.1mg/L BA for initiation of callus (Table 1). Callus initiation took place from cut edges of each explant after approximately 7 days for hypocotyl explants and after 15-20 days for leaf explants (Fig. 1a and b). Callus produced from hypocotyl explants grew faster during first 7 to 30 days of culture then stabilized at a slow growth rate in the medium containing 0.5mg/L 2, 4-D. (Monacelli *et al*., 1995). MS medium with 0.5mg/L 2,4-D alone and with coconut milk showed 40% and 90% calli formation per explants from leaf and hypocotyl. Callus easily proliferated (Fig. 2a and b). Callus produced was soft, very friable, compact, globular and lush green in color (Rao *et al*., 2006). At the beginning of culture period it was necessary to subculture after every 15 days, otherwise the calli became dark and growth ceased. The darkening of callus was probably due to the production and oxidation of phenolic compounds released by explants (Monacilli *et al*., 1995). The other growth regulator formulations showed very slow response to explants and were unable to support growth of explants and necrosis occurred after 4 weeks of culture. Hypocotyl explants and MS medium with 0.5mg/L 2, 4-D alone and with 2% v/v

**Table 1. Growth regulator formulations used for induction of callus from leaf and hypocotyls explants of *Jatropha curcus* L.**

| Growth regulator concentration (mg/L) | Leaf explants forming callus (%) | Callus response | Hypocotyls forming callus (%) | Callus response |
|---|---|---|---|---|
| 2, 4-D 0.5 Coconut milk 2% v/v | 40 | Dark green, Nodular Compact, Hard | 100 | Lush green, Compact, Nodular soft, and Friable |
| 2, 4-D 0.5 | 38 | Light to dark green, Soft, Globular, Compact | 100 | Lush green, Compact, Nodular soft, and Friable |
| 2, 4-D 0.1 $GA_3$ 0.5 Coconut milk 2% v/v | 10 | Light green, Hard, Compact | 30 | Dark green, Compact, Hard |
| 2, 4-D 0.5 $GA_3$ 0.1 BA 0.5 | 10 | Light green, Nodular Compact, Slightly soft | 28 | Light to dark green, Compact, Globular, Slightly hard |
| 2 4-D 0.5 BA 0.1 | 15 | Light to dark green, Compact, Nodular soft | 20 | Light green, Compact, Slightly soft, Globular |
| BA 0.5 2, 4-D 0.1 Coconut milk 2% v/v | 15 | Light to dark green, Compact, Nodular and soft | 15 | Dark green, Compact, Globular and soft |

coconut milk proved to be more effective for establishment of callus on a large scale in short period of time (Kawak *et al.,* 1995; Hoshino *et al.,* 1995). Kim *et al.,* (2004) reported yellowish, off-white, friable calluses in *Catharanthus roseus* by using different concentrations of 2, 4-D. Similarly, Kim *et al.,* (2005) found globular, friable, off- white callus in medium supplemented with BA, NAA and 2, 4-D respectively in *Pinellia tripartite.* Although, it has been reported that 2, 4-D is the most commonly used auxin in cereal tissue culture (Bhaskaran & Smith, 1990; Soomro *et al.,* 1993), other auxins when used with low concentrations to cytokinins have been found to be more effective for induction of callus (Gerardo Armodo *et al.,* 2001; Fang *et al.,* 2004; Zouine & Hdrami, 2004; Datta & Conger, 1999; Rao *et al.,* 2006, Pathirana & Eason 2006). In our studies, the greatest increase in fresh weight (8 to 10 fold) was observed in medium supplemented with 0.5mg/L 2, 4-D in hypocotyl explants. Contrarily Salman (2002) reported two fold increase in fresh weight by using 1.0mg/L 2, 4-D and 0.2mg/L BA in leaf segments of *Paniculata.* Similarly, the increase in fresh weight of callus was also reported by various authers, Pradhan *et al.,* (1998) in *Dalbergia sissoo;* Pattnaik *et al.,* (2000) in *Dalbergia latfolia* and Manacilli *et al.,* (1995) in *Taxus baccata,*Rao *et al.,* (2006) in Gossyonium spp. The fast growing callus and cell suspension cultures of *Jatropha curcas* were established successfully and maintained for over a period of 2 years with out any change in Growth rate for further experiments.

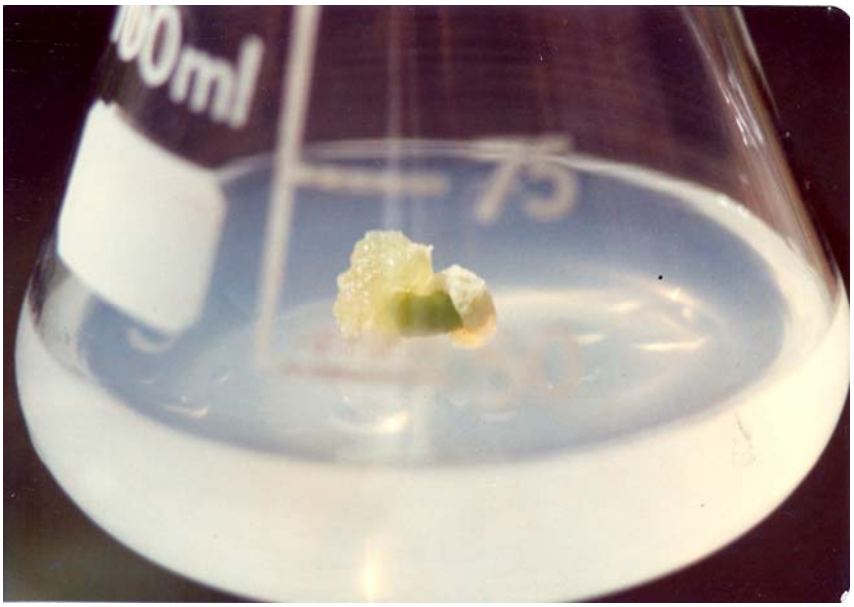


Fig. 1a. Initiation of callus of *Jatropha curcas* L., from cut edges of the hypocotyls explant after 7 days of culture on full MS medium supplemented with 0.5 mg/L 2, 4-D, 2% v/v coconut milk. The cultures were kept under 18/6 hours L/D cycle at 2000 Lux light intensity.


Fig. 1b. Initiation of callus of *Jatropha curcas* L., from cut edges of leaf explant after 15 days of culture on full MS medium supplemented with 0.5 mg/L 2, 4-D, 2% v/v coconut milk. The cultures were kept under 18/6 hours L/D cycle at 2000 Lux light intensity.

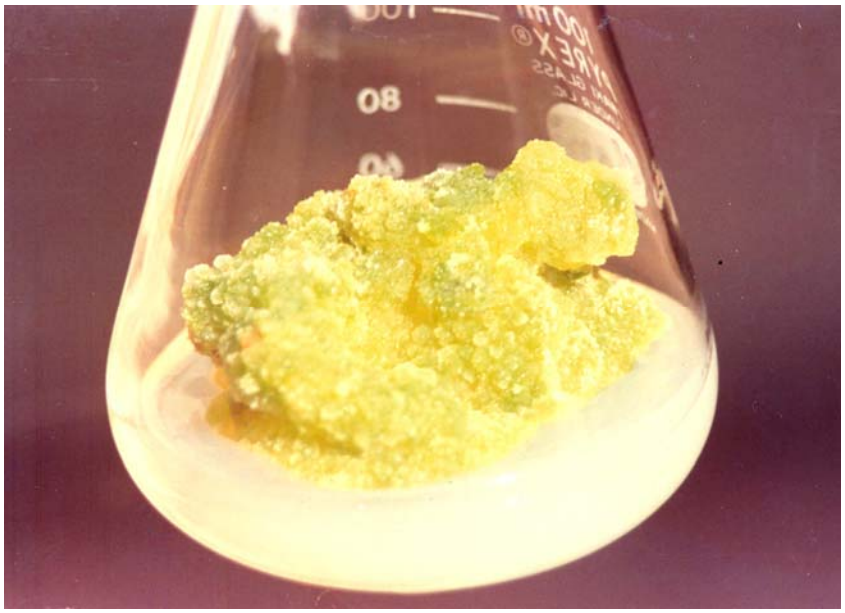

Fig. 2a. Proliferation of callus from hypocotyls explant of *Jatropha curcas* L., after 4 weeks of culture on full MS medium supplemented with 0.5 mg/L alone. The cultures were kept under 18/6 h L/D cycle light intensity.

Fig. 2b. Proliferation of callus from leaf explant of *Jatropha curcas* L., after 4 weeks of culture on full MS medium supplemented with 0.5 mg/L alone. The cultures were kept under 18/6 h L/D cycle light intensity.

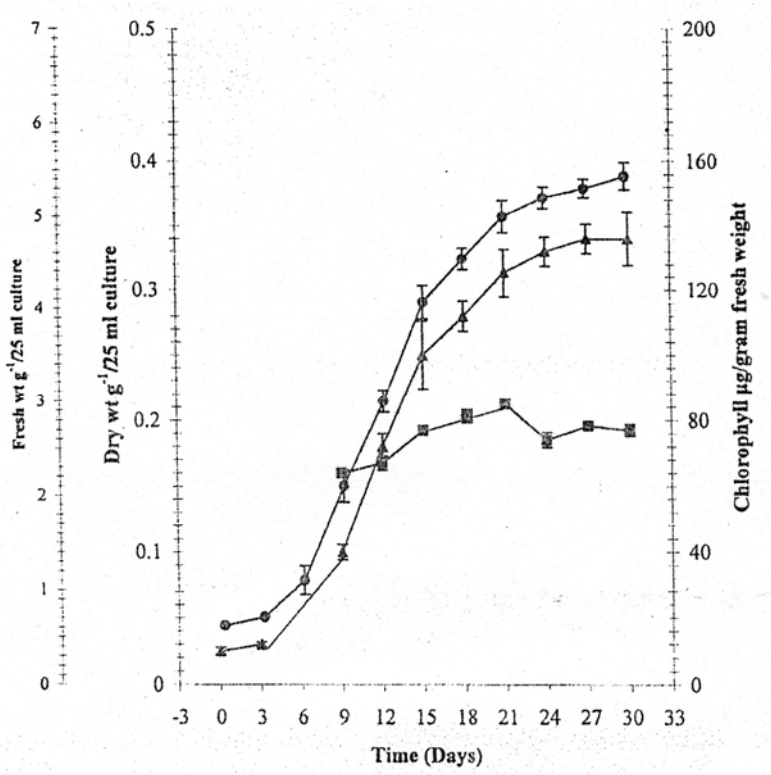


Fig. 3. Growth curve showing fresh weight (●), dry weight (▲) and chlorophyll (■) of cultured *Jatropha curcas* L., cells grown from hypocotyls derived callus in MS medium supplemented with 0.5 mg/L 2, 4-D over a period of 30 days after sub culture.

**Establishment of cell suspension culture:** Cell suspension cultures were established by using 4 weeks old callus (hypcotyl derived) as inoculum. One gram of actively growing lush green friable Callus was excised in small pieces on Whatman No.1 filter paper, meshed slightly and transferred to liquid medium supplemented with 0.5mg/L.2, 4-D alone and with coconut milk 2% v/v. Initially cell suspension culture comprises of some isolated cells and small cell aggregates of 5-8 cells. Filtering the cells through a series of sieves reduced the clusters to less than 200 µm in diameter and these cells were used for sub culturing. Hypocotyl cleaved suspension cultures were grown faster and accumulated a greater biomass in media containing 0.5mg/L 2, 4-D over a period of 30 days. Similar results were reported by Pathirana & Eason. (2006) in *Arabidopsis thaliana* suspension cultures derived from leaf callus. A majority of authors establish cell suspension culture by using 2.4-D at different concentration (Kobayashin & Esteves Vieira, 2000; Kim *et al.,* 2004-2005; Nakamura & Ishikawa, 2006; Rasika & David, 2006; Rao *et al.,* 2006). However other used auxin with low concentration to cytokinin for establishment of cell suspension cultures. (Pradhan *et al.,* 1998; Odjakova & Conger, 1999; Lopes *et al.*, 2000; Zhang *et al.,* 2002; Bargmnn *et al.*, 2006; Yu *et al.,* 2005). After 3 weeks of growth a finely dispersed homogeneous and chlorophyllous cell suspension culture was obtained (Aguado-Santacruz *et al.,* 2001). The suspension cultures comprised mainly of round, densely cytoplasmic starch containing cells with distinct nuclei. Some cells are large

elongated and highly vacuolated with sparse cytoplasm (Datta & Conger, 1999). The suspension cultures were sub cultured routinely on fortnight basis by transferring of 5ml of suspension culture into 50ml fresh medium using a wide bore pipette. For maintenance as the fine suspension culture it is necessary to subculture them because the cultures tended to form cell clusters of a few cells to aggregates.

The growth curve of suspension cultures indicated that the growth rate of cells were initially slow during first 3 days (lag phase) but as the cultures proceeded they showed a mark increase from day 6 (Fig. 3) and significantly accumulated great amount of fresh weight over a period of 15 days (log phase). Maximum increase in fresh weight was reached on day 21 which was about 5-6 fold over initial fresh weight (Nakano *et al.,* 2000; Gurel *et al*., 2003; Pathirana & Eason 2006; Mudalige & Longstreth 2006). Monacilli *et al.,* (1995) found 3 fold increase in cell suspension culture of *Taxus baccat* over a period of 40 days. The rate of growth was stable for 30 days (Stationary phase) but the rate gradually increased as the duration after the initiation of suspension cultures increased. Similarly, the cell dry weight gradually increased which almost corresponded to fresh weight. The maximum 3-4 fold increase in dry weight was observed by day 15 (Fig. 3). A five fold increase in cell cultures of *Bouteloua gracillis* was reported by Aguado *et al.,* (2001). However, Pathirana & Eason (2006) showed the dry mass of cells peaked at day 7 and then declined.

According to Widholm (1992) the total chlorophyll content in green cultures ranges from 30 to 2, 000 ug/g FW, with most cultures being below the 2000ug level. But our cultures showed the variation between 50.7 to 75.2ug/g FW in total chlophyll with in the growth cycle of cultures. The maximum total chlorophyll content was reached 80.6ug/g FW during 21 days, later a decrease in chlorophyll content was observed during the remaining period of 30 days (Fig. 3). Aguado *et al.,* (2001) founded that the total chlorophyll content varied between 18.3 and 121.6ug/g FW within the growth cycle of *Bouteloua gracilis*. However, Husemann (1984) considered a 3000% increase in fresh weight within 10 days and a high chlorophyll content (more that 70ug/g FW) as per-requirement for the initiation of photoautotrophic cell cultures. In my cultures I found that the use of growth regulator 2, 4-D is known to be more compatible with chlorophyll accumulation. Husemann & Barz (1977); Yamada *et al.,* (1978); Chaumont & Gudin (1985) reported the similar findings. The cells growing in medium containing 0.5mg/L 2,4-D were active and induced higher rates of cell division such as early prophase and late telephase after 5-7 days of sub culturing (Gurel *et al.,* 2002).

## Conclusion

In present studies, an establishment of fast growing callus and cell suspension cultures of *Jatropha curcas* L., and their long term maintenance for a period of 2 years without an apparent change in the growth rate was achieved for further experiments. It is the first report demonstrating the establishment and long-term maintenance of callus and cell suspension cultures in *Jatropha curcas* L.

### References

Aguado-Santacruz, G.A., J.I. Cabrera-Ponca, E. Ramirez-Chavez, C.G. Leon-Ramirez, Q. Rascon-Cruz, L. Herrera-Estrella and V. Olalde-Portugal. 2001. Establishment characterization and plant regeneration from highly chlorophyllous embryonic cultures of blue grams, *Bouteloua gracilis* (H.B.K) Lag.ex Steud. *Plant Cell Report*, 20: 131-136.

Arnon, D.I. 1949. Copper enzyme in isolated chloroplast. *Plant Physiol*., 24: 1-15.

Bhaskaran, S. and R.H. Smith. 1990.Regeneration in cereal tissue culture: review. *Crop Sci*., 30: 1328-1336.

Bargmann, B.O., A.M. Laxalt, B. Riet, E. Schouten, W.V. Leenwen, H.L. Dekker, C.G. Koster, M.A. Haring and T. Munnik. 2006. LePLDß1 activation and elocalization in suspension cultured tomato cells treated with xylanase. *The Plant Journal,* 45: 358-368.

Chaumont, D. and C. Gudin. 1985. Transition from photomixotrophic to photoautotrophic growth of *Asparsgus officinalis* in suspension culture. *Biomass*, 8: 41-51.

Datta, S.K. and R.K. Pandey. 1993. *Applied Botany* Abstracts, 1(2): pp.108-118.

Datta Gupta, S. and B.V. Conger. 1999. Somatic embryogenesis and plant regeneration from suspension cultures of Switchgrass. *Crop Sci*., 39: 243-247.

Dastur, J.F. 1952. *Medicinal plants of India and Pakistan*, pp.159, D.B. Taraporevela Sons and Co. Ltd., Bombay.

Escalona, M., J.C. Lorenzo, B. Gonzaler, M. Daquinta, J.L. Gonzaler, Y. Desjarodin and C.G. Borrota. 1999. Pineapple, (*Anunus Comsus* L. Merr.) Micropropagation in temporary immersion Systems. *Plant Cell Rep*., 18: 743-748.

Fang, Yu., Dongyan Zhang, B. Ai. Fengwu and An. Lijia. 2005. The accumulation of Isocamptothecin A and B in suspension cell cultures of *Camptotheca acuminate*. *Plant Cell, Tissue and Organ Culture*, 81:159-163.

Gerardo Armando Aglado-Santacruz, Jose Luis Cabrera-Ponce, Victor Olalde-Portugal, M.A. Rosario Sanchez-Gonzalez, Judith Marquez-Guzman and Luis Herrera-Estrella. 2001. Tissue culture and plant regeneration of blue grama Grass, *Bouteloua gracilis* (H.B.K) Lag ex Steud. *In vitro Cell. Dev. Biol-Plant,* 37: 182-189.

Grim, C. 1996. The Jatropha project in Nicaragua, Bagani Julu (Mali), 1, pp. 10-14. Gupta.

Gurel, S., E. Gurel and Z. Kaya. 2002. Establishment of cell suspension cultures and plant regeneration in sugar beet (*Beta vulgaris* L.). *Turkish Journal of Botany*, 26(4): 197-205.

Husemann, W. 1984. Photoautotrophic cell culture. In: *Cell culture and somatic cell genetics of plants*. (Ed.): I.K. Vasil. (Laboratory procedures and their application, vol.1). Academic Press, Orlando, 182-191.

Husemann, W. and W. Barz. 1977. Photoautotrophic growth and photosynthesis in cell Suspension cultures of *Chenopodium rubram. Plant Physiol*., 40: 77-81.

Hippolyte, I., B. Marin, J.C. Baccou and R. Jonard. 1992. Growth and rosmarinic acid Production in cell suspension cultures of *Salvia officinalis* L. *Plant Cell Report*, 11: 109-112.

Hoshino, Yoichiro, Masaru Nakano and Masahiro Mii. 1995. Plant regeneration from cell suspension derived protoplast of *Saintpaulia ionantha* Wendi. *Plant Cell Report*, 14(6): 341-344.

Ilan, A., M. Zliv and A.H. Halevy. 1995. Propagation and corn development of Brodiaca in liquid culture *Sei. Hort*., 63: 101-112.

Kawak, Sang Soo, Soo Kyung Kim, Moon Soon Lee, Kyung Hee Jung, 11–Hyun Parkand Jung Ryol Lia. 1995. Acidic peroxidases from suspension cultures of sweet potato. *Pyto-chemistry* (Oxford), 39(5): 981-984.

Kim, E.K., E.J. Hahn, H.N. Murthy and K.K.Y. Paek. 2003. High frequency of short multiplication and bulblet formation of garlic in liquid Culture. *Plant Cell Tissue and Organ Culture*, 73: 231-236.

Kim, S.W., Dong Su In., Pil Son Choi and Jang R. Liu. 2005. Plant regeneration from immature zygotic embryo-derived embryogenic calluses and cell suspension cultures of *Catharanthus roseus. Plant Cell Tissue and Organ Culture*, 76: 131-135.

Kim, S.W., Dong Su In., Kyoung Hwan Tae and Jang Ryol Liu. 2005. Somatic embryogenesis and plant regeneration in leaf and petiole explant cultures and cell suspension cultures of *Pinellia tripartite. Plant Cell Tissue and Organ Culture*, 80: 267-270.

Kobayashin, A.K. and L.G.E. Vieira. 2000. Establishment of an *invitro* system for studies on the induced resistance of cotton to *Xanthomonas Campestris* Pv. Malvacearum. *Pesq. Agropec. Bras*., 35(4): Brasilia.

Lopes, S.O., P.R. Moreno and A.T. Henriques. 2000. Growth characteristics and chemical Analysis of *Psychotria carthagenesis* cell suspension cultures. *Enzyme and Microbial Technology*, 2-4: 259-264.

Monacelli, B., G. Pasqua, A. Cuteri, A. Varusio, B. Botta and G.D. Monache. 1995. Histological study of callus formation and optimization of cell growth in *Taxus Baccata*. 159 *Cytobios*, 81: 159-170.

Nakamura, T. and M. Islinkawa. 2006. Transformation of suspension cultures of bromegrass (*Bromus inermis*) by *Agrobacteruim tumefactions*. *Plant Cell, Tissue and Organ Culture*, 84: 293-299.

Munch, E. and J. Kielfer. 1989. Schriftenreiheder GTZ. No. 209, pp. 1-32.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiol Plant*, 15: 473-497.

Mudalige, R.G. and D. Longstreth. 2006. Effects of Salinity on photosynthetic characteristics in Photomixotrophic, cell suspension cultures from *Alternanthera, philoxeroides*. *Plant Cell, Tissue and Organ Culture*, 84: 301-308.

Nakano, M., T. Sakakibara, S. Sakae and H. Saito. 2000. Decrease in the regeneration potential of long term cell suspension cultures of *Lillium formosanum* Wallaee and its restoration by the auxin transport inhibitor, 2,3,5-triiodobenzoic acid. *Plant Science*, 158: 129-137.

Nisar, E and S.I. Ali. 1986. *Flora of Pakistan*, Euphorbiaceae, pp.172.

Osjakova, M.K. and B.V. Conger. 1999. The influence of osmotic pretreatment and inoculum age on the initiation and regenerability of switchgrass suspension cultures. *In Vitro Cell. Div. Biol*., 35: 442-444.

Pathirana, R. and J.R. Eason. 2006. Establishment and characterization of a rapidly dividing diploid cell suspension culture of *Arabidopsis thaliana* suitable for cell cycle synchronization. *Plant Cell, Tissue and Organ Culture*, 85: 125-136.

Padgett, P.E. and R.T. Leonard. 1994. An amino acid from maize cell line for use in investigating, nitrate assimilation. *Plant Cell Reports*, 13: 504-509.

Pattnaik, S., C. Pradhan, S.K. Naik and P.K. Chand. 2000. Shoot organogenesis and plant regeneration from hypocotyls-derived cell suspensions of a tree legume,*dalbergia sissoo* Roxb. *In Vitro Cellular and Development Biology-Plant*, 36(5): 407-41.

Paradhan, C., S. Pattnaik, M. Dwari, S.N. Patnaik and P.K. Chand. 19980. Efficient plant regeneration from cellc suspension-derived callus of East Indian rosewood *(Dalbergia latifolia* Roxb.). *Plant Cell Reports*, 18(1-2): 138-142.

Salman, M.N. 2002. Establishment of callus and cell suspension cultures from *Gypsophila paniculata* leaf segments and study of the attachments of host cells by *Erwinia herbicola* pv. g*ypsophilae*..*Plant Cell Tissue and Organ Culture*, 69(2): 189-196.

Rao, A.Q., Hussnain S. Sarfraz, Shahzad M. Sadqib, Bokhari S. Yassir Abbas Hussain Tayyab and S. Rizuddin. 2006. Somatic embrogenesis in wild relatives of cotton (*Gossipium* spp.). *Journal of Zhejiang University Science*, B 291-298.

Risika, G. Mudalige and David J. Logstregth. 2006. Effect of salinity on photosynthetic characteristics in photomixtrophic cell-suspension cultures from *Alternanthera Philoxeroides*. *Plant cell Tissue and Organ Culture*, 84: 301-308.

Soomro, R., S. Yasmin, G.S. Markhand, B. Ahmed and A.Q. Mahar. 1993. Induction and growth of callus derived from *Vigna radiate* L., hypocotyl explants. *Scientific Sindh, Annual Jour. Res*., (1): 159-166.

Sujatha, M. and Mukta. 1996. Morphogenesis and plant regeneration from tissue cultures of *Jatropha curcas*. *Plant cell Tissue and Organ Culture*, 44: 135-141.

Widholm, J.M. 1992. Properties and uses of phytoautotrophic plant cell cultures. *Int. Rev. Cytil*., 132: 109-175.

Yamada, Y., F. Sato and M. Hagimori. 1978. Phtoautotrophism in green cultures cells. In: *Frontiers of plant tissue culture*. (Ed.): T.A. Thorp. University of Calgary Printing Services, Calgary, app. 453-461.

Yoshifumi, T. 1982. Development study on Jatropha curcas oil as a substitute for diesel egine oil in Thailand. *Interim Report. Agric. Engineering Div. Department of Agriculture, Thailand*.

Zhang, D., F. Bai, and L. An. 2005. The accumulation of isocamptothecin A and B in suspension cell culture of *Camptotheca acuminata*. *Plant Cell, Tissue and Organ Culture*, 81: 159-163.

Zhang-Shouying, que GuoNing, S.Y. Zhang and G.N. Que. 2002. Establishment of cell Suspension culture and isolation of protopolast of *Pinus serotaina*. Forest Research, Bejjing, 15(2): 247-251.

Zhong, J.J., K.B. Konstantinov and T. Toshida. 1994. Computeraided on line mnitoring of physiological variables in suspended cell cultures of a *Perillafrutescens* in a bioreactor. *J. Ferment. Bioeng*., 77: 445-447.

Zouine, J. and E.I. Hadrami. 2004. Somatic embryogenesis in *Phoenix dactylifera* L: Effect of exogenous supply of sucrose on proteins, phenolics and peroxidases activities during the embryogenic cell suspension culture. *Biotechnology*, 3(2): 114-118.

(Received for publication 14 February 2006)