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# *IN VITRO* CULTURE OF KINNOW EXPLANTS

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**Abstract**

Kinnow mandarin is the most adaptable *Citrus* scion variety in Punjab, Pakistan. The explant tissues responded to culture media. BA and 2-4, D were essential for good callusing. The reproductive tissues such as nucellus have tendency of embryogenesis, while vegetative tissues like seedling leaves regenerated as shoot organogenesis. The seeds formed callus in MS medium supplemented with BA+2, 4-D each @ 1mg/litre and callus regeneration was observed in MS +GA+BA each @ 1mg/litre +2, 4-D (0.25mg/litre). Seedling leaves formed callus in MS supplemented with BA+GA each @ 1mg/litre +2, 4-D (0.5mg/litre) + proline (5mg/litre) and callus regenerated in BA + GA each @ 1mg/litre +NAA (0.5mg/litre) + Proline (5mg/litre). Nucellus regenerated in BA (1mg/litre) + 2, 4-D (0.5mg/litre) + Glutamine (5mg/litre). Buds were initially cultured in BA (1mg/litre) + GA (1mg/litre) +Glutamine (5mg/litre) and buds developed shoots in BA (2mg/litre) +NAA (0.5mg/litre) +GA (1mg/litre). Nucellus at 100 DAP (Days after Pollination) responded best (68%) at 60 Gray gamma radiation exposure. $LD_{50}$ dose for mature seeds was slightly less than 100 Gray. Apical meristem explants were sensitive to gamma (γ) radiation doses. The maximum survival of explants (77%) was in control (No γ-radiation exposure) followed by 15 Gray (66% survival). The regenerated shoots were grafted on rough lemon seedlings.

## Introduction

*Citrus* has leading position among the fruits of Pakistan in which major share is of Kinnow mandarin as it is the most adaptable variety among *Citrus* cultivars in the climate of the province of Punjab which is the major (>95%) producer of *Citrus* fruit (Anon., 2008). Kinnow has inherited heat tolerance from the cultivar King which helps it to survive in harsh hot summer of Punjab, where maximum temperatures are around 48°C. Kinnow has inherited polyembryony from cultivar King. Polyembryony accompanied by high ovule fertility becomes responsible for unwanted apomictic seedy trait. Kinnow plants have different embryony status (Altaf & Rehman, 2008), ovule and pollen fertility and pollen self incompatibility which results in different seed numbers (0-56) in its populations. Because of the variability within the cultivar, the explants and the medium requirements mentioned by various workers is different (Praveen, 2003; Singh, 2006; Gill *et al*., 1994; Altaf, 2006a; Jaskani *et al*., 2005 and Bhatti *et al*., 2007). Specific objective of this work was to develop tissue culture plants using various explant sources and the ultimate goal is to utilize natural and induced variability to obtain novel characteristics in Kinnow mandarin.

## Materials and Methods

**Explant callusing and regenerations:** The explants used were seeds, seedling leaves, nucellus and mature plant buds. The plant material was sterilized using 0.1% $HgCl_2$ solution, then washed with sterile distilled water and finally cultured in MS medium (Murashige & Skoog, 1962) containing growth regulators BA, 2,4-D, GA, NAA @ 0.1-

4 mg/litre and amino acids: proline and glutamine (1-10 mg/litre). The pH of medium was adjusted to 5.8 and the medium was solidified with 1% agar. The cultures were kept at normal day light at 25±2°C. The objective of the work was to find out the best explant and their suitable initial culture media with regeneration potential.

**Radiation of explants and culture conditions:** The fruits were collected after 60, 70, 80, 90 and 100 DAP and exposed to 0, 30, 60, 90 and 120 Gray gamma radiations with Cobalt $^{60}$ irradiator. The Nucelli were aseptically isolated and cultured by the method given by Altaf (2006b). The immature (August) and mature (December) seeds were radiated with 0, 25, 50, 75 and 100 Gray, gamma radiation. The seeds were sterilized and cultured by the procedure mentioned by Altaf & Rehman (2008). The seed germination was recorded after 35 days. The sprouts were radiated with 0, 15, 35, 45 Gray of Gamma radiations. Two centimeters apical portion of sprouts was sterilized in alcohol for 30 seconds, washed with sterile water and then sterilized with 0.1% $HgCl_2$ for 30 seconds, washed thoroughly with distilled water and cultured in MS medium supplemented with BA+GA(each @ 1mg/litre) + Glutamine @ 5mg/litre. The *In vitro* survival of explants was noted after one month.

## Results and Discussion

Vigor and quality of callus induction is specific with the plant genotype and the culture medium. The callus texture of Kinnow is variable on the same medium and with the same explant from different orchards. Callus from seeds (Figs. 1&2) leaves (Fig. 3) and nucellus (Fig. 4), was proliferated and was studied for regenerations. There were various embryogenic forms in the same culture. However, leaf explants derived callus produced regenerations in the presence of BA (Table 1). There were shoot organogenesis in some cultures. None of the leaf derived callus produced shoots and embryos within the same callus culture line. There were qualitative and quantitative differences in callusing and also in callus regeneration responses. If embryos were incubated horizontally on the medium, regeneration at the apical end occurred in some by indirect organogenic pathway after callus formation in low concentrations of cytokinins and auxins. Embryos developed much faster when separated to individual units than when left to grow together in clumps. Development of embryos into plantlets with multiple shoots and roots was because of the poor separation of embryos early in the development. The other cause of multiple shoots was the growth of cotyledon buds. Development of primary shoot and root was because of apical dominance. Polyembryony from seeds after removal of seed coat was enhanced. Contaminations are the major problem in bud culture of mature trees. The harsh sterilization treatment damage the growing points of buds which affect the culture growth and regeneration potential of these buds. Plants were regenerated from seeds, seedling leaves, nucellus and bud cultures *via* callus formation.

*Citrus* has several natural factors as cause of variability (Ribeiro & Machado, 2007). Radiation can create variability from which useful variations can be selected (Brunner, 1995). The nucellar regeneration was highest at 100 DAP (Fig. 7). Nucellus has natural competence and determination for embryogenesis (Fig. 5). The mutated cells of nucellus, if survived as embryonic plants can be useful for studying solid mutations. The survival of mature seeds in response to radiation was more than double as compared to immature seeds when germinated in MS basal medium (Fig. 6). The $LD_{50}$ was near to 100 Gray for mature seeds and slightly less than 75 Gray in immature seeds (Fig. 8). These results are in agreement with Dhatt *et al*., (2000) who found that $LD_{50}$ dose of gamma radiation in

case of Kinnow seeds was slightly less than 10Kr (I Kr=10Gary). The apical meristem survival decreased with increasing dose of gamma radiation. The maximum *In vitro* survival in culture was in control followed by 15 Gray. Apical meristem is the most sensitive tissue (Fig. 9) in response to radiation and mature seeds are comparatively hard tissue. All healthy plant regenerations were grafted on rough lemon rootstock.

**Table 1. Explant and the culture media for plant regeneration.**

| Explant | Initial culture | Regeneration media |
|---|---|---|
| Seed | BA+ 2, 4-D each @ 1 mg/litre | GA + BA (each @ 1 mg/litre) + 2, 4-D @ 0.25 mg/litre |
| Seedling leaf | BA+ GA (each @ 1 mg/litre) + 2, 4-D @ 0.5 mg/litre + proline @ 5 mg/litre | BA + GA (each @ 1 mg/litre) + NAA @ 0.5 mg/litre + Proline @ 5 mg/litre |
| Nucellus | BA @ 0.5 mg/litre + 2, 4-D @ 2 mg/litre | BA @ 1 mg/litre + 2, 4-D @ 0.5 mg/litre + Glutamine @ 5mg/litre |
| Bud | BA @ 1 mg/litre + GA @ 1mg/litre + Glutamine @ 5 mg/litre | BA @ 2 mg/litre + NAA @ 0.5 mg/litre + GA @ 1 mg/litre |

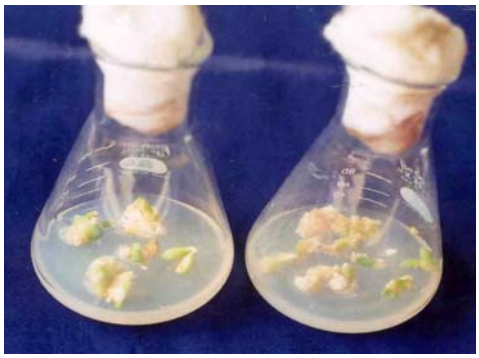

Fig. 1. Seed callus.

Fig. 2. Seed callus regenerations.

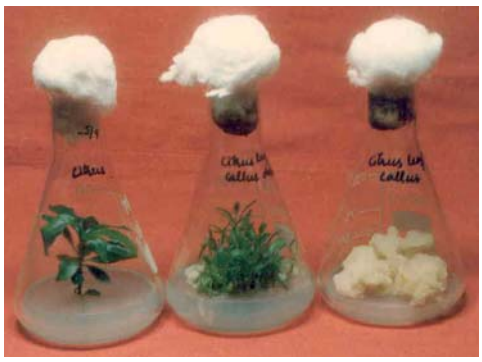

Fig. 3. Leaf callus regenerations.

Fig. 4. Nucellus regenerations.

Fig.5. Nucellus regeneration.

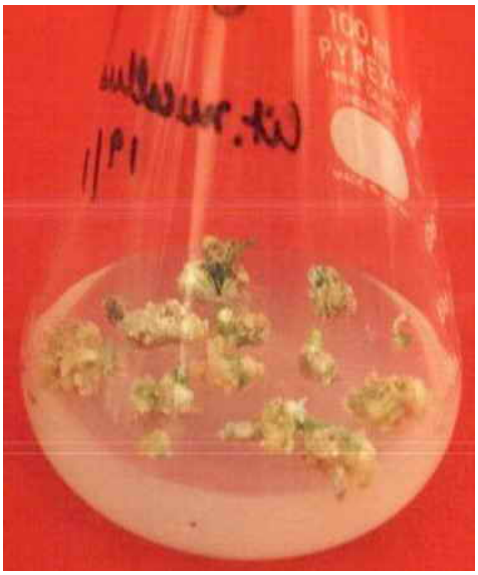

Fig. 6. Seed germination.

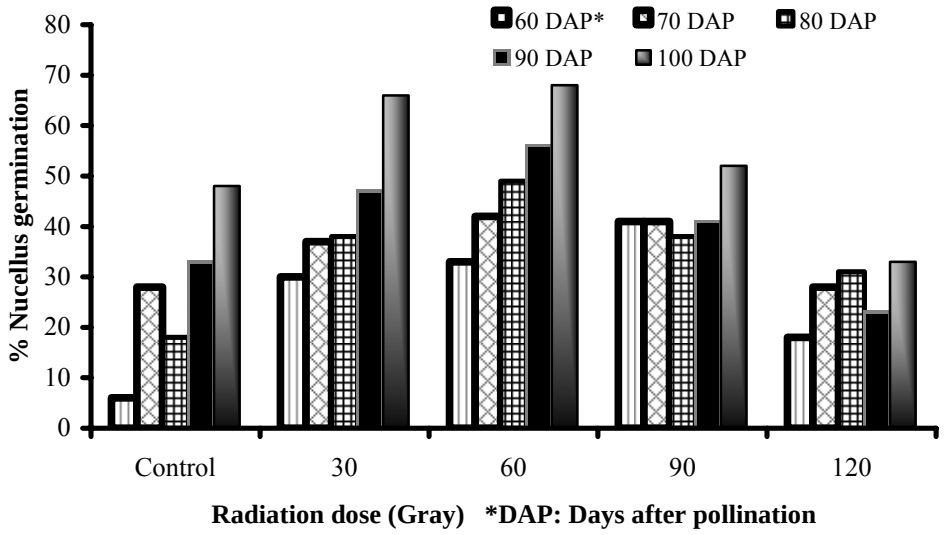


Fig.7. Nucellus regeneration.

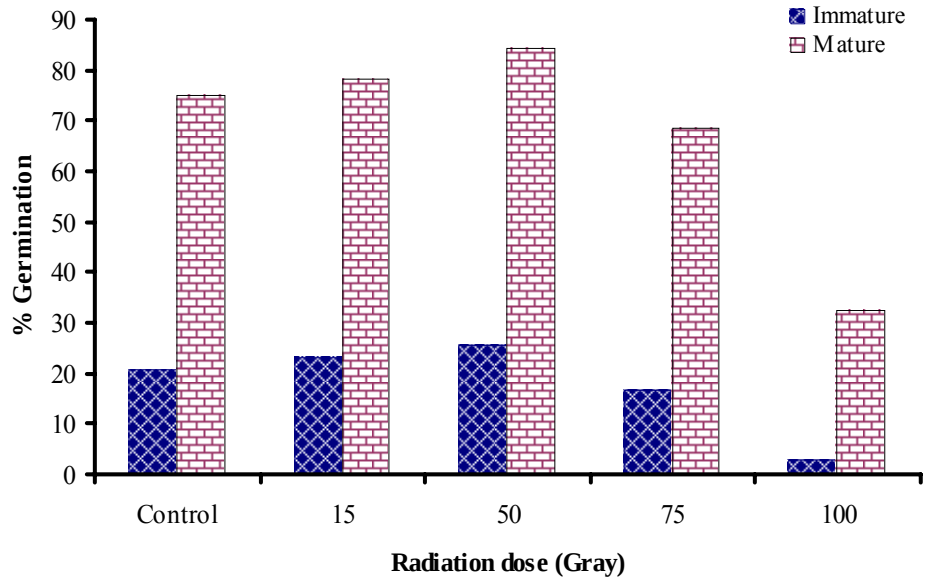


Fig. 8. Immature and mature seed germination.

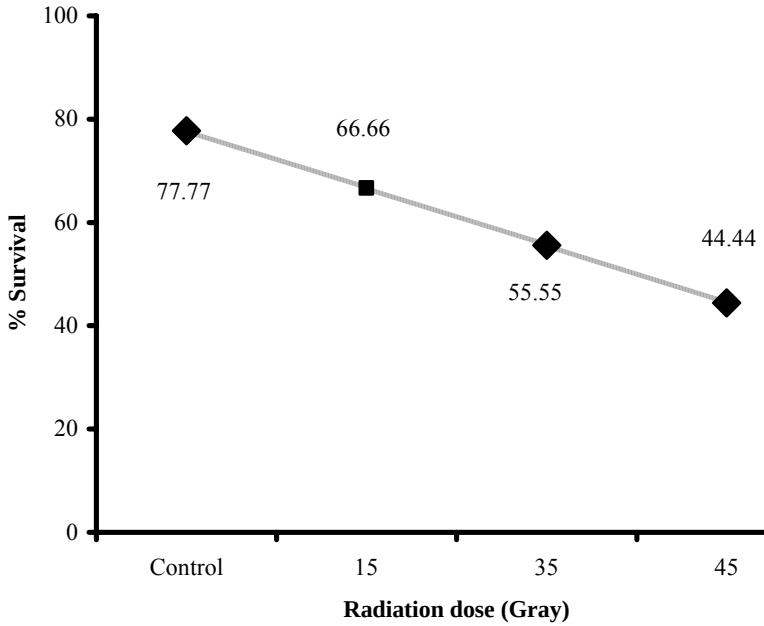


Fig. 9. Shoot apex survival after gamma radiation exposure.

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