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PROTOPLAST ISOLATION AND REGENERATION FROM LEAVES AND NUCELLAR EMBRYOS OF KINNOW MANDARIN (*CITRUS RETICULATA* BLANCO)

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Abstract

Citrus is one of the most dominant horticultural fruit crops in the world. For the genetic improvement of Kinnow mandarin (*Citrus reticulate* blanco), protoplast isolation and somatic hybridization strategy is suitable. In this study, Kinnow mandarin protoplasts were isolated to get single species regeneration. Protoplasts were obtained from explants like *In vitro* and field grown leaves and nucellar embryos. Enzymatic protoplast isolation was performed and then yield, and viability testing was done. After initial testing of protoplast their regeneration was achieved. Nucellar embryos gave best regeneration but protoplasts from leaves were unable to regenerate. This study showed that 16 hours incubation was found best for optimum viable protoplasts yield of 5.4 x 10^4 protoplasts/gm FW (fresh weight) from nucellar embryos and 17.3 x 10^4 protoplasts/gm FW (fresh weight) of non-viable protoplasts yield from leaves.

Key words: Protoplast isolation, Root stock improvement, Enzymatic hydrolysis, Nucellar embryos, Protoplast regeneration, Yield testing, Viability testing.

Introduction

In plants genetic improvement programs, somatic hybridization by means of protoplast fusion is the established method to fuse physical cells (entire or fractional) of various plant species, genera or cultivars. To get desired characters together in an offspring or at times novel hereditary blends, allotetraploid somatic hybrids or substantial half breeds, physical cross breeds or somatic hybrids or symmetric hybrids and cybrids have been employed. This technique is very convenient in breeding plants and to get desired gene transfer from one plant to another by overcoming many problems related to conventional breeding, such as sexual mismatch between different genera, female and male sterility and polyembryonic property. Protoplast isolation from *Citrus* × *Poncirus* hybrids and *Severinia* has been reported (Calovic *et al*., 2019; Grosser & Chandler, 1987). Similarly, protoplast isolation from various citrus cultivars has been reported in other studies (Gloria *et al.*, 2000; Guo *et al*., 2013, Vardi & Galun, 1989). Protoplast technology status has been reviewed (Davey *et al*., 2005; Xing & Tian, 2018).

In citrus, protoplast somatic hybridization has been reported by several researchers (Grosser & Gmitter, 1990; Grosser *et al*., 2000) and in potato (Przetakiewicz *et al*., 2007). Key assessments present focus on somatic hybridization and organelle inheritance (Kumar & Cocking, 1987; Grosser *et al*., 2004). Recovery of substantial half and half utilizing protoplast combination is regularly quickened by cross breed deftness (Guo & Grosser, 2005), while cybrids may produce via donor-recipient method (Vardi & Galun., 1989; Melchers *et al.*, 1992). Cytoplast-protoplast fusion is also possible spontaneously through intergeneric or interspecific and intraspecific symmetric hybridization (Song & Dang, 2006; Grosser *et al.*, 1996). Physical crossover of natural seedless cultivar Satsuma mandarin (*Citrus unshiu*) cytoplasm to seedy cultivars for development of seedlessness has been done (Guo *et al*., 2004).

Somatic hybridization for citrus is delivering major allotetraploid reproducing parents for the interploid crosses in the development of seedless triploid plants (Grosser & Gmitter, 2005). Protoplast isolation and somatic hybridization techniques were found to be very useful for the root stock improvement at tetraploid level and helped in the development of resistant root stocks and achieved marked potential for controlling various tree sizes as well as zygotic extraordinary progenitor species (Grosser *et al.*, 2003). Varieties of substantial cross breeds have been created that focused on the disease resistant qualities. In somatic hybridization joining of chloroplast or mitochondrial genome of one parent with nuclear genome of second parent is also possible (Guo *et al.*, 2004; Smith & Dudits, 1989; Dudits, 1980). Factors affecting yield and viability of protoplasts include source of material, pre-enzyme treatments, enzyme treatment, osmoticum etc. By using protoplast isolation, it is possible to develop somatic hybrids through their fusion to create novel germplasm that chains many good qualities of fused species. This study is in continuation of the studies on citrus multiplication (Kazmi *et al.*, 2018) and improvement (Grosser & Gmitter, 2005).

Material and Methods

Protoplast isolation and somatic hybridization: Protoplast isolation from nucellar embryos was performed due to high regeneration ability of nucellar embryos.

Nucellar embryo production: Nucellar embryos were induced by cutting Kinnow mandarin seeds longitudinally and placed on MT basal medium (Murashige & Tucker, 1969) with 0.5mg/l BAP and 0.5mg/l Kinetin (Kazmi *et al.*, 2018). Nucellar embryos were multiplied and regenerated on the same medium for three months for protoplast isolation and fusion. The established cultures were used for protoplast isolation.

Explant preparation and enzyme incubation: Protoplast isolation from nucellar embryos was chosen for their high regeneration ability (Kazmi *et al*., 2015). Protoplast isolation from leaves was also performed for comparison. Rapidly growing 1 gm nucellar embryos and leaves were weighed for protoplast isolation. Both explants were cut into fine segments and placed in sterile 50 ml Erlenmeyer flask with few drops of cell and protoplast washing medium CPW solution (Table 1) with 13% mannitol called (CPW 13) was poured immediately to prevent cells from drying. Afterwards, filter sterilized enzyme solution containing 1% cellulase and 1% macerozyme solution (Table 2) was poured in equal volume to CPW 13 solution, incubated for different time intervals of 14hrs, 15hrs, 16hrs and 17hrs.

Table 1. Composition of the CPW solution used for protoplast purification in (mg/l).

	KH_2PO_4	KNO_3	$CaCl_2 \cdot 2H_2O$	$MgSO_4 \cdot 7H_2O$	KI	$CuSO_4 \cdot 5H_2O$
Conc.	27.2	101	1480	246	0.16	0.025

PH of the washing solution is 5.6 (Carimi, 2005)

Table 2. Enzyme solution for citrus protoplast isolation.

Component	Concentration (mg l-1)
Mannitol	127,000
$CaCl_2$	1.33
MES*	1.17
NaH_2PO_4	0.17
Cellulase Onozuka RS	10,000
Macerozyme	10,000
Pectolyase Y-23	2,000

MES = 2-(*N*-Morpholino) ethanesulfonic acid. The pH of the solution is adjusted to 5.6±0.1. The solution is sterilized by passage through a 0.22µm filter unit and stored at 4°C (Carimi, 2005)

Protoplast isolation: The enzyme and CPW 13 solution mixture along with chopped explants was placed on shaker at 80 rpm for 20 minutes for homogenization. After shaking, protoplasts were isolated by passing the mixture through nylon sieve of 45µm and filtrate was collected. The filtrate was centrifuged at 980 rpm for seven minutes and supernatant was discarded after first centrifugation cycle. CPW 13 solution was poured into the glass tube containing pallet, mixed gently and again centrifuged at 980 rpm for 7 minutes. Three subsequent washes with the CPW 13 solution were performed; the pallet was then suspended in CPW 13 solution and poured on the tube containing CPW 26 solution containing (1× CPW solution with 26% sucrose concentration). The tube was centrifuged at 550 rpm for 4 minutes. Protoplast ring was formed at the interface of two solutions; this ring was collected gently with the help of Pasteur pipette and transferred into separate glass tube. After ring collection, protoplasts were cultured in a BH3 medium (Kao & Michayluk, 1975) for regeneration. The same procedure was repeated for leaves protoplast isolation.

Yield testing: Cell counting with the help of haemocytometer was performed under the compound microscope and protoplast yield was calculated with the help of formula given below (Siang *et al*., 2010).

$$\text{Protoplast yield} = \frac{\text{Total cell count} \times \text{Total volume of cell suspension}}{4 \times \text{Weight of fresh tissues (g)}}$$

Viability testing: The viability of protoplasts was monitored using Fluorescein diacetate (FDA) with a final concentration of 5mg/ml (Nadel, 1989). Images were taken through fluorescent microscope (Nikon TE 2000E).

Regeneration of nucellar embryos and leaves protoplasts: Protoplast isolated from nucellar embryos were cultured onto BH3 medium. After two weeks, culture plates were observed under fluorescent microscope and growth pattern was recorded. Protoplasts were sub-cultured after one week. After one-month, callus formation was visible and after two months, subsequent multiplication was achieved. For shooting purpose, callus was transferred to shooting media with MT basal medium (Murashige & Tucker, 1969) containing 0.5mg/l BAP and 0.5mg/l Kinetin along with 30gm/l sucrose supplemented with 0.5 gm/l malt extract.

Regeneration of leaves protoplast: In leaves regeneration experiment only microcolonies were observed but they failed to form callus and were unable to regenerate.

Results

Nucellar embryos protoplast yield: The protoplast isolated from nucellar embryos gave highest yield at 16 hrs incubation time, the suspension had highest yield of viable protoplast which was 5.4×10^4 protoplast/gm FW. Analysis of variance resulted with high significance value at (p<0.000). In 18 hrs of incubation, protoplast yield was 2.1×10^4 protoplasts/g FW, at this treatment large size protoplasts were seen along with debris (Fig. 3). This result was obtained because of more cell digestion, more debris were obtained and protoplast isolated were less viable with low yield.

Viability testing of nucellar embryos: After protoplast yield testing, their viability was also analyzed. It was found that many protoplast cells were viable in fluorescein diacetate stain under blue light (Fig. 1).

Leaves protoplast yield: The protoplast isolated from leaves gave highest yield at 16 hrs incubation time, the suspension contains high yield of protoplast which is 17.3×10^4

protoplast/gm FW with more viability. Analysis of variance resulted with high significance value at (P=5.97E-06). In the treatment of 18 hrs of incubation protoplast yield was 12×10^4 protoplasts/g FW, at this treatment large size protoplasts were seen along with many debris. This result was obtained due to more enzymatic treatment time because of more cell digestion more debris were obtained and protoplast isolated were less viable with low yield. In 14 hrs incubation treatment, low yield cells were obtained 8.5×10^4 protoplasts/g with less viability (Fig. 4).

Viability testing of leaves: The viability of leaves was analyzed under fluorescent microscope. It was observed that few protoplast cells were viable in Fluorescein diacetate stain as compare to nucellar embryos (Fig. 2).

Discussion

Protoplast isolation and regeneration from nucellar embryos of Kinnow mandarin: Protoplasts were successfully isolated after enzyme treatment from chopped nucellar embryos and cultured on BH3 medium where they divide and form micro colonies. For callus formation they were cultured on combination of protoplast growth medium containing MT basal medium supplemented with 205.4gm/l sucrose and BH3 medium formulated (Kao & Michayluk, 1975; Grosser & Gmitter, 1990) in 1:1 ratio at which these isolated protoplasts form callus. The callus was further transferred on shooting medium and showed high embryogenic ability, as it was regenerated to form many plantlets. This is a novel finding and can be very beneficial for citrus genetic improvement programs because protoplasts isolated from nucellar embryos were found to be highly regenerative. The study findings are in agreement with the previous reports (Gloria *et al.*, 1999; Niedz, 2006; Xu *et al*., 2017). The protoplast isolation from chopped nucellar embryonic tissues is the new aspect which can be beneficial for protoplast isolation and fusion technology in plants genetic improvement studies.

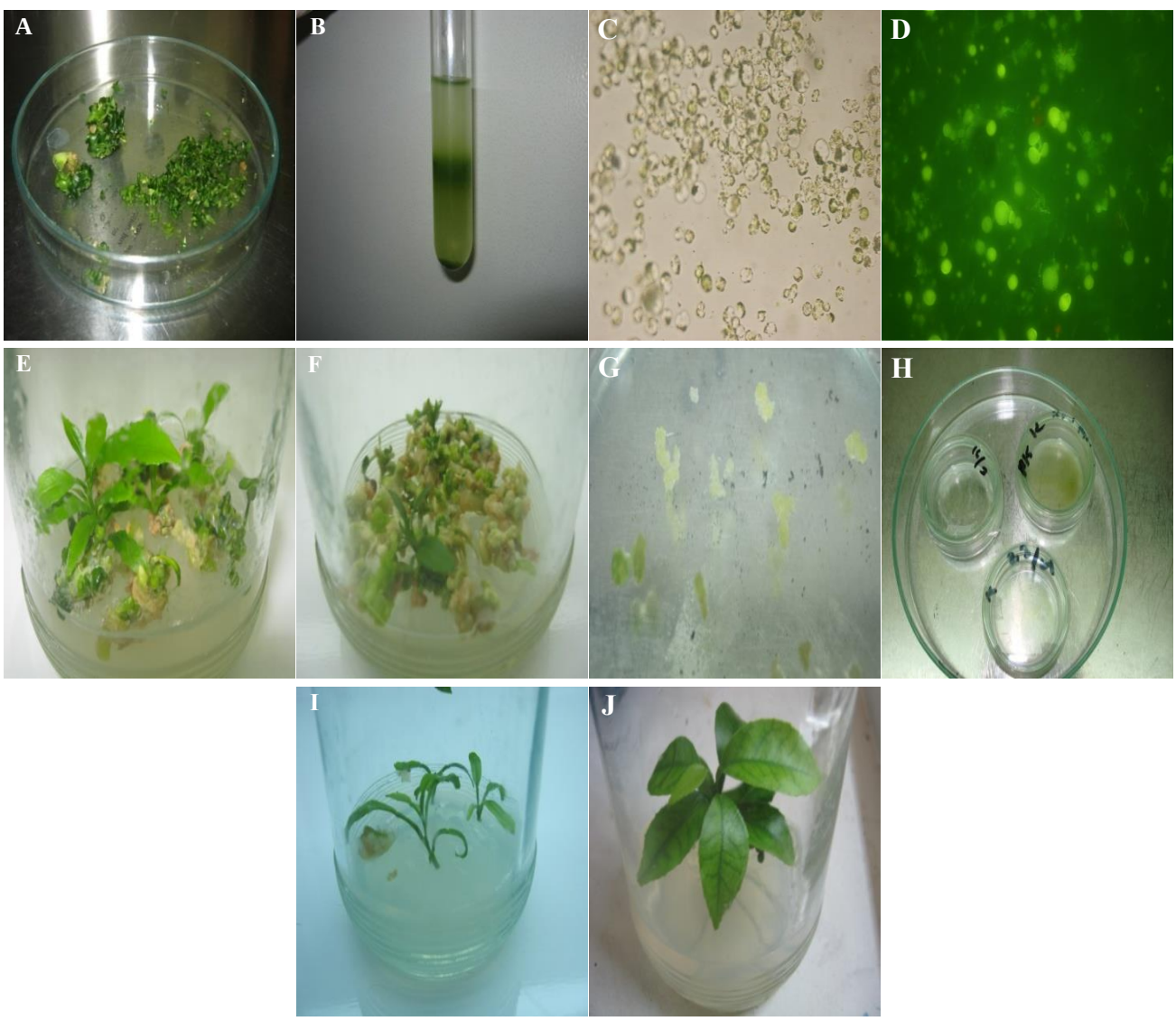

Fig. 1. Protoplast isolation from nucellar embryos.
A- Chopped nucellar embryos; B- Protoplast ring in the middle; C- Bright field image of nucellar protoplast; D- FDA stained image of protoplast under blue fluorescence; E- Nucellar protoplast cultured in BH3 medium; F- Formation of callus from nucellar protoplast; G- Small shoots formation from callus; H- Shoot growth; I- Separated shoots for further growth; J- Rooting of shoots.

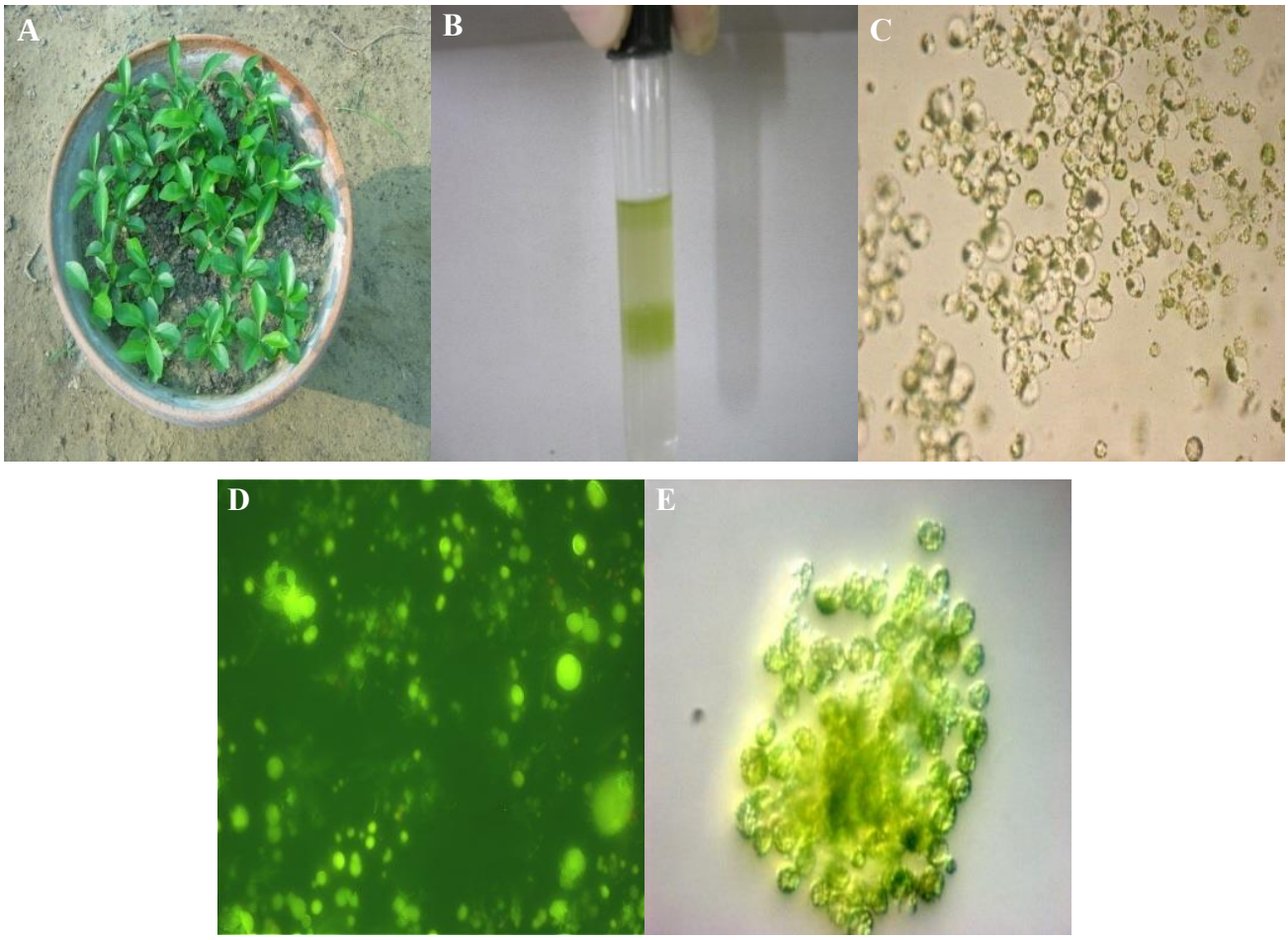

Fig. 2. A- Green house grown plants of Kinnow mandarin; B- Leaves protoplast ring formed; C- Bright field image of leaves derived protoplast; D- Leaves protoplast FDA stained image under blue fluorescence; E- Leaf protoplasts cultured in BH3 medium.

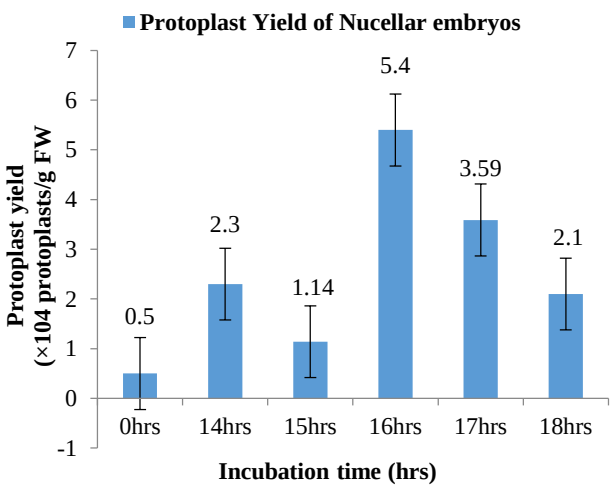

Fig. 3. Protoplast yield of nucellar embryos.

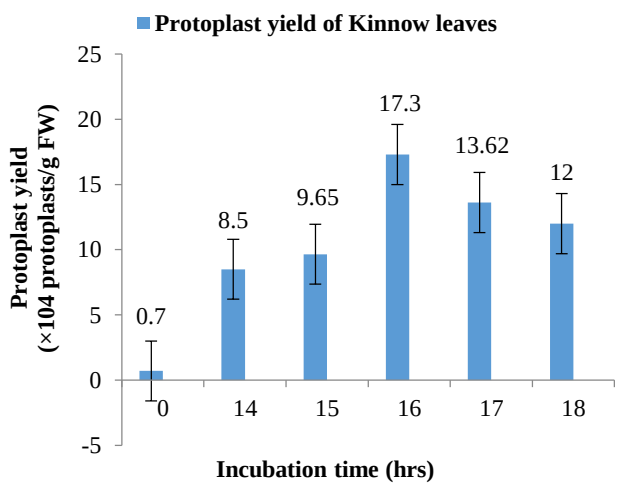

Fig. 4. Protoplast yield of kinnow mandarin leaves.

Protoplast isolation and micro colonies formation from leaves of Kinnow mandarin: The protoplasts of Kinnow mandarin leaves were isolated through enzyme treatment and in the experiment of time duration, required for best protoplast isolation. 16 hours were found suitable for maximum number of viable protoplast isolation. Isolated protoplasts were cultured in a 1:1 combination of BH3 and MT medium with 205.6 gm/l sucrose. After subculture micro colonies were found to appear in the microscopic images of the culture but they failed to regenerate and form callus.

The enzyme mediated protoplast isolation from leaves of Citrus species is well established and findings of this study agree with previous reports (Guo *et al.*, 2000; Dong *et al.*, 2007). Isolated protoplast from leaves of 'Shatian' pummelo (*C. grandis* L. Osbeck) and 'Bingtang' orange (*C. sinensis* L. Osbeck) were utilized for fusion with Satsuma mandarin '*Citrus*

unshiu'. Embryogenic cell lines of sweet orange (*Citrus sinensis*) cultivar 'Early Gold' were used in a callus cell suspension culture and fused with *In vitro* grown leaves derived protoplast of *Citrus micrantha* (Khan & Grosser, 2004). In most of the studies leaf parent was used to transfer specific character of that plant to embryogenic parent, which can regenerate to form embryogenic callus parent carrying specific character of mesophyll parent.

Conclusion

Novel approach of this study is the protoplast isolation from nucellar embryos and their high regeneration ability. The findings of this study agree with available literature on protoplast isolation studies on Citrus plants. Incubation time (12-17 hours) reported through literature (Kumar & Cocking, 1987) was found suitable for protoplast isolation from Citrus cultivars. In somatic hybridization studies, protoplasts of plants which contain desired gene can be used to transfer that character into highly regenerative nucellar embryo protoplasts to get desired characters in root stocks.

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References

Calovic, M., C. Chen, Q. Yu, V. Orbović, F.G. Gmitter and J.W. Grosser. 2019. New somatic hybrid mandarin tetraploids generated by optimized protoplast fusion and confirmed by molecular marker analysis and flow cytometry. *J. Amer. Soc. Hort. Sci*., 144(3): 151-163.

Carimi, F. 2005. Somatic Embryogenesis Protocol: Citrus. In *Protocol for Somatic Embryogenesis in Woody Plants*, by P.K.Gupta and S.M. Jain, pp 321-343. Palermo, Italy: *Springer* Netherlands.

Davey, M.R., P.J. Anthony, B. Power and K.C. Lowe. 2005. Plant protoplast technology: Current status. *Acta. Phy. Plan.*27(1):117-129.

Dong, C.X., J. Fu, X.X. Deng and W.W. Guo. 2007. Production and molecular characterization of potential seedless cybrid plants between pollen sterile Satsuma mandarin and two seedy Citrus cultivars." *Plant Cell Tiss Organ Cult*., 90(3): 275-283.

Dudits, D., O. Fejer, G. Hadlaczky, C. Koncz, G.B. Lazar and G. Horvath. 1980. Intergeneric gene transfer mediated by plant protoplast fusion. *Mol. Gen. Genet*., 179: 283-288.

Gloria, F.J.M., M. Filho, F.D.A. Alves and B.M.J. Mendes. 2000. Plant regeneration from protoplast of Brazilian Citrus Cultivars. *Pes. Agr.pec. Bra*., 4: 727-732.

Gloria, M.D., M. Filho, C.G.B. Demétrio and B.M.J. Mendes. 1999. Embryogenic calli induction from nucellar tissue of citrus cultivars. *Scient. Agric*., 56(4): 1111-1115.

Grosser, J.W. and F.G. Gmitter 1990. Protoplast fusion and Citrus improvement. *Plt. Bred. Rev*., 8: 339-374.

Grosser, J.W., P. Ollitrault and O.O. Fuster. 2000. Somatic hybridization in citrus: an effective tool to facilitate variety improvement. *In Vitro Cell. Dev. Biol.-Plant*. 36: 434-449.

Grosser, J.W., F.G. Gmitter, N. Tusa, G.R. Recupero and P. Cucinotta. 1996. Further evidence of a cybridization requirement for plant regeneration from citrus leaf protoplasts following somatic fusion. *Plant Cel. Rep*., 15(9): 672-676.

Grosser, J.W., J.H. Graham, C.W. McCoy, A. Hoyte, H.M. Rubio, D.B. Bright and J.L. Chandler. 2003. Development of "tetrazyg" rootstocks tolerant of the diaprepes/ phytophthora complex under greenhouse conditions. *Proc. Fla. State Hortic. Soc*., 116: 262-267.

Grosser, J.W., X.D. Guo and J.W. Cai. 2004. Somatic Cell cybrids and hybrids in plant improvement. In *Molecular Biology and Biotechnology of Plant Organelles: Chloroplasts and Mitochondria*, by Henry Daniell Christine Chase. Dordrecht: *Springer*. pp 635-659.

Grosser, J.W. and F.G. Gmitter. 2005. Applications of Somatic Hybridization and Cybridization in Crop Improvement, with Citrus as a Model. *Thinking Outside the Cell.* Verlag: *In Vitro Cell. Dev. Biol. Plant*., 41(3): 220-225.

Grosser, J.W. and J.L. Chandler. 1987. Aseptic isolation of leaf protoplasts from Citrus, Poncirus, Citrus × Poncirus hybrids and Severinia for use in somatic hybridization experiments. *Sci. Hort*., 31(3-4): 253-257.

Guo, W.W., S.X. Xiao and X.X. Deng. 2013. Somatic cybrid production via protoplast fusion for citrus improvement. *Sci. Hort.*, 163: 20-26.

Guo, W.W., Z. Chang-He., Y.H. Lin and X.X. Deng. 2000. Intergeneric somatic hybrid plants between Citrus and *Poncirus trifoliata* and evaluation of their root rot resistance. *Act. Bot. Sin.*, 42(7): 668-672.

Guo, W.W. and J.W. Grosser. 2005. "Somatic hybrid vigor in Citrus: Direct evidence from protoplast fusion of an embryogenic callus line with a transgenic mesophyll parent expressing the GFP gene." *Pl. Sci*., 168: 1541-1545.

Guo, W.W., D. Prasad, Y.J. Cheng, P. Serrano, X.X. Deng and J.W. Grosser. 2004. "Targeted cybridization in citrus: transfer of Satsuma cytoplasm to seedy cultivars for potential seedlessness." *Pl. Cel. Rep.*, 22: 752-758.

Kao, K.N. and M.R. Michayluk. 1975. Nutritional requirements for growth of *Vicia hajastana* cells and protoplasts at a very low population density in liquid media. *Pl.*, 126(2):105-110.

Khan, I.A. and J.W. Grosser. 2004. Regeneration and characterization of somatic hybrid plants of *Citrus sinensis* (sweet orange) and *Citrus micrantha*, a progenitor species of lime. *Eup*., 137(2): 271-278.

Kumar, A. and E.C. Cocking. 1987. Protoplast Fusion: A Novel Approach to Organelle Genetics in Higher Plants. *Am. J. Bot.*, 74: 1289-1303.

Melchers, G., Y. Mohri, K. Watanabe, S. Wakabayashi and K. Harada. 1992. One-step generation of cytoplasmic male sterility by fusion of mitochondrial-inactivated tomato protoplasts with nuclear-inactivated *Solanum* protoplasts. *Proc. Natl. Acad. Sci.*, *USA* 89: 6832-6836.

Murashige, T. and D.P.H. Tucker. 1969. Growth factor requirements of citrus tissue culture. *Proc. Fir. Int. Cit. Sym*., 3: 1155-1161.

Nadel, B.L. 1989. Use of Fluorescein Diacetate in citrus tissue culture for determination of cell viability and the selection of mutants. *Scient. Hort*., 39(1): 15-21.

Niedz, R.P. 2006. Regeneration of somatic embryos from sweet orange (*C. sinensis*) protoplasts using semi-permeable membranes. *Plant Cell Tiss .Org. Cul*., 84(3): 353-357.

Przetakiewicz, J., A.N. Orczyk, K. Dominik and W. Orczyk. 2007. "Tetraploid somatic hybrids of potato (*Solanum tuberosum* L.) obtained from diploid breeding lines. *Cell Mol. Biol. Lett*., 12(2): 253-267.

Smith, M.A. and P.D. Dudits. 1989. Analysis of chloroplast and mitochondrial DNAs in asymmetric somatic hybrids between tobacco and carrot. *Theo & Appl Gen*., 77(5): 641-644.

Kazmi, S.K., S. Khan, A.A. Mirbahar and N. Kabir. 2018. Micropropagation of Nucellar embryos and their histological comparative study for regeneration ability with other Explants of Kinnow Mandarin (*Citrus reticulata* Blanco). *Pak. J. Bot*., 50(1): 345-353.

Kazmi, S.K., S. Khan, A.A. Mirbahar and H.A. Kayani. 2015. Comparative protein production profiling of (*Citrus reticulata* Blanco) among tissue culture stages by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). *Th. Nat. Pro. Jr*., 5: 186-190.

Song, J.K. and X.X. Deng. 2006. Induction and genetic identification of embryogenic calli from hybrids of shatian pummelo. *Agr. Sci.Ch*., 5(8): 591-595.

Siang, T.C., P.S. Lee., A.L.P. Kiong and M. Mahmood. 2010. Optimisation of protoplast isolation protocols using *In vitro* leaves of *Dendrobium crumenatum* (pigeon orchid). *Afr. Jr. Agr. Res*., 5(19): 2685-2693.

Vardi, A. and E. Galun. 1989. Isolation and culture of *Citrus* protoplasts. In: (Ed.); Bajaj, Y.P.S. Plant Protoplasts and Genetic Engineering I. *Bio. Agr. Fort*., vol 8. *Springer*, Berlin, Heidelberg.

Xing, T. and L. Tian. 2018. Protoplast as a tool to address questions in plant physiology. *Jr. Pl. Dev.*, 25: 99.

Xu, X., X. Xu, Y. Zhou, S. Zeng and W. Kong. 2017. Identification of protoplast-isolation responsive microRNAs in *Citrus reticulata* Blanco by high-throughput sequencing. *Pl. One*. 12(8):1-16.

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