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AN EFFICIENT METHOD FOR THE ESTABLISHMENT OF CELL SUSPENSION CULTURES IN POTATO *(SOLANUM TUBEROSUM* L.)

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Abstract

Cell suspension cultures offers an *In vitro* system that can be used as a tool for various studies involving mutant selection, mass propagation, protoplast isolation, gene transfer and selection of cell-lines which are resistant to various biotic or abiotic stresses. Research work on the development of cell suspension cultures was carried out to establish the most efficient method in Potato (cv. Desiree). Healthy, well-proliferating tissues from different types of callus cultures (compact, friable, embryogenic or non-embryogenic) were inoculated on various media combinations, i.e., MS, MS2 or AA liquid medium containing 18.09 µM 2, 4-D. A fixed quantity (0.5-1.0 g) of callus tissue from 60-day-old callus cultures was transferred to 10-25 ml of liquid medium in 100 ml Erlenmeyer flask. Cultures were placed on an orbital shaker and agitated at different speeds (75, 100 or 125 rpm) under 16-h photoperiod at 25 ± 2°C. Medium was changed after every 3 days and fractionated tissue was filtered after every 6 days through sterile mesh (100-800 µm) to develop a cell-line by transferring resulting suspension to fresh medium under the same conditions. Results indicated that eight-week-old translucent, friable, off-white callus cultures were an excellent starting material for the initiation of homogeneous cell suspension cultures as compared to other tested sources. Of the three tested media (MS, MS2 or AA medium containing 18.09 µM 2, 4-D), MS2 was found to be a better medium for the initiation of cell suspension cultures. Cell suspension cultures, placed in 16-h photoperiod at 25 ± 2°C and agitated at 120 rpm using a gyratory shaker showed excellent results. Several other factors influencing quick establishment of cell suspension cultures in this cultivar are also discussed in this communication.

Key words: Callus, Cell suspension cultures, *In vitro*, Potato.

Introduction

Potato (*Solanum tuberosum* L.) is an important commercial food crop of the world and ranks fourth after maize, wheat and rice with annual production of 342 M tones (Anon., 2014). It belongs to the family *Solanaceae* which includes 90 genera and 2800 species. It is reported to have far greater nutritive value as a food crop and is consumed at the rate of 11.0 kg per capita per annum (Anon., 2014). Besides being an important vegetable, it also supplies at least 12 essential minerals other than starch (12-20%), protein (1.87%), fiber (1.80%), fats (0.1%), vitamin C, and high phosphate contents with small amount of calcium and ash (Irfan, 1992). Potato has been recognized as a crop of high potential after cereals that can meet future food demands. In the year 2013-2014, a world-over total area under this crop was 19.837 M ha with the yield of 16.992 tons/ha. Total area under cultivation in Pakistan is 23.63 M ha out of which potato is grown on over 131.90 thousand hectares with an annual production of 19.90 tons/hectare (Anon., 2014). The per hectare yield of potato in Pakistan is very low as compared to developed countries of the world (Malik 1995, Farhatullah *et al.*, 2002) due to several reasons like poor agricultural practices, susceptibility to several diseases and pests, non-availability of healthy certified seeds, and soil salinity.

With the rapid development of cell engineering, studies using suspension cells as starting plant material have gained momentum over the past several years (Evans *et al.*, 2003, Mazareia *et al.*, 2011, Queiros *et al.*, 2011). A fine cell suspension line is not only a good target tissue for gene transfer (Zheng *et al.*, 2005), but also offers a suitable system to study cytology, molecular biology (Chen *et al.*, 2002) or to work with protoplast cultures and production of secondary metabolites (Spela *et al.*, 2005) and somatic embryogenesis. Therefore the establishment of simple and reproducible protocols for the establishment of cell suspension cultures in economically important potato cultivars may provide with an opportunity to make further progress in above-mentioned areas of research.

Plant physiologists and plant biochemists have also recognized the merits of using various single cell systems over intact organs and whole plants for studying cellular metabolism (Queiros *et al.*, 2011). The free cell system permits quick administration and withdrawal of diverse chemicals and radioactive substances (Gnanam & Kulandaivelu, 1969, Edwards & Black, 1971). The cloning of single cells permits crop improvement through the extension of the techniques of microbial genetics to higher plants. Large scale cultivation of plant cells *In vitro* provides a viable alternative for the production of vast arrays of commercially important phytochemicals.

Cell suspension culture medium and conditions that varies from species to species is the major problematic area in establishing efficient cell suspension lines. Further, Bacterial and fungal contamination is also a hurdle in the establishment of cell suspension (Dodds & Roberts, 1995). An opaque, dense white or pink colouration that appears very rapidly (within 24hours) is indicative of yeast and bacterial contamination. Fungal contamination can be seen in the form of balls of mycelium (Reinert & Yeoman, 1982).

Here we report the development of an efficient method of initiation and development of cell suspension cultures in potato cv. Desiree (commercially important). The different cell types among the potato suspension cultures provide an opportunity to gain further insights into their potential application in selection of tolerant cell lines against different stress conditions.

Materials and Methods

Callus Induction and proliferation: To optimize an efficient callus induction protocol in potato (*Solanum tuberosum* L. cv. Desiree), various media combination and explant source were used. Murashige & Skoog (MS 1962) medium supplemented with different concentrations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) alone or in combination with 6-Benzyladenine (BA) or Naphthaleneacetic acid (NAA) (media designated as C1 to C13) was used. Internodal explants (ca. 1.0 cm long) were used for callus induction. Calluses were sub-cultured after every 15 days. The incubation conditions included darkness at 25 ± 2°C.

Cell Suspension cultures: Healthy, well-proliferating tissues from different types of callus cultures (compact, friable, embryogenic or non-embryogenic) were inoculated on different media combinations, i.e., MS, MS2 (Vargas *et al.*, 2005) or AA liquid medium (Muller & Grafe 1978) containing 18.09 µM 2, 4-D for the establishment of cell suspension cultures. A fixed quantity (0.5-1.0 g) of callus tissue from 60-day-old callus cultures was transferred to 10-25 ml of liquid medium in 100 ml Erlenmeyer flask. Cultures were placed on an orbital shaker and agitated at different speeds (75, 100 or 125 rpm) under 16 h photoperiod at 25 ± 2°C. Medium was changed after every 3 days and fractionated tissue was filtered after every 6 days through sterile (100-800 µm) mesh to develop a cell-line by transferring resulting suspension to fresh medium under the same conditions.

Results

Standardization of medium for callus induction and proliferation: Amongst various concentrations of auxins used in MS medium, 2, 4-D at 18.09 µM was quite effective favoring 90% callus induction after 12 days of inoculation. Calluses were morphologically off-white, friable and granular (Plate 1A&B). Decrease in the concentration of 2, 4-D reduced the rate of callus induction. As evident from the data given in Table 1, when the concentration of 2, 4-D was decreased from 18.09 to about one-half (9.04 µM), rate of callus formation correspondingly decreased from 90 to 40%. Interestingly, no callus induction was observed in all the replicated culture vessels using MS medium with 4.52 µM 2, 4-D.

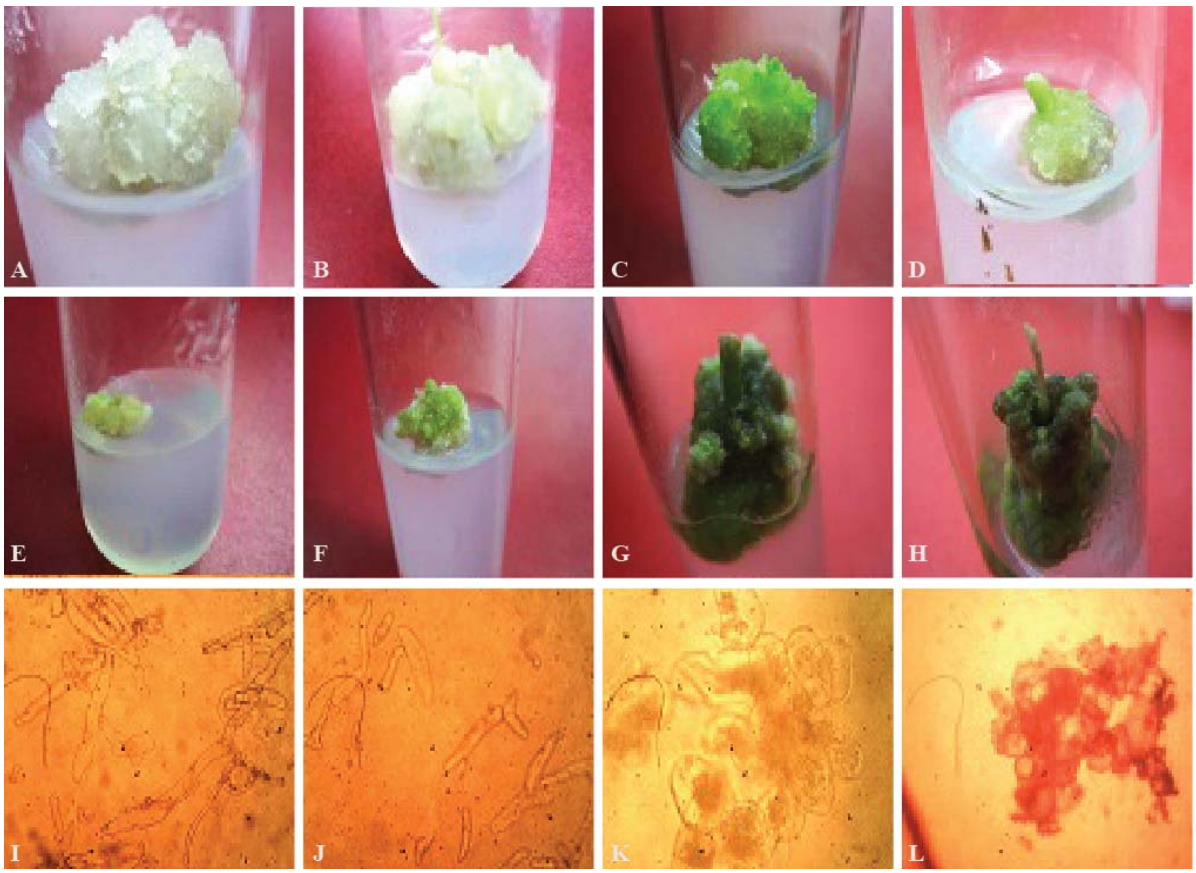

Plate 1. Morphology of potato callus cultures (cv. Desiree) initiated from internodal segments on MS basal medium supplemented with different growth regulators at day 60. A&B: Well-proliferating callus cultures on MS medium supplemented with 18.09 µM 2, 4-D (1.5 & 1.2X). C&D: Callus cultures on MS medium supplemented with 13.5 µM 2, 4-D and 2.22 µM NAA (1 & 1.5X). E&F: Callus cultures on MS medium supplemented with 13.5 µM 2, 4-D and 1.07 µM NAA (1.2 &1X). G&H: Callus cultures on MS medium supplemented with 10.0 µM BAP and 1.07 µM NAA (2X). I: Elongated and poorly-dividing cell suspension of cv. Desiree with thick walls (100X). J. Elongated cells in suspension cultures of Desiree derived from compact-green callus cultures (100X). K Globular, rounded cells with good division efficiency (100X). L. Clusters of rapidly-dividing rounded cells with smaller diameter (100X).

Table 1. Effect of different growth regulators supplemented to MS medium on callus induction and proliferation in *Solanum tuberosum* L. cv. Desiree

Media designation	Medium and Growth regulators	Concentration of growth regulators (µM)	Time to callus induction (Days)	Callus induction (%)
C1	MS + 2, 4 –D	4.52	-	0
C2		9.04	19 ± 1.009	40
C3		13.57	19 ± 0.894	60
C4		18.09	12 ± 1.094	90
C5	MS + 2, 4-D + BAP	13.5 + 1.11	13 ± 1.394	60
C6		13.5 + 2.22	14 ± 0.304	65
C7		13.5 + 4.43	18 ± 0.794	52
C8	MS + 2, 4-D + NAA	13.5 + 1.07	14 ± 0.990	90
C9		13.5 + 2.14	13 ± 1.314	80
C10		13.5 + 3.21	21 ± 1.322	65
C11	MS + BAP + NAA	10.0 + 1.07	13 ± 0.994	89
C12		10.0 + 2.20	13 ± 1.334	70
C13		10.0 + 3.30	20 ± 1.340	70

Data are the means ± S.E. from 30 replicate culture vessels per treatment

Table 2. Optimum conditions for the initiation/establishment of cell suspension cultures of potato cv. Desiree

Parameters	Optimum Conditions
Source material	Eight-week-old translucent, friable, off-white calluses
Culture medium	MS2 medium (Vargas *et al*., 2005)
Growth regulator/s	18.09 µM 2, 4-D
Source material/medium ratio	0.5 g callus/10 ml medium
Culture vessels	Erlenmeyer flasks, capacity 100 ml
Agitation rate (rpm); Optima orbital shaker, OS-752, Japan	120 rpm
Temperature	25 ± 2°C
Light conditions	16 h photoperiod using fluorescent-white tube-lights (35 µmol m^{-2} s^{-1})
Sub-culturing interval	Every 3-5 days
Sieving mesh size for the first two subcultures	450 µm

Three different combinations of 2, 4-D and BA were used to find out their effect on callus induction. Among these combinations, 2, 4-D at a concentration of 13.5 µM with 2.22 µM BA proved effective with 65% callus induction response after 14 days of inoculation. Usually the calli were off-white to green, friable and loose (Plate 1C & D). When concentration of BA was decreased or increased from 2.22 µM, rate of callus induction was reduced in the tested cultivar.

Of auxin-auxin combinations, 2, 4-D and NAA were also used for callus induction. MS medium supplemented with 2, 4-D at a concentration of 13.5 µM with 1.07 µM NAA showed excellent results with 90% callus induction response after 14 days of inoculation (Plate 1E & F). With further increase in the concentration of NAA, i.e., 2.14 to 3.21 µM with the same concentration of 2, 4-D (13.5 µM), callus induction response was reduced from 80% to 60%.

Best callus induction response was observed by using a combination of BA (10.0 µM) and NAA (1.1µM) supplemented to MS medium (Plate 1G & H). At this concentration, 89% callus induction was obtained after 13 days of explants inoculation. Callus cultures were green with white patches and compact. By increasing the concentration of NAA to 3.3 µM, the rate of callus formation was reduced to 70%.

Optimization of conditions for the initiation of cell suspension cultures: The summary of conditions determined for the initiation of cell suspension cultures in potato cultivar Desiree is given in Table 2.

Source material: Eight-week-old translucent, friable, off-white callus cultures were an excellent starting material for the initiation of homogeneous cell suspension cultures as compared to other tested sources, e.g., compact green, compact white or friable-yellow callus cultures (data not given). It was also noted during this study that elongated cells with thick walls were quite poorly-dividing (Plate 1 I & J). On the other hand, globular or rounded cell suspensions had better division efficiency (Plate 1 K & L) in potato.

Medium: Of the three tested media (MS, MS2 or AA medium containing 18.09 µM 2, 4-D), MS2 was found to be a better medium for the initiation of cell suspension cultures. The division efficiency of suspension cells was also comparatively better on this medium.

Culture conditions: Cell suspension cultures, placed in 16-h photoperiod at 25 ± 2°C and agitated at 120 rpm using a gyratory shaker yielded excellent results. It was also observed that filtration through nylon or stainless-steel sieve (450 µm) helped to remove the larger cell aggregates that could otherwise clog smaller pore-size meshes. The cells collected after sieving through 450 µm mesh proved to an excellent source material for the establishment of cell suspension cultures in potato.

Discussion

During the present investigation, different media combinations were used for callus induction. Internodal segments were chosen as explants for the initiation of callus since this explant source had shown good callusing potential in many earlier studies involving potato (Svetek *et al.*, 1999; Turhan 2004; Vargas *et al.*, 2005; Gopal *et al.*, 2008). In this study, 2, 4-D at a concentration of 18.09 µM was proven to be quite effective for callus induction and proliferation in this tested potato cultivar. This effective role of 2, 4-D in callus induction in potato has also been reported in many previous studies (Khatun *et al.*, 2003; Vargas *et al.*, 2005; Naz & Khatoon, 2014). It is evident from the literature that 2, 4-D increases the endogenous auxin levels in explants (Michalczuk *et al.*, 1992) which might influence cell enlargement and cell divisions more efficiently than other growth regulators. On the contrary, different combinations of growth regulators (auxin & cytokinin) were also used for callus induction in different potato cultivars (Svetek *et al.*, 1999; Rahnama *et al.*, 2003; Nasrin *et al.*, 2003; Rahnama & Ebrahimzadeh, 2004; Queiros *et al.*, 2007; Jan & Khatoon, 2014; Sajid & Aftab, 2014). This varied response for callus induction on different media might be due to the genotypic variation.

As mentioned, cell suspension cultures are ideally suited to study various factors that affect on their growth and differentiation (Evans *et al.*, 2003). During this study, it was observed that friable callus cultures were an excellent starting material for the establishment of cell suspension cultures in this cultivar of potato. The use of friable callus for the initiation of cell suspension cultures is considered to be very important and a primary step (Bhojwani & Razdan 2004, Liang *et al.*, 2006). In line with this, friable callus cultures segregated into free cells relatively easily as compared to the compact ones. As far as the growth media were concerned, MS2 medium proved to be quite effective for the initiation of cell suspension cultures. Vargas *et al.* (2005) also observed good results on this medium in potato. This study shows that other media may also be used to establish cell suspensions in potato. For example Aftab *et al.* (1996) selected AA medium for the establishment of cell suspension cultures in sugarcane. Interestingly, the same medium when tested for the initiation of cell suspension cultures in potato gave good results though to a lesser extent in comparison with MS2 medium and thus not continued any further. Likewise, source material to medium ratio was considered to be an important factor for the establishment of cell suspension cultures (Aftab & Iqbal, 1999). The only tested ratio in this study (0.5 g fresh weight of callus in 10 ml liquid medium) gave quite satisfactory results and hence continued to be used throughout the study period. Liang *et al.* (2006) had proposed 0.75 g callus mass in 20 ml medium as an effective ratio of cells to medium for the establishment of cell suspension cultures in *Orthosiphon stamineus* Benth. So slight variation on the basis of plant in question can easily be found in the literature. Regarding agitation rate, 100-120 rpm was considered optimum for the establishment of efficient cell suspension cultures in various plant groups (Evans *et al*., 2003). It was therefore observed during this study that agitating the suspensions at 120 rpm on a gyratory shaker yielded good results for the establishment of cell suspensions in potato.

In conclusion, the reported method though simple in approach reveals several important factors for the establishment of cell suspensions in potato cv. Desiree. Adhering to the standardized conditions as mentioned for this particular cultivar of potato may yield good results in other lab conditions as well. We are quite optimistic that the reported protocol should prove useful to the research community for various downstream studies, especially for the selection of resistant plant species against various stresses.

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References

Aftab, F. and J. Iqbal. 1999. Plant regeneration from protoplasts derived from cell suspension of adventive somatic embryos in sugarcane (*Saccharum sp. hybrid* cv. CoL-54 and cv. CP-43/33). *Plant Cell Tiss. Org. Cult.*, 56: 155-162.

Aftab, F., Y. Zafar, K.A. Malik and J. Iqbal. 1996. Plant regeneration from embryogenic cell suspensions and protoplasts in sugarcane (*Saccharum* spp. hybrid cv. CoL-54). *Plant Cell Tiss. Org. Cult.*, 44: 71-78.

Anonymous. 2014. Food and agriculture organization of the United Nations. http://www.potato2014.org/en/index.html.

Bhojwani, S.S. and M.K. Razdan. 2004. Cell culture. In: Plant tissue culture theory and practices. Elsevier Publishers Netherlands, pp. 63-64.

Chen, S.L., S.X. Dai, J.K. Li, S.S. Wang, P. Andrea and H. Aloys. 2002. Isolation of protoplast and ion channel recording in plasma membrance of suspension cell of *Populus euphratica*. *Forest. Study in China,* 4(1): 1-4.

Dodds, J. H. and L.W. Robers. 1995. Cell suspension. In: *Experiments in plant tissue culture Cambridge University* Press, UK, Pp. 92-97.

Edwards, G.E. and C.C. Black. 1971. Photosynthesis in mesophyll cells and bundle sheath cells isolated from *Digitaria sanguinalis* (L). Scop. Leaves. In: *Photosynthesis and photorespiration.* (Eds.): M.D. Hatch, C.B. Osmond and R.O. Slatyer. New York: John Wiley Inc., 153-158.

Evans, D.E., J.O.D. Coleman and A. Kearns. 2003. Role of callus in embryogenesis, organogenesis, and cell culture. In: *Plant Cell Culture. Oxford Brookes University*, Oxford, UK Bios Scientific Publishers, pp. 64-65.

Farhatullah, Rashid, M. and Raziuddin. 2002. *In vitro* effect of salt on the vigor of potato (*Solanum tuberosum* L) plantlets. *Biotechnology*, 1: 73-77.

Gnanam, A. and G. Kulandaivelu. 1969. Photosynthetic studies with leaf cell suspensions from higher plants. *Plant Physiol*., 44(10): 1451-56.

Gopal, J., K. Iwama and Y. Jitsuyama. 2008. Effect of water stress mediated through agar on *In vitro* growth of potato. *In Vitro Cell Develop. Biol.,* 44: 221-228.

Irfan, A. 1992. Proceedings of the workshop on Seed Potato Health. Seed Potato Health Laboratory Project, Sahiwal. Federal Seed Certification Department. Ministry of Food and Agriculture Cooperation, pp. 1-2.

Jan, T. and K. Khatun. 2014. *In vitro* regeneration of *salvia santolonifolia*. *Pak. J. Bot.*, 46(1): 325-328.

Khatun, N., M.A. Bari, R. Islam, S. Huda, N.A. Siddique, M.H. Rahman and M.U. Mollah. 2003. Callus induction and regeneration from nodal segment of potato cultivar Diamant. *J. Biol. Sci.*, 3: 1101-1106.

Liang, L.F., C.L. Ken and B.P. Lim. 2006. Selection of cell-lines for the production of rosmarinic acid from cell suspension cultures of *Orthosiphon stamineus* Benth. *In Vitro Cell Develop. Biol.,* 42: 538-542.

Malik, N.J. 1995. Potato in Pakistan. World mate, Islamabad, Pakistan.

Mazareia, M., H. Al-Ahmada, M.R. Rudisa, B.L. Joycea and C.I. Stewart. 2011. Switchgrass (*Panicum virgatum* L.) cell suspension cultures: Establishment, characterization and application. *Plant Sci.,* 181: 712-715.

Michalczuk, L., T.J. Cooke and J.D. Cohen. 1992. Auxin levels at different stages of carrot somatic embryogenesis. *Phytochemistry*. 31: 1097-1103.

Muller, A.J. and R. Grafe. 1978. Isolation and characterization of cell-lines of *Nicotiana tabacum* lacking nitrate reductase. *Mole. Gen. Genetics,* 161: 67-76.

Murashige, T. and F.A. Skoog. 1962. Revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, 154: 73-497.

Nasrin, S., M.M. Hossain, M.F. Khatun, Alam and M.R.K. Mondal. 2003. Induction and evaluation of somaclonal variation in potato (*Solanum tuberosum* L.). *Online J. Biol. Sci*., 3: 183-190.

Naz, S. and K. Khatoon. 2014. The effect of auxin on callus induction in *Achyranthus aspera. Pak. J. Bot.,* 46: 2203-2207.

Queiros, F., F. Fidalgo, I. Santos and R. Salema. 2007. *In vitro* selection of salt-tolerant cell lines in *Solanum tuberosum* L. *Biol. Plant*., 51: 728-734.

Queiros, F., J.A. Rodrigues, J.M. Almeida, D.P.F. Almeida and F. Fidalgo. 2011. Differential responses of the antioxidant defense system and ultrastructure in a salt-adapted potato cell line. *Plant Physiol. Biochem*., 49: 1410-1419.

Rahnama, H. and H. Ebrahimzadeh. 2004. The effect of NaCl on proline accumulation in potato seedlings and calli. *Acta Physiol. Plant.,* 26: 263-270.

Rahnama, H., E. Ebrahimzadeh and B. Ghareyazie. 2003. Antioxidant enzymes responses to NaCl stress in calli of four potato cultivars. *Pak. J. Bot.,* 35: 579-586.

Reinert, J. and M.M. Yeoman. 1982. Plant cell and tissue culture: A laboratory Manual. Springer-Verlag Berlin, pp 14-15.

Sajid, Z.A. and F. Aftab. 2014. Plant regeneration from *In vitro* selected salt tolerant callus cultures of *Solanum tuberosum* L. *Pak. J. Bot*., 46(4): 1507-1514.

Spela, B., H. Matjaz and C. Marjana. 2005. Establishment of cell suspension cultures of Yew (*Taxus*×*Media Rehd*.) and assessment of their genomic stability. *In Vitro Cell Develop. Biol.*, 41(3): 338-344.

Svetek, J., K. Borut, V. Barbara and S. Milan. 1999. Lateral domain diversity in membranes of callus and root cells of potato as revealed by EPR spectroscopy. *Physiol. Plant.,* 105: 499-505.

Turhan, H. 2004. Callus induction and growth in transgenic potato genotypes. *Afr. J. Biotech*., 3: 375-378.

Vargas, T.E., E. De-Garcia and M. Oropeza. 2005. Somatic embryogenesis in *Solanum tuberosum* from cell suspension cultures: histological analysis and extracellular protein patterns. *J. Plant Physiol.,* 162: 449-456.

Zheng, Q.F., G.B. Hu, D.C. Chen and Z.R. Huang. 2005. A study of cell suspension culture and genetic transformation of litchi and longan. *J. Fruit Sci*., 22 (2): 125-128.

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