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EMBRYOGENIC CALLUS INDUCTION, SOMATIC EMBRYOGENESIS, REGENERATION AND HISTOLOGICAL STUDIES OF KINNOW MANDARIN (*CITRUS RETICULATA BLANCO* L.) FROM NUCELLAR EMBRYO AND EPICOTYL REGION

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Abstract

Citrus is one of the most dominant horticultural fruit crop in the world. The objective of this study was to develop an efficient protocol for *invitro* embryogenic callus induction and regeneration of Kinnow mandarin. In this study, different concentrations of 2,4-D (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L) along with constant 0.5mg/L BAP were used to check the effective response of epicotyl segments and nucellar tissues on callus induction and regeneration. It was observed that MS medium containing 2.5 mg/L 2,4-D and 0.5mg/L BAP supplemented with 0.5gm/L malt extract was suitable for embryogenic callus induction from both epicotyl segments and nucellar embryonic tissues. Callus embryogenesis was achieved on simple MS medium where as regeneration was obtained on MT medium containing 0.5mg/L BAP and 0.5mg/L Kinetin along with 0.5gm/L malt extract. It was observed that the nucellar embryos are the best explants for efficient callus induction and regeneration. In order to ensure the efficient regeneration ability of nucellar callus histological studies were performed, which showed that nucellar embryos and its calli have more chloroplasts as compared to epicotyl segments, which enhanced regeneration ability of nucellar embryos.

Key words: Indirect regeneration, Nucellar embryos, Toluidine Blue O staining

Introduction

Among the economically important fruit crops citrus is one of the most widely used. Citrus belongs to the family Rutaceae and are native to Southeast Asia (Indonesia and China), but now extensively grown almost throughout the world under tropical and sub-tropical conditions where the soil and climatic conditions are favorable for its growth and yield (Shah, 2004). Its annual production in the world is about 105 million tons (Randhawa *et al*, 2012). The top six citrus producing countries are China, Brazil, United States of America, India, Mexico and Spain (Anon., 2013). In addition to it citrus fruits are nutritious and are good source of carbohydrates, dietary fiber, vitamins as well as minerals. Its flavor is among the most popular fruit flavors for beverages. They are equally precious for people with micronutrient deficiencies as well as those who are concerned about the problems of over nutrition, obesity and diet-related chronic diseases. Thus citrus is an ideal component of low-fat, sodium-restricted diets (Economos & Clay, 1998).

Citrus fruits account for about 40% of the total fruit production in the Pakistan where it is cultivated over an area of 194,500 ha with an annual production of about 1982.2 million tons (Pakistan Bureau of Statistics, 2010-11). More than 95% of the citrus fruits are produced in the Punjab province and 70% of the entire crop plantation is Kinnow mandarin (Niaz *et al,* 2004). Kinnow mandarin is a hybrid between the two varieties, namely *Citrus nobilis* and *Citrus deliciosa*. This cross was made by Dr. H.B Frost, at the Citrus Research Center, University of California, Riverside, USA in 1915. It was introduced, acclimatized and released by researchers working at the campus of the University of Agriculture Faisalabad (Khan, 1992). Due to Kinnow mandarin's exceptional taste, easy peeling property, beautiful bright orange color and pleasant fragrance, it was readily accepted by the growers (Khan, 1992). Kinnow mandarin export can be increased by introducing a seedless variety because in international market such varieties are in great demand and Pakistani Kinnow variety have 20-25 seeds per fruit. There is a report about retaining seedless varieties in Pakistan (Khalil *et al*., 2011), but its propagation is not very fast through traditional methods of propagation. To overcome this problem, it is necessary to propagate Kinnow mandarin through tissue culture techniques, such as direct and indirect micropropagation from callus culture and efficient regeneration so that large number of plants can be obtained in less time. This is a substantial current need of time to conduct the research and develop high temperature and disease resistant as well as salt and drought tolerant varieties, especially root stocks are needed to develop and propagate on a large scale to give support and sustainability to this important crop. For these aspects of research, micropropagation by using tissue culture technique is the best choice.

There are various previous reports available for direct and indirect micropropagation of citrus varieties including micropropagation of rough lemon through callus formation and regeneration in the presence of 2,4-D and BAP was performed by (Ali & Mirza, 2006). Two years

old callus cultures have been used to continuously generate genetically uniform *Citrus acida* plantlets (Chakraverty & Goswami, 1999). Callus cultures have also been grown from leaf, epicotyl, cotyledon and root segments of *in vitro* grown nucellar seedlings of *Citrus reticulata Blanco* 'Local Sangtra' by using very high concentration of NAA (10mg/l) and kinetin 0.5mg/l (Gill *et al*., 1995). Effect, of different carbohydrates on somatic embryogenesis of citrus plants is reported by using calli from 'Ponkan' mandarin (*Citrus reticulata, Blanco*), 'Cravo' mandarin (*C. reticulata*), 'Itaborai' sweet orange (*C. sinensis L. Osbeck*.), 'Valencia' sweet orange (*C. sinensis*) by (Ricci *et al*., 2002).

In some citrus species including Kinnow mandarin, multiple embryos have been found in addition to zygotic embryos representing the phenomenon called nucellar polyembryony. Polyembryonic seed formation in citrus is one of the apomictic processes that was found to occur in the ovules of angiospermic species (Koltunow, 1993). During polyembryonic seed formation, non-zygotic nucellar embryos start growing directly from maternal nucellar wall surrounding the embryo sac while the zygotic embryo development. These nucellar embryos carry the same genetic makeup as it is present in their female parent (Koltunow *et al.*, 1996). A significant role in the evolution of cultivated forms can be played by tetraploid genotypes arising from chromosome doubling of nucellar cells of apomictic citrus as parents in interploid crosses or directly as new rootstocks (Aleza *et al*., 2011).

Genetic transformation through callus is quite easy and high frequency transgenic plants can be obtained from callus since in the case of direct regeneration, frequency of untransformed plants is quite high. Furthermore genetic transformation by protoplast isolation and fusion, agro bacterium mediated genetic transformation, induced mutation through gamma rays or chemical mutagenesis can also become efficient through callus formation and regeneration. Nucellar embryos or nucellar tissues and epicotyl segments can give an efficient callus induction and regeneration systems. The aim of this research is to induce embryogenic callus from epicotyl segments and nucellar embryos from seeds of Kinnow mandarin and compare the regeneration ability of callus obtained from both sources on histological basis.

Materials and Methods

Seeds sterilization and germination: Seeds were collected from commercially available export quality Kinnow mandarin. Prior to sterilization, the seeds were washed thoroughly under running tap water for 5 min and dipped in commercially available washing liquid for 10 min. Then they were sterilized in 70% commercial bleach containing 2 drops of tween-20 as a wetting agent in 200ml distilled water for 20 minutes. The seeds were later washed by four rinses with autoclaved distilled water to remove the traces of bleach. After sterilization seed coats were removed with scalpel.

Simple MT media formulated by (Murashige & Tucker, 1969) supplemented with 50gm/L sucrose and 0.5gm/L malt extract at pH 5.7 adjusted using 1N HCl and 1N KOH was used for germination of seeds. Four to five seeds were placed in each jar, where the jars were placed under dark condition at 25ºC. After three days seeds were germinated with 100% germination potential, as epicotyl region of each seedling became elongated. During germination under dark condition it was observed that nucellar embryos were sprinkled out from some seeds also reported by (Koltunow *et al*., 1996).

Callus formation: After four weeks, germinated seedlings had elongated epicotyl region cut into segments to be initiated on MS media supplemented with growth regulators having different concentrations of 2,4- D (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L) along with constant 0.5 mg/L BAP with 0.5 gm/L malt extract at PH 5.7. Media codes assigned were MS0, MS1, MS2, MS3, MS4, MS5, MS6. Nucellar embryos, which were sprinkled out from seeds, were removed and cut into segments and placed on same media combinations as for epicotyl segments described above. All cultures were incubated at 25±2^{o}C with a 16/8 hrs photoperiod. After three days callus formation from epicotyl region and nucellar embryonic tissues was observed. During next three weeks callus induction data was recorded and scored for statistical analysis.

Callus regeneration: After callus induction, nucellar embryonic tissues were placed on simple MS medium for somatic embryogenesis for four weeks and epicotyl calli were placed on MS media supplemented with 2mg/L 2,4-D and 0.5mg/L kinetin for somatic embryogenesis as described by (Jain *et al*, 1995).

Plant regeneration was achieved by transferring callus on MT media supplemented with various combinations of BAP (0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0mg/L) and constant kinetin with 0.5mg/L. MT1 had 0.5mg/L kinetin only, while each combination was fortified with 0.5g/L malt extract. Media codes assigned were MT0, MT1, MT2, MT3, MT4, MT5, MT6, MT7, MT8. Jars were placed in a growth room with 16 hrs photoperiod at 25±2ºC, during next four weeks data was recorded for number of shoots and their length.

Rooting of plantlets: Regenerated plantlets with healthy shoots were transferred to rooting media containing half strength MT basal medium supplemented with growth regulators IBA 0.5mg/L and NAA 0.1mg/L along with 0.5 gm/L activated charcoal and 30 gm/L sucrose (Liu, 2005). The cultures were incubated at 25 ± 2°C while under white fluorescent tube lights having photoperiod for 16/8 hrs.

After four weeks in rooting media the plantlets developed full roots, and they were transferred in the small plastic pots to acclimatize in the green house under humid environment. This procedure has been successfully repeated many times for micropropagation of Kinnow mandarin plants.

Statistical analysis: Data was collected for callus induction as well as regeneration of both kinds of explants and was analyzed with one way analysis of variance using Minitab software version 11 and bar graphs were constructed.

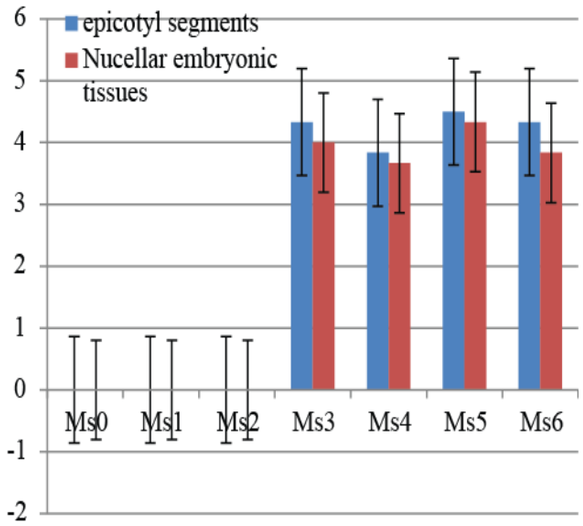
Histological staining: Histological studies were carried out for both nucellar embryos and epicotyl segments along with induced calli. Toluidine Blue O, a polychromatic dye, was used to stain these samples. After hand sectioning, the samples were stained with two drops of 0.1% Toluidine blue O for ten minutes in 10ml distilled water. These sections on slides were observed under Nikon TE 2000E fluorescent microscope in bright field mode using 10X and 40X magnification and images were captured with the NIKON NIS-Elements camera and its software.

Results

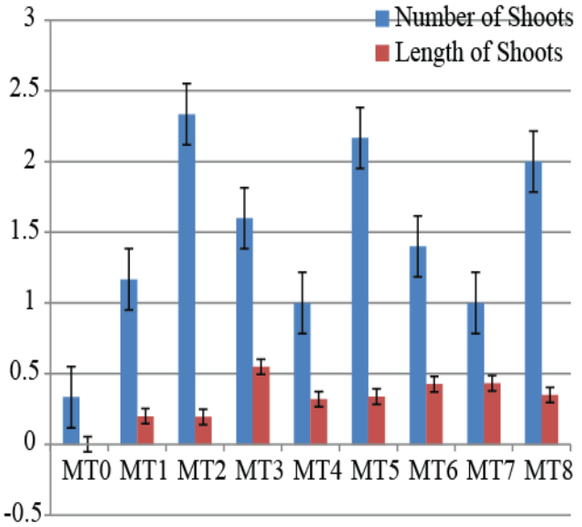
Callus growth: Analysis of variance revealed highly significant differences among the numerous callus induction media ($p<0.001$). It was observed that cream colored calli were induced from both types of explants in all combinations of 2,4-D excluding MS0, MS1 and MS2 media.

The epicotyl region showed highest calli growth in the MS5 medium as shown in (Graph. 1). Thus, as compared to other media highly significant growth was observed ($p<0.001$) in MS5 media containing 2.5mg/l 2,4-D and 0.5mg/l BAP. Similarly as in the case of nucellar embryo highest calli growth were observed on MS5 medium containing 2.5mg/l 2,4-D and 0.5mg/l BAP and analysis of variance revealed highly significant result ($p<0.001$). Since the calli induced from nucellar embryonic tissues were later found more embryogenic as compare to epicotyl segments (Fig. 2B and Fig. 2D).

Plantlet regeneration: Nucellar calli were used for regeneration study and it was observed that MT2 media containing 0.5mg/l BAP and 0mg/l kinetin induced statistically significant ($P=0.189$) highest mean number of shoots as compared to other media combinations (Graph. 2). Whereas on MT3 media containing 0.5mg/l BAP and 0.5mg/l Kinetin the regenerated plantlets were attained the highest mean length of shoots as shown in (Graph. 2) having high significance level of ($p<0.001$).



Graph. 1. Callus growth pattern of Epicotyl segments and Nucellar embryonic tissue on different media.



Graph. 2. Growth pattern for number and length of shoots produced on different media compositions from nucellar callus.

Histological studies: Histological analysis of nucellar embryos and epicotyl segments along with their induced callus was performed after Toluidine blue O staining. It was observed that in nucellar embryos, the epidermis and chlorenchyma cells appeared bluish green in color where as vessels appeared dark purple and parenchyma cells looked greenish blue (Fig. 3c). In calli induced from nucellar embryos it was observed that dark purple cells formed from parenchymatous cells having condensed chloroplast within them (Fig. 3d). In epicotyl segment the cross section appeared just like stem segment showing epidermis with dark green color and blue colored lignified sclerenchyma as describe by (O'Brien *et al*, 1964). Phloem looked light brown and xylem appeared dark blue in color (Fig. 3a). Callus induced from epicotyl segment appeared colorless and only nucleus could be seen stained blue (Fig. 3b).

Discussion

Epicotyl segments and nucellar tissues both showed callus induction. In both cases the callus was soft, friable and embryogenic in the media with 2.5mg/l 2,4-D and 0.5mg/l BAP. These findings are in disagreement with (Nafees *et al*, 2009) since they used BAP and 2,4-D combinations from 0 to 0.4mg/l and also with (Chakraverty & Goswami, 1999) as they found 1mg/l 2,4-D and 0.5mg/l BAP (90.2%) suitable for callus induction as well as 1mg/l BAP for (75.7%) somatic embryogenesis. In this study it was observed that calli from nucellar tissues were found more embryogenic resulting to produce more plants as compared to calli induced from epicotyl segments (Fig. 1E and Fig. 2E), although callus induced from epicotyls was more(Fig. 1) but less regenerative. These findings are in contrast with (Gill *et al*., 1995) since they found epicotyl parts more embryogenic as

compare to other plant parts. In our approach before putting on regeneration, nucellar callus was placed on hormone free simple MS medium along with 0.5gm/L malt extract for somatic embryogenesis, so that it readily accepts MT media for regeneration. Callus induction from other parts of the Kinnow mandarin like leaves, stem segments was also tested, but leaves failed to induce callus which is in disagreement with (Chakraverty & Goswami, 1999). They reported embryogenic calli from the leaves of *Citrus acida Roxb* which may be due to cultivar difference. This phenomenon of different genetic behavior to same media composition was also studied by (Mendes-da-Glória *et al.*, 1999).

During regeneration stages, many shoots developed along with many thin leaves on MT media supplemented with 0.5mg/l BAP but during acclimatization, these plants were not very successful. Thus to increase leaves width for successful acclimatization these shoots were placed on MT media along with 0.5mg/l kinetin and 0.5mg/l BAP (Fig. 2). During acclimatization, the farmyard manure was mixed with the soil to provide nutrients to the plants.

In Toluidine blue O staining the parenchymatous cells appeared reddish purple as described by (O'Brien *et al*, 1964). Histological study highlighted the reasons for less regeneration ability of callus induced from epicotyl segments, as compared to that from nucellar embryonic tissues. Nucellar induced callus was found intensely rich in green chloroplast bodies present in parenchymatous cells as shown in (Fig. 3B) at 40X magnification. Their presence is a proof of high photosynthetic activity and apparently, due to this characteristic plants regenerate more rapidly and are higher in number.

Fig. 1. Indirect regeneration of Kinnow mandarin from Epicotyl regions.
A. Epicotyl segments grown in a dark condition B. Epicotyl segments on callus media C. Callus growth D. Light regeneration E. Dark regeneration F. Separated plantlets on shooting media G. Rooting of plantlets H. Acclimatization

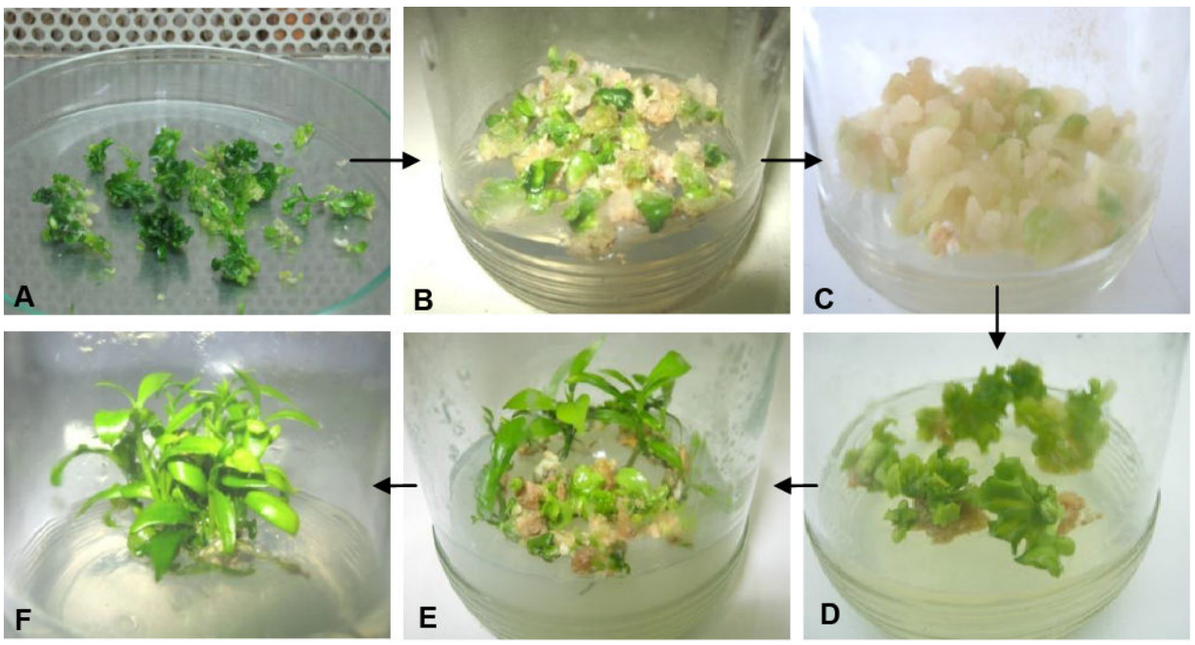


Fig. 2. Indirect regeneration of Kinnow mandarin from Nucellar Embryos.
A. Nucellar embryos B. Callus growth in light C. Callus growth in dark condition D. Somatic embryogenesis E. Shoots elongation F. Regeneration from callus.

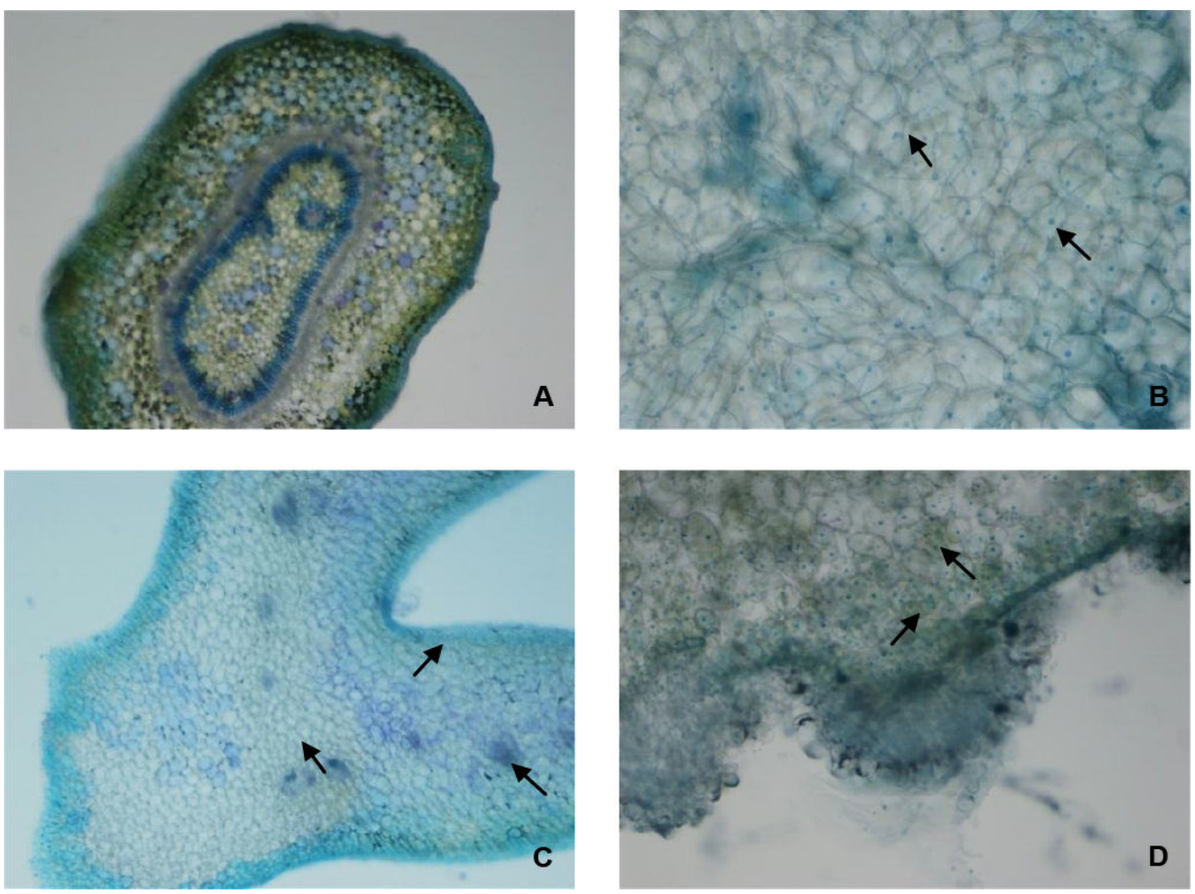


Fig. 3. Microscopic images of stained region of epicotyl segment and nucellar region of Kinnow mandarin.
A. Epicotyl segment stained section B. Embryogenic callus of epicotyls segment C. Nucellar embryo stained section D. Embryogenic callus of Nucellar embryo.

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