Pak. J. Bot., 50(1): 345-353, 2018.

MICROPROPAGATION OF NUCELLAR EMBRYOS AND THEIR HISTOLOGICAL COMPARATIVE STUDY FOR REGENERATION ABILITY WITH OTHER EXPLANTS OF KINNOW MANDARIN (*CITRUS RETICULATA BLANCO*).

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Abstract

Polyembryony is the most beneficial and distinct character in citrus seeds. This characteristic can be beneficial in citrus improvement programs. Nucellar embryos developed from nucellar wall in citrus seeds along with zygotic embryo are found to have high plants regeneration ability in comparison to zygotic embryos. Under *In vitro* culture conditions, nucellar embryos were detected and multiplied on MT media with 0.5ml of coconut water along with 50g/L sucrose. These nucellar embryonic tissues were also regenerated on MT media supplemented with 0.5 mg/L BAP and 0.5 mg/L kinetin. Many shoots were regenerated from nucellar embryonic tissue; these shoots were rooted on MT media supplemented with IBA 0.5mg/L and NAA 0.1 mg/L in addition to activated charcoal 0.5gm/L. The resulting plantlets were acclimatized in the green house. After micropropagation, histological studies of nucellar embryonic tissues were carried out under fluorescence microscope to examine their high regeneration ability in comparison with usual plant parts, like seeds, shoots as well as with tissue culture stages including embryogenic and non-embryogenic callus. It was found that nucellar embryos have more regeneration ability as compared to usual plant parts and other tissue culture stages.

Key words: Nucellar embryos, Apomictic, Fluorescence microscopy, Somatic embryogenesis, Toluidine blue O staining.

Abbreviations: *TAX RED-(Texas Red),* DIC-**(**differential interference contrast), BAP-(6-Benzylaminopurine), NAA-(Naphthalene acetic acid), IBA-(Indole-3-butyric acid), MT- (Murashige and Tucker), TBO- Toluidine Blue O.

Introduction

Citrus stands first in the area and production among fruit trees throughout the world. Citrus plants are cultivated in various tropical and subtropical climatic conditions. This fruit crop is widely used in food, medicine and cosmetic industries. It is well known fact that citrus and citrus products are rich source of vitamins, minerals and dietary fiber (non-starch polysaccharides) that are essential for normal growth, development and overall nutritional well-being (Economos & Clay, 1999). For hundreds of years, herbalists trained in Traditional Chinese Medicine (TCM) and have been using mature mandarin orange peel, known as *chen pi* or *ju pi* in Chinese medicine, to improve liver and stomach digestion, relieves intestinal gas and bloating, as well as to resolve phlegm. Mandarin essential oil has very sharp floral fragrance, and is generally preferred for therapeutic purposes. It is one of the safest oils often recommended for children and used during pregnancy (Matesz, 2010).

In Pakistan citrus is the most important fruit crop which is cultivated on an areaofabout 206,569 hectares, more than 98% citrus is produced from Punjab province (183,210 hectares) because of favorable conditions and adequate water supply (Memon, 2017). Introduction of Kinnow mandarin has given great rise to the citrus industry; many varieties of Sweet oranges has been cultivated like Blood red, Mosami, Grape fruit, Duncan, seedless Marsh, Foster and Shamber, other commercial cultivars are Eureka lemon, Kaghzi lime and Sweet lime (Chaudhary, 1994).

Most of the citrus varieties are polyembryonic, and contain seeds, which develops from nucellar portion of embryo sac just after pollination. These embryos are of maternal origin and are tetraploid. In apomictic citrus plants, tetraploidy occurs frequently in nucellar cells through chromosomal doubling, it is due to both genotypic and environmental factors. It occurs usually in marginal climatic areas. Tetraploid citrus genotypes have potential usage as rootstocks and are widely used as parents in breeding programs, like seedless triploid cultivars (Aleza *et al*., 2011). Sometimes *Citrus reticulata* seeds show extra ordinary polyembryonic ability by sprinkling nucellar tissues out of them, these tissues have ability to multiply in large number and regenerate into many plantlets (Kazmi *et al.*, 2015b).

After maturation, decline of regenerative potential was observed in woody plants (Durzan, 1990). In citrus species, the rapid *In vitro* multiplication rate was observed in the juvenile phase explants of stock material in the micropropagation of polyembryonic citrus plants. Juvenile tissue explants include nucellar embryos, somatic embryos or young seedlings (Sim *et al*.,1989). It was reported that in comparison of regeneration potential of explants collected from juvenile citrus tissues and explants collected from adult plants the best

regeneration frequency was found in juvenile tissues (Carimi & Pasquale, 2003). These characteristics recognize nucellar embryos as strong candidates for direct utilization in citrus improvement programs like protoplast isolation and fusion, genetic transformation, induced mutation, gene editing etc.

Regeneration ability of citrus is satisfactory from internodal sections; one node give rise to one plant only. Due to nucellarpoly embryonic property of citrus, it was found that regeneration ability was enhanced and large number of plants were germinated from these nucellar embryonic tissues which were identical to their mother plant. The comparative protein production profiling of tissue culture stages along with nucellar embryos was previously studied by Kazmi *et al*. (2015b). In this study comparative, histological analysis of these nucellar embryos helped to find out visually enhanced regeneration ability in comparison to the other tissue culture stages and usual plant parts. Their comparison with embryogenic and non-embryogenic callus, seeds and *In vitro* grown plants was observed under different fluorescent light filters. Staining was done with polychromatic dye toluidine blue O to observe presence of chloroplast content for regeneration ability.

Materials and Methods

Sterilization: For this experiment, commercially available good quality Kinnow mandarin (*Citrus reticulatablanco)* fruits were selected to collect their seeds. Seeds were first washed with commercially available washing liquid and sterilized with 70% commercial bleach for 20 min. Seeds were then rinsed three to four times with autoclaved distilled water. Seed covers were removed in Petri dishes with scalpel and placed in MT media jars with sugar concentration of 50 g/L (Murashige & Tucker, 1969). Seeds could germinate in dark condition at 25±2ºC, after one month full plantlets grew with elongated epicotyl portion and small leaves.

Embryonic multiplication: After one month plantlets became elongated from epicotyl portion due to dark condition, some plantlets gave out polyembryonic nucellar regenerative tissues or embryos. They were initially small and then became bigger gradually.

Highly regenerative nucellar embryos were transferred on MT media containing 0.5g/L malt extract. These embryos started regeneration in a short time. After this observation, these embryos were tested for multiplication on four media combinations having MT basal media with 50g/L sucrose carrying different growth supplements as follows: 0.5 g/L casein hydrolysate, 0.5ml of coconut water, 0.5g/L adenine sulphate, 0.5g/L malt extract. Simple MT media was used as a control, pH of the media was adjusted to 5.7 with 1N HCl and 1N KOH, *In vitro* cultures were maintained on 16/8hrs photoperiod at 25±2ºC.

Shooting: When nucellar embryonic bodies started multiplication and became bigger, than these were transferred to simple MT media for shoot formation. For elongation and development of shoots, these were placed in hormone containing MT media with 0.5mg/L kinetin and 0.5 mg/L BAP.

Rooting: After shoots development, plants were placed on ½ strength MT media supplemented with IBA 0.5mg/L and NAA 0.1 mg/L in addition to 0.5g/L activated charcoal and sucrose 30g/L as described by (Liu, 2005). For root formation, fully developed plants were acclimatized under green house.

Embryogenic and non-embryogenic callus induction: Non-embryogenic callus formation was induced from longitudinal cut epicotyl segments grown in the dark place, which were placed on MS media (Murashige & Skoog, 1962) with 2 mg/L 2,4-D. Embryogenic callus was also induced from epicotyl sections, which were germinated and elongated in dark, then transversely cut and placed on MS media along with 1.5 mg/L 2,4-D and 0.5 mg/L BAP with 0.5 mg/L Malt extract (Kazmi *et al*., 2015a).

Morphological and histological analysis: During histological study, different micropropagation stages and nucellar embryo images were captured with a digital camera. These tissue culture stages were analyzed under stereomicroscope and images were taken through Nikon DS-5M camera. Tissue sections were made by hand sectioning using razor blade, and were observed under fluorescent microscope Nikon TE 2000E for studying some distinct characteristics. The comparative study between nucellar embryos, embryogeniccalli, non-embryogeniccalli, seeds and whole plants was carried out and their results were correlated to the regenerative nature of each specimen. Toluidine blue O (TBO) a polychromatic dye was used for staining of all above mentioned specimens. Thus 0.1% solution of TBO was prepared in 100ml distilled water and sections of all specimens were stained for 10 minutes in 10 ml distilled water with three drops of toluidine blue O solution before the study.

Table 1. Multiplication of nucellar embryos on different media combinations.

S. No.	Treatments	Appearance	Growth Pattern	Texture
1.	Nem1	Dark green big embryos	Somatic embryos increased in size	Small thick leaves formed
2.	Nem2	Leaves formed	Clear regeneration of long shoots	Small thin leaves formed
3.	Nem3	Healthy green embryos	Embryos multiplication observed	Small sized globular shaped somatic embryos formed
4.	Nem4	Some regeneration but browning started	Only small regeneration and multiplication observed but browning was dominant	Cells browning was dominant
5.	Nem5	Mixed appearance	More regeneration and less multiplication	Embryos formed were so small and globular in shape

Multiplication and micro propagation of nucellar embryos

Fig. 1. Germinated plants in dark (A). Embryos sprinkled out of seeds (B). Greening of nucellar embryos started in light (C). Shoots raised in simple MT media (D). Multiplication of embryos (E).Regeneration of embryos (F). In start the short thick leaves raised (G). Shoots started elongation (H). Roots formed in plants simultaneously from nucellar embryos (I). Plantlets ready to acclimatize (J). Plants after acclimatization in green house (K).

Results and Discussion

Tissue culture: Nucellar embryos were vigorously germinated *In vitro*, and produced many plantlets. After germination they were separated and placed on shooting and rooting media for one month each. These *In vitro* grown plantlets were then transferred and acclimatized in the semi-controlled environment of green house, these plants were of maternal origin because nucellar wall was female reproductive part of a flower. When nucellar embryos appeared from germinated seeds of Kinnow mandarin in dark conditions, some of them were found to have regeneration and multiplication ability when transferred on simple MT media with 0.5g/L malt extract and 50g/L sucrose. After appearance of nucellar embryos, the multiplication experiment was conducted with the following results (Fig. 1; Table 1). Media combination NEM 1 was showing increased in growth of nucellar embryos, On NEM 2 media, regeneration was achieved and NEM3 media, a formulation of simple MT media with 50g/L sucrose along with 0.5ml/L coconut water was found suitable for multiplication of nucellar embryos. NEM 4 media was found giving dominant browning with less multiplication and regeneration. NEM 5 media gave rise to mix appearance with more regeneration and less multiplication. During the multiplication stage, nucellar embryos showed all stages of somatic embryonic development. Their regeneration was observed on MT media fortified with 0.5mg/L BAP and 0.5mg/L Kinetin along with 0.5g/L malt extract, this combination of two cytokinins was found very suitable in regeneration. Shoots developed rapidly from nucellar embryos and their rooting was achieved on ½ strength MT media supplemented with IBA 0.5mg/L and NAA 0.1 mg/L along with 0.5g/L activated charcoal and sucrose 30g/L was used as described by (Liu, 2005). Activated charcoal was used in tissue culture to improve cell growth and development. In addition to roots development, activated charcoal is involved in various stimulatory and inhibitory activities. The release of substances naturally present in activated charcoal which promotes growth, alteration and darkening of culture media, and adsorption of vitamins, metal ions and plant growth regulators, including abscisic acid and gaseous ethylene (Thomas, 2008).

Histological Analysis

Digital camera and stereomicroscopic images: These nucellarpoly embryonic highly regenerative tissues were analyzed under stereomicroscope and found to have all characteristic shapes which occur during somatic embryogenesis like globular, heart, torpedo and cotyledon as shown in Fig(2) B also described by (Ling *et al*., 2008). These were highly regenerative and possessed rapid multiplication property. Different tissue culture stages like embryogenic callus, non-embryogenic callus, *In vitro* grown plants as well as seeds were observed under stereomicroscope. Their images were very different from each other due to morphological and physiological difference. Under stereo microscope, *In vitro* grown nucellar embryos were found to have hairs on their surface as shown in Fig. (3) B. In *In vitro* grown embryogenic and non-embryogenic callus cultures, morphological differences were very clear. Embryogenic callus was bright cream in color and somatic embryos were visible on its surface. In case of non-embryogenic callus which was quite dark cream in color, was more soft and friable. *In vitro* grown plantlets with complete roots, shoots and small leaves were acclimatized for the development of cutaneous layer or hardening under green house.

Fluorescent microscopic study: Nucellar embryos were multiplied *In vitro* and transversely hand sectioned, thin slices were studied under florescent microscope. These sections were observed with different fluorescent colors as the property of plant cells that they gave fluorescent colors automatically as shown in Fig (4) D, this is called plant auto fluorescence. During histological study, nucellar embryos were observed to have all characteristic shapes of somatic embryos and dense chlorophyll content at 40× magnification, which indicated more ability to germinate. In blue light, they appeared as many beans packed in a case, representing lignin walls of blue color. Green color indicated presence of phenolics and cutaneous hairs were also present on the surface of embryos (Figs. 5-8). Chlorophyll appeared red and lignin blue. Cutin and suberin were silvery white, phenolic compounds other than lignin vary from green to blue as described by (Yeung, 1998). In bright field of microscope, the images appeared dark green colored with uniform appearance. DIC images showed light and dark colors of green. Bright red color proved the presence of heavy chlorophyll content. Lignin content present in nucellar embryo was represented by blue color. Less green color, indicated less phenolic compounds. Merge image was a combination of all fluorescencent images. Bright field image of nucellar embryos illustrates cutaneous hairs on its surface. Merge image of all fluorescent filters showed blue fluorescent color, indicative of lignin. 40x image of nucellar embryos confirmed dense chloroplast content. 20x images were indicated lignified walls and sieve tubes with purple color and parenchymatous cells with dense chloroplast. Bright field image of non-embryogenic callus presented light green color on peripheral parts showed all tissue organelles. DIC image looked light green in color as in the bright field. TAX red filter showed light red fluorescence which indicated less chlorophyll content. Blue filter DAPI presented less blue coloration in center and more blue color on peripheral region of non-embryogenic callus which explained presence of lignin. FITC filter showed red and a green fluorescence pattern which indicated chlorophyll content and some phenolic compounds. Merge picture was combination of all above mentioned filter images showing combined fluorescent color pattern.

In center of the stem, the pith parenchyma stained purple and green. TAX Red filter showed bright red color, which indicated presence of chlorophyll content in sclerenchyma cells. In the FITC Filter, green color was also present, which showed the presence of phenolic compounds. DIC image clearly demonstrated the cellular arrangements in the stem. DAPI filter showed fluorescence of epidermis and vascular bundles indicating lignin. Merge picture was a merger of all fluorescent images and indicating hairs on the surface and heavy chlorophyll content along with phenolic compounds in vascular bundles. Bright field image showed silvery white appearance of cutin and suberin. Bright field image illustrated irregular mass of embryogenic callus. DAPI

image confirmed presence of lignin in scattered parts. TAX Red indicated presence of chlorophyll content. FITC image showed presence of phenolic compounds. Merge image demonstrated collective pattern of all filters which resulted in red color as a more dominant one, which confirmed presence of more chlorophyll content. 20X image explained presence of microtubules in the callus. 40X bright field image illustrated presence of chloroplast bodies and nucleus in the embryogenic callus. Bright field image showed scattered cells in the seeds. DIC image demonstrated clear morphology of scattered cells. DAPI filter image indicated presence of lignin in the seed. TAX Red filter showed presence of chlorophyll content in the seed. FITC filter image explained appearance of phenolic compounds. Merge image showed presence of more green color representing dominant phenolic compounds.

Digital camera images

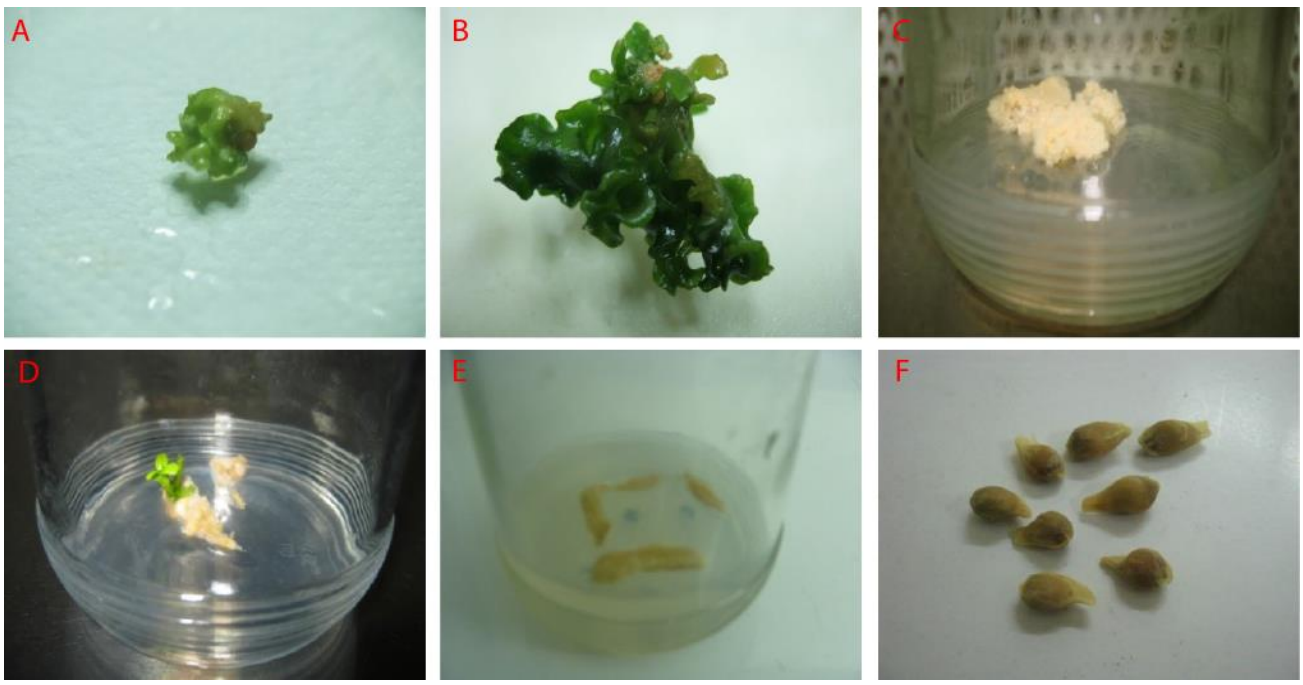

Fig. 2. Nucellar embryos (A).Nucellar embryonic tissues just before formation of new plantlets (B).Embryogenic callus growth for the histological studies (C).Regeneration from embryogenic callus (D).Non embryogenic callus formation to study histology (E). Mature seeds of Kinnow mandarin (F).

Stereomicroscopic images of embryos

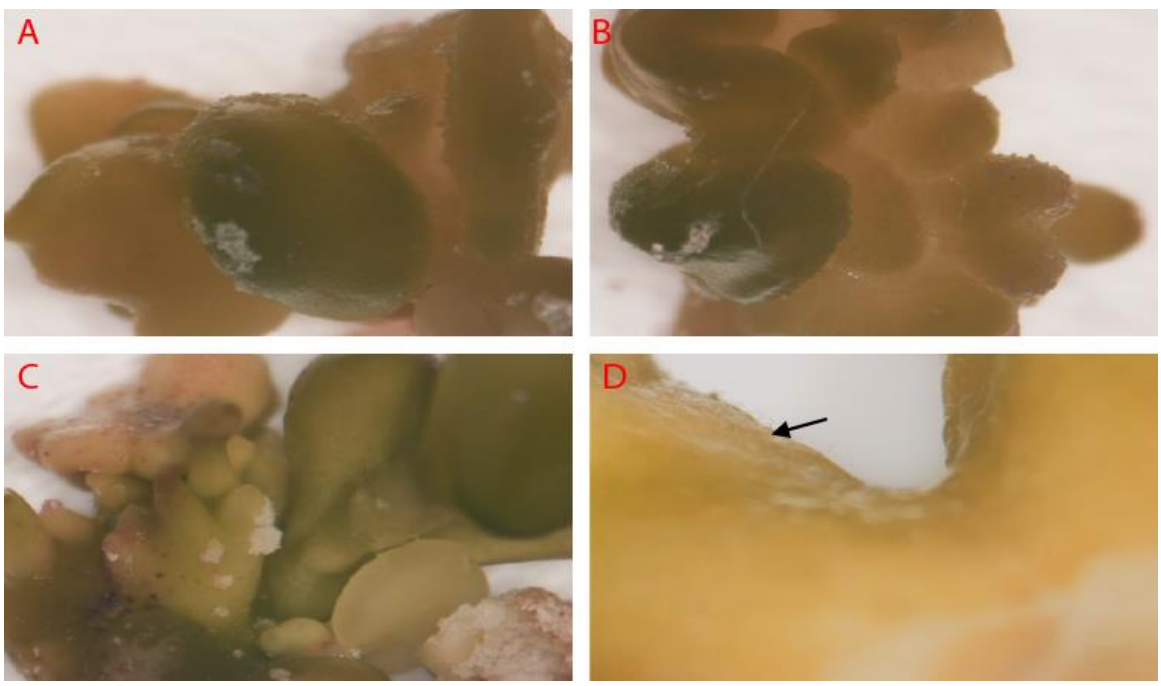

Fig. 3. Globular shapes of nucellar embryos (A). Heart shapes of nucellar embryos (B). Torpedo shapes of nucellar embryos (C). Cutaneous hairs are present on the surface of nucellar embryos (D).

Fluorescent microscopic pictures of nucellar embryo

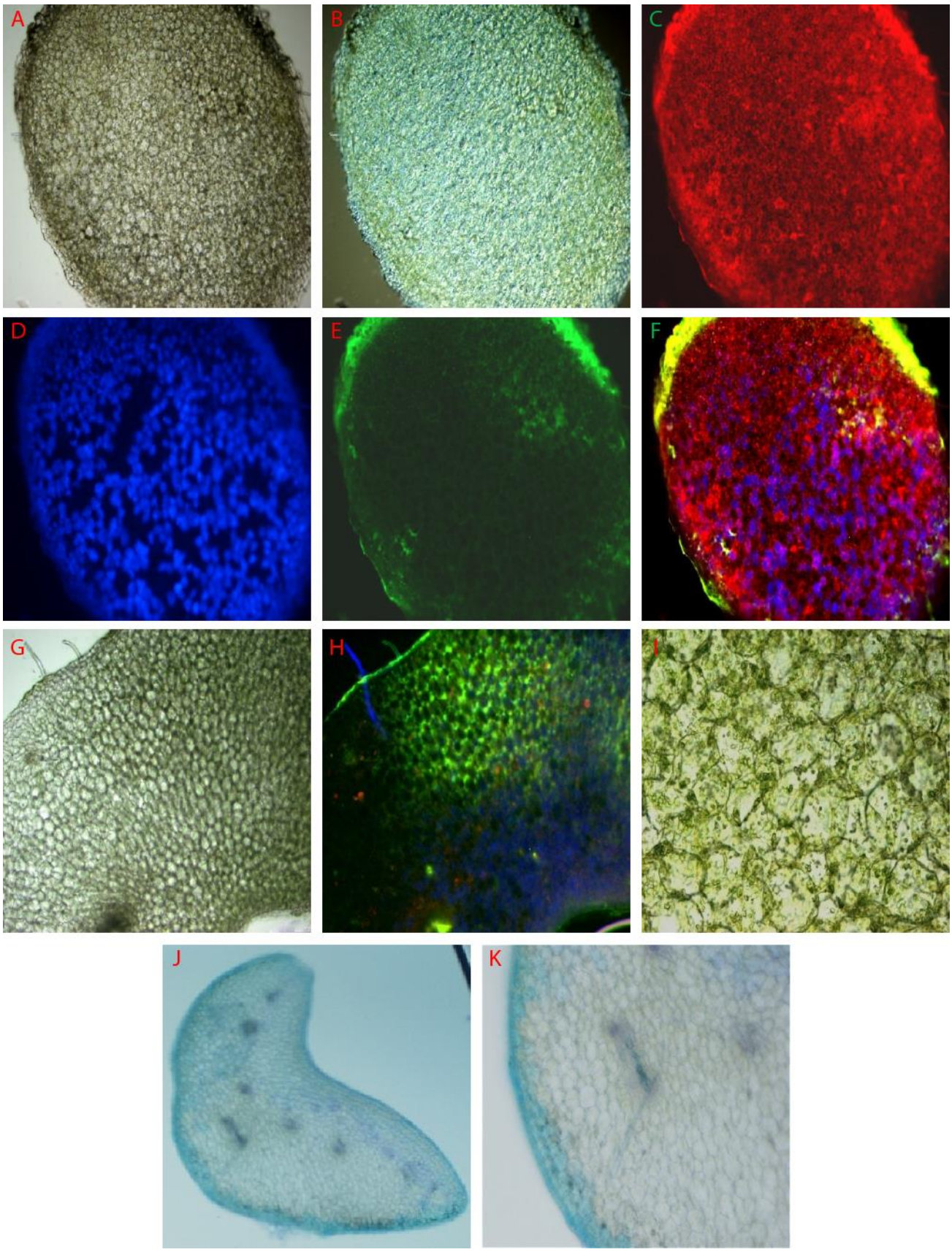

Fig. 4. In bright field image dark green color appeared (A). DIC image with light and dark green color (B). Bright uniform red color (C). Nucellar embryo with blue colored beads (D). Less green color appeared (E). This image is a merge of all previous fluorescence images (F). Bright field image of nucellar embryo with cutaneous hairs (G). Merged image of all fluorescent filters (H). 40X image of embryo (I). TBO staining of nucellar embryo (J). 20X image showing lignified walls and sieve tubes (K).

Non-embryogenic callus and plant shoot fluorescent images

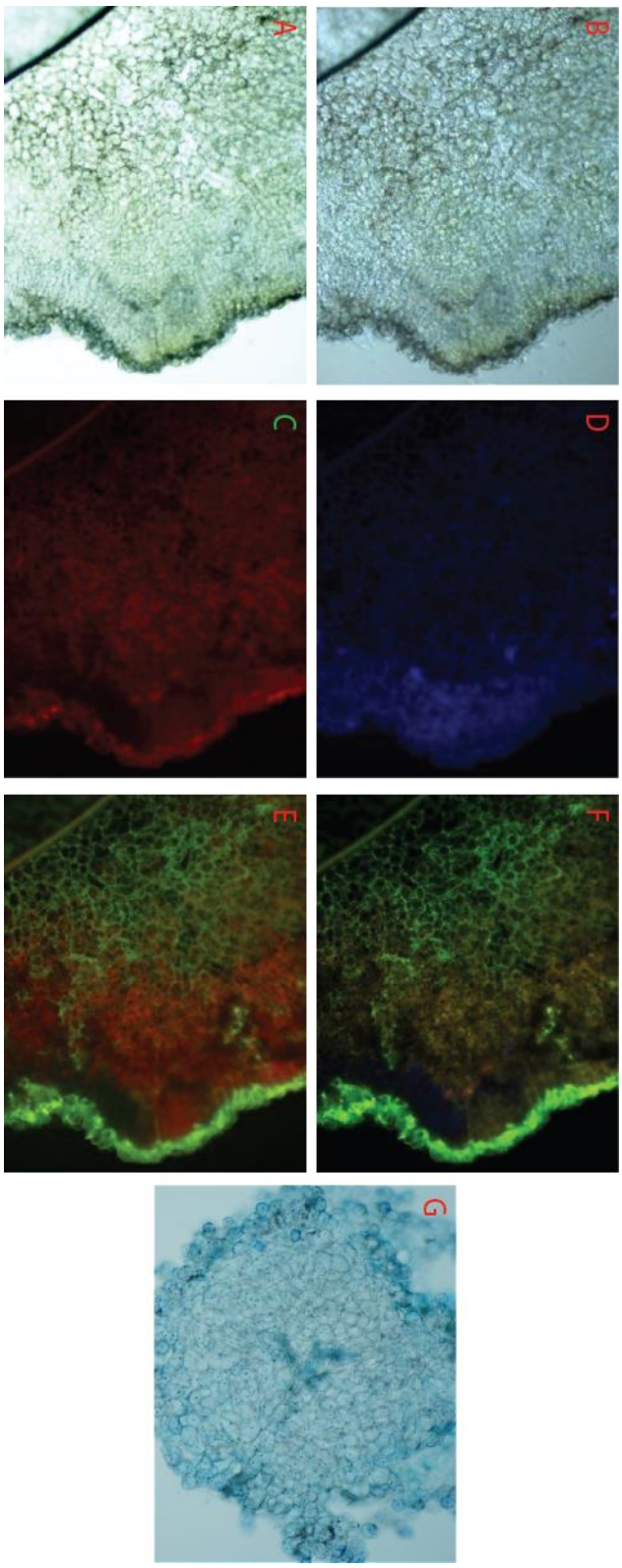

Fig. 5. Bright field image of non-embryogenic callus (A).DIC image with light green color as in bright field (B).Tax Red filter showed light red fluorescence with less chlorophyll content (C). Blue filter DAPI showed less blue coloration in center and more blue color on peripheral region (D). In FITC filter red and a green florescence pattern indicate chlorophyll content and phenolic compounds (E). A merge of all above mentioned filter images showed combine fluorescent pattern (F). TBO staining of non-embryogenic callus stained nucleus in blue color (G).

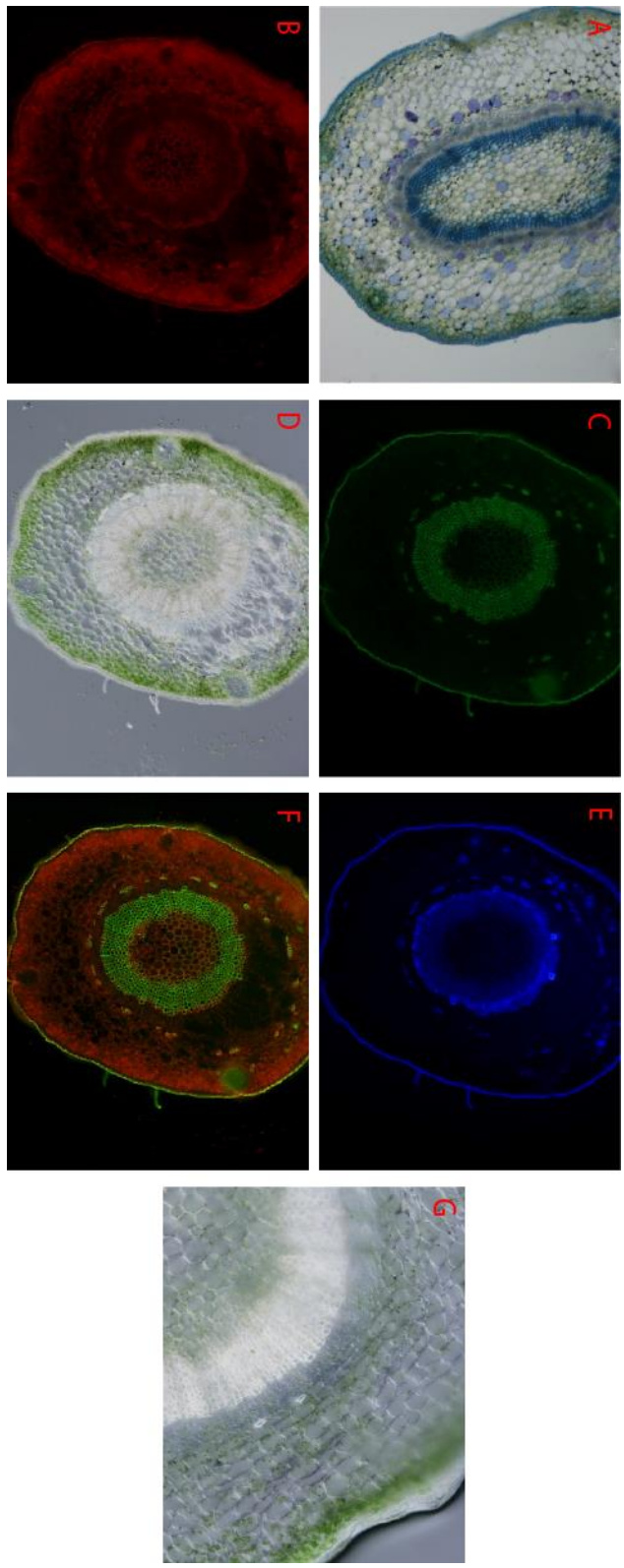

Fig. 6. TBO staining of plant stem displayed different colors for different parts (A). Tax Red filter showed bright red color which indicates presence of chlorophyll content in sclerenchyma cells (B). In the FITC Filter, green color was also present which indicates presence of phenolic compounds (C). DIC image clearly showed the cellular arrangement in the stem (D). DAPI filter showed fluorescence of epidermis and vascular bundles indicating lignin (E). This picture is a merge of all fluorescent images (F). Bright field image showed silvery white appearance of cutin and suberin (G).

mages of embryogenic callus and seeds

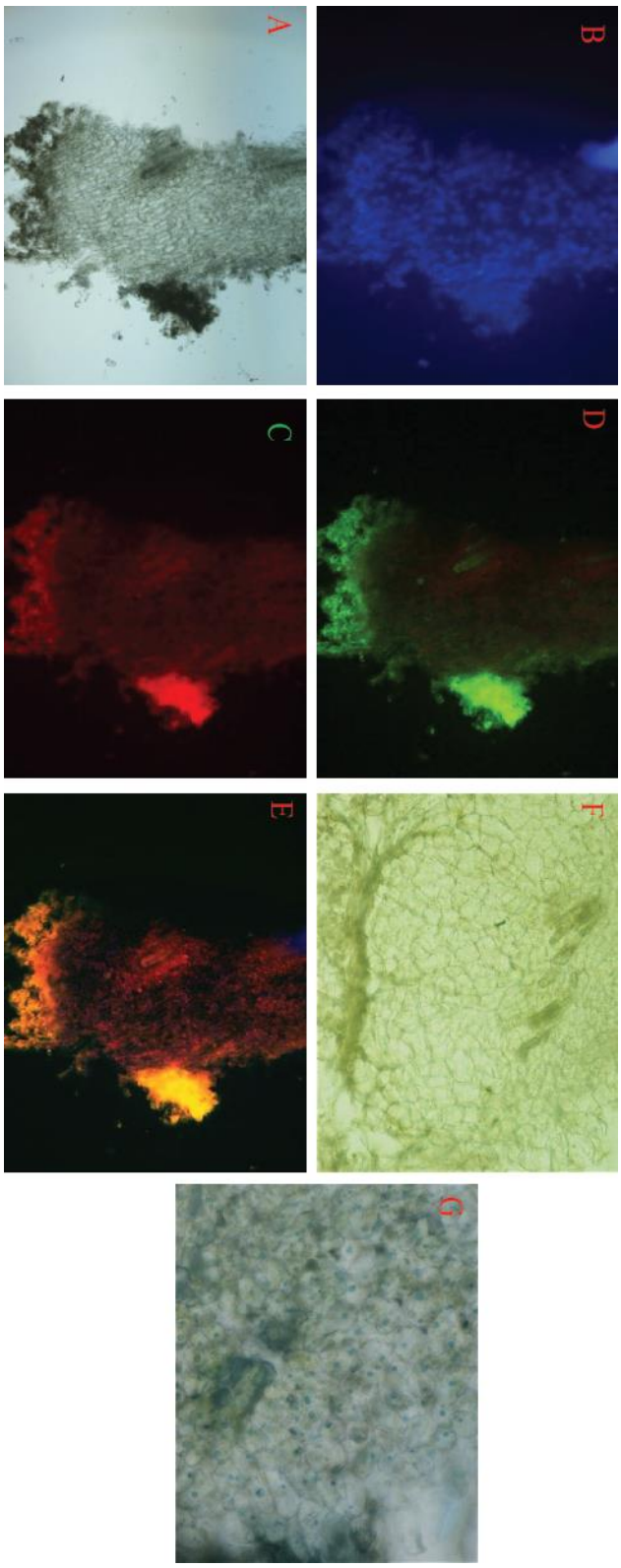

Fig. 7. Bright field image displayed irregular mass of embryogenic callus (A). DAPI image showed presence of lignin in scattered parts (B). TAX Red appeared to have chlorophyll content (C). FITC image showed presence of phenolic compounds (D). Merge image showed collective pattern of all filters which results in red color as a more dominant one which indicate presence of chlorophyll content (E). 20X image presence of microtubules in the callus was found (F). 40X bright field image, Chloroplast bodies and nucleus in the embryogenic callus were found (G).

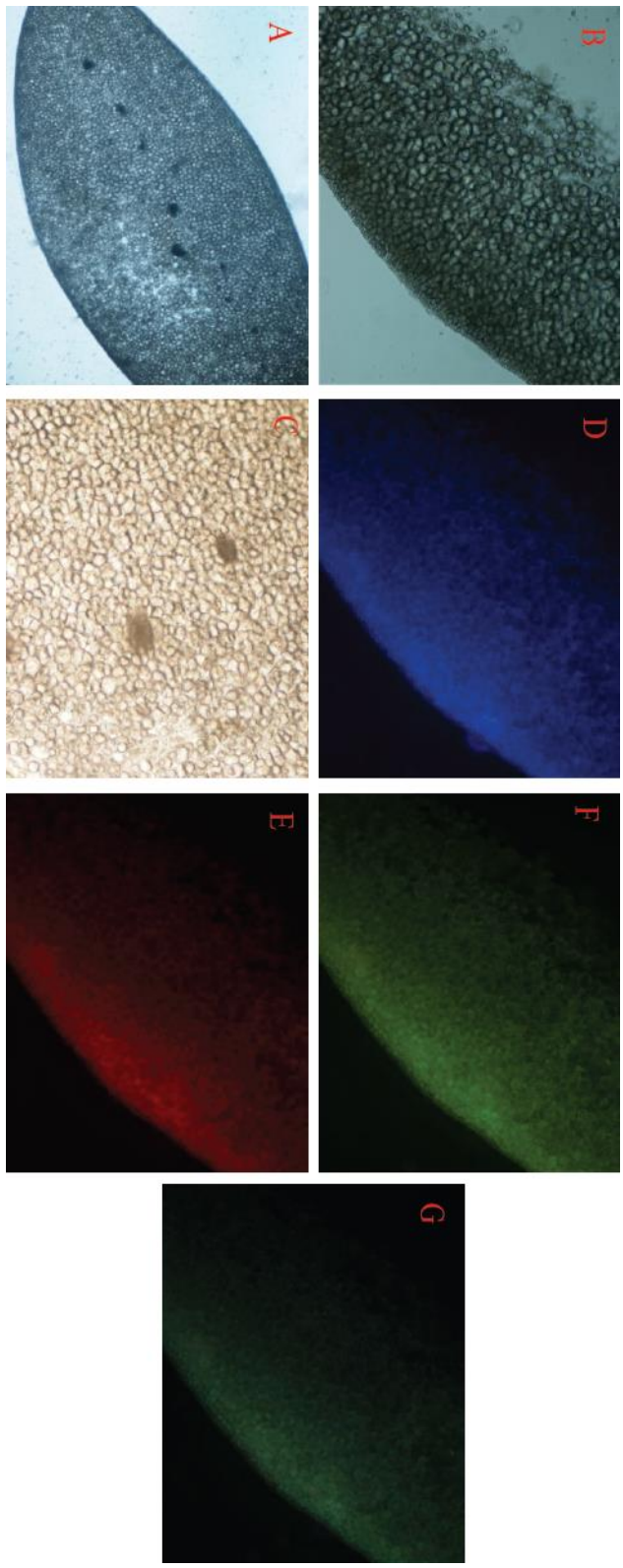

Fig. 8. TBO staining of seed illustrated dark blue coloration with uniformly distributed cells (A). Bright filed image showed scattered cells in the seeds (B). DIC image showed clear morphology of scattered cells (C). DAPI filter showed presence of lignin in the seed (D). Tax Red filter, presence of chlorophyll content in the seed was evident (E). FITC filter showed appearance of phenolic compounds (F). Merge image witnessed presence of more green color showing dominant phenolic compounds (G).

Toluidine blue O staining: TBO is a polychromatic cationic dye, it binds to negatively charged groups. The solution of toluidine blue O is blue in color but after binding to plant cells, it differentially stains different charged groups. A pinkish purple color appeared when the dye reacted with carboxylated polysaccharides such as pectic acid while green, greenish blue or bright blue was observed with polyphenolic substances such as lignin and tannins, whereas purple or greenish blue appeared with nucleic acids (O'Brien *et al*., 1964). Toluidine blue O staining of nucellar embryos showed blue green color of tracheary elements of lignified walls and sclerenchyma, sieve tubes and companion cells appeared purple and parenchymatous cells were of green in color with dense chloroplast content. Toluidine blue O staining of non-embryogenic callus was pictured as blue colored nucleus and chloroplast bodies were not much visible. Toluidine blue O staining of plant stem displayed different colors for identifying distinctive parts, as blue color for epidermis was detected due to presence of lignin and tannins. Cortical region appeared green, while sclerenchyma seemed to be light grey and xylem and phloem appeared dark blue which is the distinct character of the vessel elements. The secondary wall contains lignin therefore, mature vessel elements were stained blue with TBO (Yeung, 1998). Embryogeniccalli after TBO staining were confirmed by dark blue color image with scattered uniform cells. Thus, TBO staining of seed also gave dark blue coloration with uniformly distributed cells. After toluidine blue O staining of non-embryogeniccalli, images were less bright as in the case of embryogeniccalli due to lack of chloroplast bodies, only blue colored nucleus was visible. In nucellar embryos the blue green color was present in whole embryo and purple colored filaments along with microtubules were also visible.

Conclusion

It is concluded from this study that nucellar embryos were found to be tetraploid in nature due to the presence of multiple chloroplasts and high chlorophyll content; which is also the reason behind high regeneration ability of nucellar embryos as compared to other explants. Histological analysis revealed structural differences among different tissue culture stages, identifying enhanced regeneration ability of nucellar embryos, which is useful for plant micropropagation and transformation experiments. These protocols can help to get large number of plants from nucellar embryos, rootstock multiplication and crop improvement can become enhanced, easier and cost effective.

Acknowledgement

We are thankful to Higher Education Commission of Pakistan for providing funding for this research, also to our group members for their support and encouragement.

References

Aleza, P., Y. Froelicher, S. Schwarz, M. Agustı,´ M. Herna´ndez, J. Jua´rez, F. Luro, R. Morillon, L. Navarro and P. Ollitrault. 2011. Tetraploidizationevents by chromosome doubling of nucellar cells are frequent in apomictic citrus and are dependent on genotype and environment. *Annals of Botany*, 108: 37-50.

Carimi, F. and F. De, Pasquale.2003. "Micro propagation of Citrus," In: *Micropropagation of Woody Trees and Fruits*, (Eds.): Jain, S.M. and K. Ishii. Kluwer Academic Publishers, Netherlands, pp. 589-619.

Chaudhary, M.I. 1994. Fruit Crops. In: *Horticulture*. (Ed.): Malik, M.N. National Book Foundation, Islamabad. 422.

Don, Matesz. 2010. Ancient healing: Traditional uses of peel. http://www.motherearthliving.com/health-and-wellness/citrus-peel-medicine.aspx.Accessed 20th Jan 2016.

Durzan, D.J. 1990. Genetic manipulation of forest trees. Implications for physiological processes. In: *Molecular Basis of Plant Aging*. (Eds.): Rodriguez, R.R., R. Sánchez-Tamés and D.J. Durzan. Plenum Press, N.Y. pp. 303-309.

Economos, C. and W.D. Clay. 1999. Nutritional and health benefits of citrus fruits. http://www.fao.org/docrep/x2650T/x2650t03.htm. Accessed 20th Jan 2016.

Kazmi, S.K., S. Khan, N. Kabir, A.A. Mirbahar, M. Raziq and N. Kauser. 2015a. Embryogenic callus induction, somatic embryogenesis, regeneration and histological studies of Kinnow mandarin (*Citrus reticulate blanco* L.) from nucellar embryo and epicotyl region. *Pak. J. Bot*., 47(1): 305-310.

Kazmi, S.K., S. Khan, A.A. Mirbahar and H.A. Kayani. 2015b. Comparative protein production profiling of (*Citrus reticulate blanco*) among tissue culture stages by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). *The Natural Products Journal*. 5: 186-190.

Ling, A.P.K., S.W. Leong, H. Sobri and I. Rusli. 2008. Induction of somatic embryos from different explants of *Citrus sinensis*. *J. Plant Sci*., 3: 18-32.

Liu, J. 2005. Protoplast isolation and culture of woody plants. In: *Protocol for Somatic Embryogenesis in Woody Plants*. (Eds.): Jain, S.M. and P.K Gupta Springer. The Netherlands, 553-566.

Memon, N.A. 2017. Citrus fruit (Kino). Exclusive on Kino. *Pakistan Food Journa*l. Jan-Feb. 29-31.

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

Murashige, T. and D.P.H. Tucker. 1969. Growth factor requirements of citrus tissue culture. *Proc. First International Citrus Symposium*. 3: 1155-1161.

O'Brien, T.P., N. Feder and M.E. McCully. 1964. Polychromatic staining of plant cell walls by toluidine blue O. Protoplasma, 59: 368-373.

Sim, G.E., C.J. Goh and C.S. Loh. 1989. Micropropagation of *Citrus mitis* Blanco-multiple bud formation from shoot and root explants in the presence of 6-benzylarninopurine. *Plant Sci*., 59: 203-210.

Thomas, T.D. 2008. The role of activated charcoal in plant tissue culture. *Biotechnology Advances*, 26: 618-631.

Yeung, E.C. 1998. A beginner's guide to the study of plant structure. In: (Ed.): Karcher, S.J. *Tested Studies for Laboratory Teaching*. 19: 125-141.

(Received for publication 7 August 2016)