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# Establishment of an efficient callus induction method from leaf and stem in kinnow mandarin (*Citrus reticulata* Blanco.) and citron (*Citrus medica* L.)

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**Kinnow mandarin (*Citrus reticulata* Blanco.) is a highly adaptable variety among citrus cultivars in the climate of the region of Panjab, Pakistan and citron (*Citrus medica* L.) was the first of the citrus fruits known to become visible in the Mediterranean Basin. In Sylhet region of Bangladesh, it is commonly known as Zara lemon. The establishment of *in vitro* technique for seed germination and callus induction has been done for kinnow and citron in this experiment. In case of seed germination, ½ MS media supplemented with 6-benzylaminopurine (BAP) (0.5 mg/L)+ 1-naphthaleneacetic acid (NAA) (2.0 mg/L)+KIN (1.0 mg/L) shows best seed germination response (90%) for kinnow and BAP (1.0 mg/L)+NAA (0.5 mg/L) shows best (92%) for citron. For callus induction, 5 weeks old plantlets were used as a source of leaf and stem explants for both. For callus induction from leaf and stem of kinnow mandarin, 2,4-D (1.0 mg/L) shows best result (90%) for leaf while 2,4-dichlorophenoxyacetic acid (2,4-D) (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) shows best callus response (95%) for stem explants. In case of citron, 2,4-D (1.0 mg/L) shows best callus response (80%) for both leaf and stem explants.**

**Key words:** *In vitro,* kinnow mandarin, citron, seed germination, callus induction.

## INTRODUCTION

Citrus is one of the leading tree fruit crop in the world. The genus citrus includes more than 162 species belonging to the family Rutaceae. Kinnow mandarin (*Citrus reticulata Blanco.*) is the most far and wide-planted mandarin hybrid in Pakistan. Kinnow has inherited heat tolerance from the cultivar King which helps it to survive in ruthless hot summer of Punjab. This "easy peel" citrus has assumed special economic importance and export demand due to its high juice content, special flavor, and as a rich source of vitamin C. Citron is a scented citrus fruit; it is a small tree about 2.44 to 4.57 m, having large fruit (20 to 22.5 cm long) that resembles pineapple in shape. It is botanically classified as *Citrus medica L.* and was the first of the citrus fruits to come into view in the Mediterranean region. In Sylhet region of Bangladesh it is locally known as Zara lemon and available in the local

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market. While the lemon or orange are peeled to consume their pulpy and juicy segments, the citron's pulp is dry, containing a small quantity of insipid juice, if any. The main content of a citron fruit is the thick white rind, which adheres to the segments, and cannot be separated from them easily. From ancient through medieval times, the citron was used mainly for medical purposes: especially to combat seasickness, pulmonary troubles, intestinal ailments, and other disorders. Citron juice with wine was considered as an effective antidote to poison. Because of the variability within the cultivar, the explants and the medium requirements are different, mentioned by various workers (Praveen et al., 2003; Singh et al., 2006; Gill et al., 1994; Jaskani et al., 2005; Bhatti et al., 2007).

The present investigation was undertaken to find out the suitable explant source along with the best suited concentration of plant growth regulators for callus induction and mass propagation of *C. reticulata* and *C. medica* through *in vitro.* Although, tissue culture and micro propagation protocols have been described for a number of citrus species and explants sources (Grinblat, 1972; Chaturvedi and Mitra, 1974; Barlass and Skene, 1982; Edriss and Burger, 1984; Duran Vila et al., 1989). Micro propagation has many advantages over conventional methods of vegetative propagation (cutting or seed) and its application in Horticulture, Agriculture and Forestry is currently expanding worldwide (Jeong et al., 1995). Embryogenic callus was successfully induced in six relatives of Citrus with combination of 5.0 mg/L BA, 2.5 mg 2,4-D and 600 mg/L malt extract in Murashige and Tucker (MT) medium (Jumin and Nito, 1995).

## MATERIALS AND METHODS

The research project was conducted at the plant Genetic Engineering Laboratory, Department of Genetic Engineering and Biotechnology, Shahjalal University of Science and Technology (SUST), Sylhet-3114, Bangladesh. Mature seeds of kinnow mandarin and citron were germinated to produce young plantlets. 5 weeks old plantlets were then used as a source of explant for callus induction following callus induction, and were maintained in MS medium with supplementation of 2,4-D (1.0 mg/L).

### Explant collection

Hundred seeds of kinnow mandarin and hundred seeds of citron were collected from Citrus Research Institute, Jointapur, Sylhet. Fresh healthy seeds of kinnow mandarin and citron were used as an explant for *in vitro* germination in half strength Murashige and Skoog (MS) regeneration medium. Callus development was done by using leaf and stem from germinated plantlets.

### Sterilization of the explant

First of all, the seeds were dipped into 95% alcohol for 1 min. Then rinsed for four times with sterile distilled water. Afterwards, the seeds were dehusked and surface sterilized with 70% alcohol for 30 s. Furthermore, surface sterilization was done with 5% sodium Hypochlorite for 5 min. Then rinsed with sterile water for 3 to 4 times. In addition, it immersed into 2 to 3 drops of tween-20 for 15 min. Then washing of the explant took place with distilled water for 3 to 4 times. Before inoculation soaking of the explant was done on the filter paper.

### Media for seed germination

Freshly collected 160 seeds of two varieties were cultured on half strength MS (Murashige and Skoog, 1962) basal medium enriched with hormonal concentration of BAP (1.0 mg/L)+NAA (0.5 mg/L) , BAP (0.5 mg/L)+NAA (2.0 mg/L)+KIN (1.0 mg/L), 2,4-D (2.0 mg/L)+BAP (1.0 mg/L)+NAA (0.5 mg/L), and ½ MS medium without growth regulator. Approximately, 10 seeds were cultured in each hormonal combination on separate test tube for seed germination and each combination has two replications in a separate time frame (a gap between 15 days). pH of the media was adjusted to 5.8 to 6.0. Visual observation was taken for each replication with 10 samples for every 7 days and effect of hormonal combination on seed germination was recorded.

### Media for callus induction

Five weeks old leaf and stem were used for callus induction in the case of kinnow mandarin and citron. Leaf and stem cuttings of kinnow mandarin and citron plantlet cultured on MS (Murashige and Skoog, 1962) basal medium supplemented with hormonal concentration of 2,4-D (1.0, 1.5, 2.0, 4.0) mg/L alone and 2,4-D (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) in combination. For callus proliferation media with 2, 4-D (1.0 mg/L) was used. In every case 3% sucrose was added. pH of the media was adjusted to 5.8 to 6.0. Visual observation was taken every 7 days and effect of different treatment was quantified on the basis of percentage of callus induction.

### Explant inoculation

Each explant was inoculated in test-tube by the help of a sterile forceps in the laminar air flow chamber. After the inoculation of explants, the test-tubes were transferred to the culture room and incubated at 25°C. The photoperiod was maintained as 16 h light and 8 h darkness by 40 W white florescent tubes light with intensity from 2000 to 3000 lux. Data was recorded every week for three months.

### Statistical analysis

All the data were recorded at regular interval for analysis and reckoned under statistical basis. Arithmetic mean (A.M.) and standard deviation (S.D.) were evaluated by analyzing data with Microsoft excel 2007. Standard error (S.E.) was calculated by dividing standard deviation by square root of the total 20 replication for a single variety in each hormonal concentration. In case of our experiment error related to contamination was calculated properly and expected values were taken from the calculation.

## RESULTS AND DISCUSSION

### Direct regeneration from embryonic seed

Citrus seeds have a very short life because they are amenable to injured by drying during storage and thus

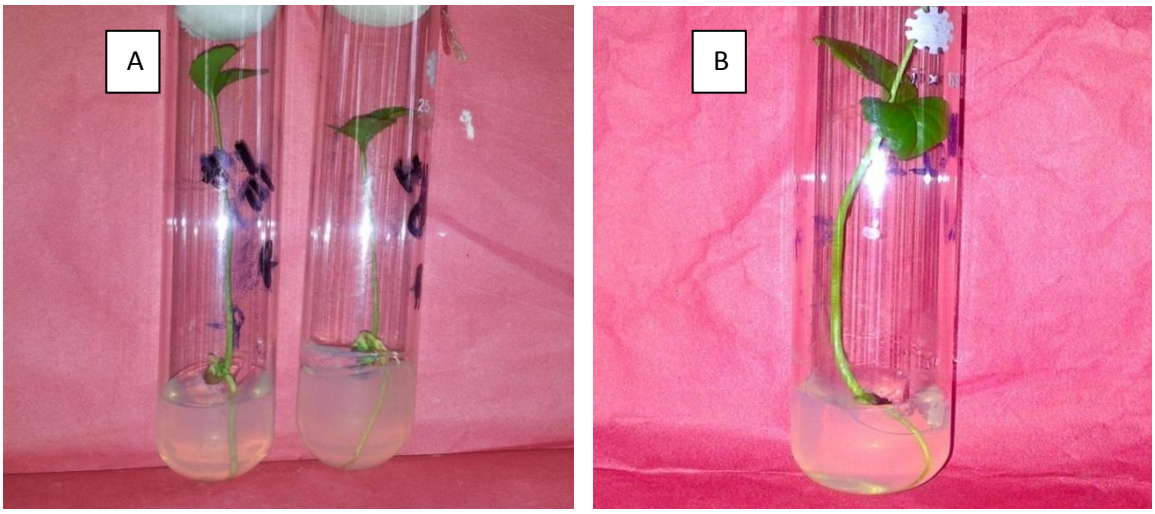


**Figure 1.** Plantlets were obtained using BAP (1.0 mg/L)+NAA (0.5 mg/L) for kinnow mandarin (A) and citron (B).

**Figure 2.** Callus of kinnow on ½ MS medium supplemented with 2, 4- D (2.0 mg/l) +BAP (1.0 mg/l) +NAA (0.5 mg/l).

lose their viability (Johnston, 1968). This is why freshly isolated seeds from kinnow and citron were used. Removal of seed coat showed an early response for shoot formation. In case of sweet orange, highest (70%) shoot formation was obtained from seeds without seed coat (Azim et al., 2011). In this experiment direct regeneration from seed was done for obtaining 5 weeks old plantlets of kinnow and citron species. Half strength MS media supplemented with BAP (0.5 mg/L)+NAA (2.0 mg/L)+KIN (1.0 mg/L) shows (90%) germination in case of kinnow mandarin and in case of citron media supplemented with BAP (1.0 mg/L) +NAA (0.5 mg/L) shows (92%) best result for germination of plantlets (Figure 1). Altaf et al. (2009) reported that, the seeds formed callus in MS medium supplemented with BA and 2,4-D each at 1 mg/L. In our experiment, in hormonal combination of 2,4-D (2.0 mg/L) +BAP (1.0 mg/L)+NAA (0.5 mg/L) citron shows 70% seed germination response, where seed callus found for kinnow mandarin (Figure 2). This implies that, citron may not require BAP and NAA along with 2,4-D in this concentration for callus induction from their seed thus callus induction requires different concentration of 2,4-D and other plant hormones for citron (Table 1).

**Table 1.** Effect of different hormonal combination for seed germination after 5 weeks of observation (20 replications for each combination for a single species).

| Species | Treatments (mg/L) | Growth rate | Average germinated plant height for 20 replications (cm) | Average leaf number of all the replica | Seed Germination percentage (%) |
|---|---|---|---|---|---|
| Kinnow mandarin | BAP(1.0)+NAA(0.5) | Average | 2 | 2±1 | 85 |
| | BAP(0.5)+NAA(2.0)+KIN(1.0) | Good | 2.5 | 3±1 | 90 |
| | 2,4-D(2.0)+BAP(1.0)+NAA(0.5) [Callus obtained] | --- | ---- | ---- | ---- |
| | MS basal | Low | 1.5 | 2±1 | 70 |
| Citron | BAP(1.0)+NAA(0.5) | Excellent | 3.0 | 3 ±1 | 92 |
| | BAP(0.5)+NAA(2.0)+KIN(1.0) | Good | 2.5 | 2±1 | 85 |
| | 2,4 D(2.0) +BAP(1.0)+NAA(0.5) | Average | 1.5 | 2±0 | 75 |
| | MS basal | Average | 2 | 2±1 | 80 |

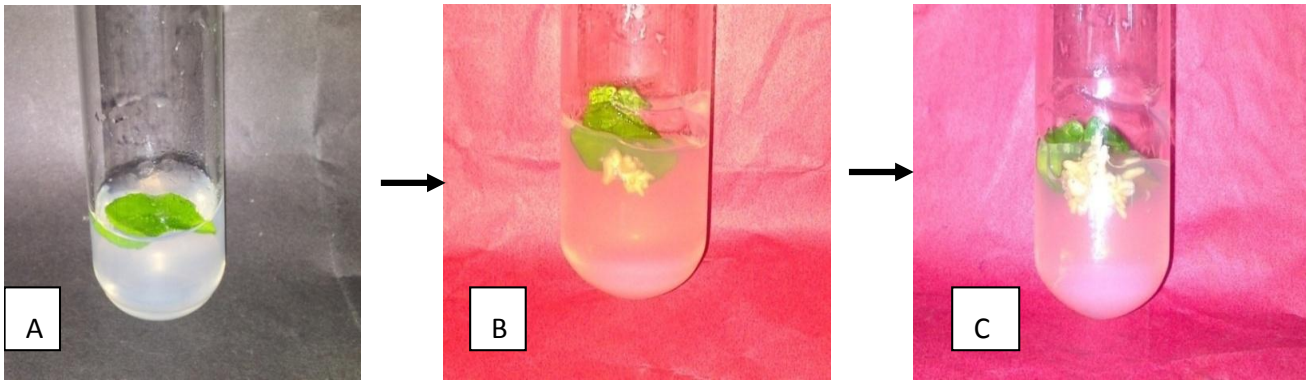


**Figure 3.** Callus induction from kinnow leaf in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 21 days; C = Callus after 45 day.

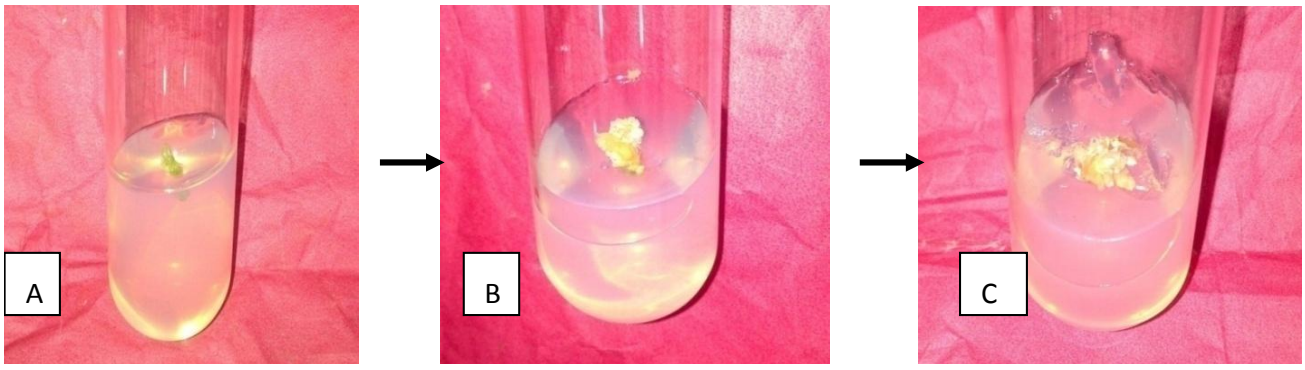


**Figure 4.** Callus induction from kinnow stem in 2, 4-D (1.0 mg/l) +BAP (0.5 mg/l)+NAA(0.25 mg/l). A = Initial stage; B = callus after 21 days; C = callus after 45 days.

## Callus induction from leaf and stem of kinnow mandarin and citron

In this experiment, callus induction was done from leaf and stem explants that were taken from 5 weeks old plantlets. After 4 to 6 weeks of inoculation high efficiency callus was produced (Figures 3, 4, 5 and 6). Altaf et al. (2009) reported that hormonal combination for good callus induction for seedling leaf of kinnow mandarin is BA+ GA (each at 1 mg/L) + 2,4-D at 0.5 mg/L + proline at

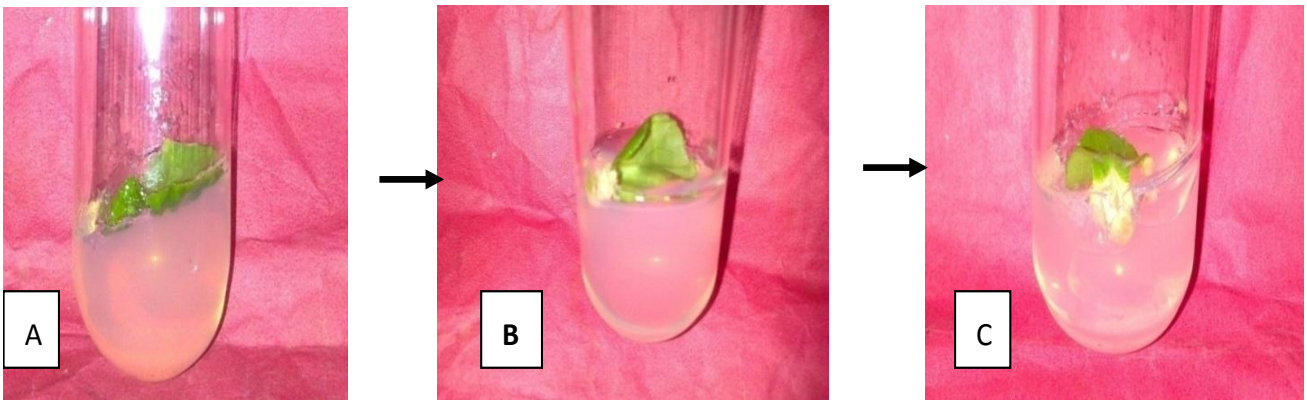


**Figure 5.** Callus induction from citron leaf in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 30 days; C = callus after 45 day.

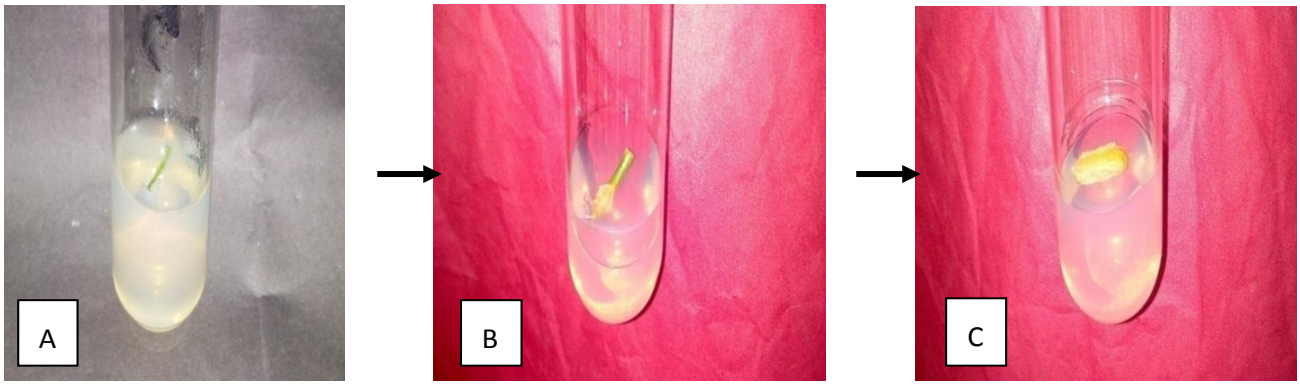


**Figure 6.** Callus induction from citron stem in 2,4-D (1.0 mg/l). A = Initial stage after inoculation; B = callus after 30 days; C = callus after 45 days.

**Table 2.** Effect of different hormonal combination for callus induction from leaf and stem explant of kinnow mandarin, after 45 days of observation (total of 20 replications for each combination).

| Explant for callus | Treatments (mg/L) | Average number of calli | Color of callus | Average callus type |
|---|---|---|---|---|
| Leaf | 2,4-D(1.0) | 7±2 | Yellowish whitish | Nodular compact |
| | 2,4-D(1.5) | 5±1 | Yellowish white | Nodular compact |
| | 2,4-D(2.0) | 3±1 | Whitish | Smooth compact |
| | 2,4-D(4.0) | 2±2 | Whitish | Smooth compact |
| | 2,4-D(1.0)+BAP(0.5)+NAA(0.25) | 2±1 | Whitish | Smooth compact |
| Stem | 2,4-D(1.0) | 3±2 | Yellowish white | Nodular compact |
| | 2,4-D(1.5) | 2±1 | Yellowish white | Nodular compact |
| | 2,4-D(2.0) | 5±1 | Yellowish white | Nodular compact |
| | 2,4-D(4.0) | 2±2 | Whitish | Nodular compact |
| | 2,4-D(1.0)+BAP(0.5)+NAA(0.25) | 6±2 | Yellowish white | Nodular compact |

5 mg/L. So, in our experiment callus induction has been done for both leaf and stem with a little adjustment of BAP and NAA along with 2,4-D and good callus performance has been found for stem rather than leaf. Approximately 90% of the callus was nodular compact, while 10% was smooth and compact in case of kinnow (Table 2). The color of the callus produced was whitish and yellowish white. The lower concentration of 2,4-D

**Table 3.** Effect of different hormonal combination for callus induction from leaf and stem explant of Citron after 45 days of observation (Total 20 replications for each combination).

| Explant for callus | Treatments (mg/L) | Average number of calli | Color of callus | Type |
|---|---|---|---|---|
| Leaf | 2,4-D(1.0) | 4 ±2 | Yellowish whitish | Nodular compact |
| | 2,4-D(1.5) | 3 ±1 | Yellowish white | Nodular compact |
| | 2,4-D(2.0) | 2 ±2 | Yellowish white | Smooth compact |
| | 2,4-D(4.0) | 2 ±1 | Whitish | Smooth compact |
| | 2,4-D(1.0)+BAP (0.5)+NAA (0.25) | 1 ±1 | Whitish | Smooth compact |
| Stem | 2,4-D(1.0) | N/A | Yellowish white | Smooth compact |
| | 2,4-D(1.5) | N/A | Yellowish white | Smooth compact |
| | 2,4-D(2.0) | N/A | Yellowish white | Smooth compact |
| | 2,4-D(4.0) | N/A | Whitish | Smooth compact |
| | 2,4-D(1.0)+BAP (0.5)+NAA (0.25) | N/A | Yellowish white | Smooth compact |

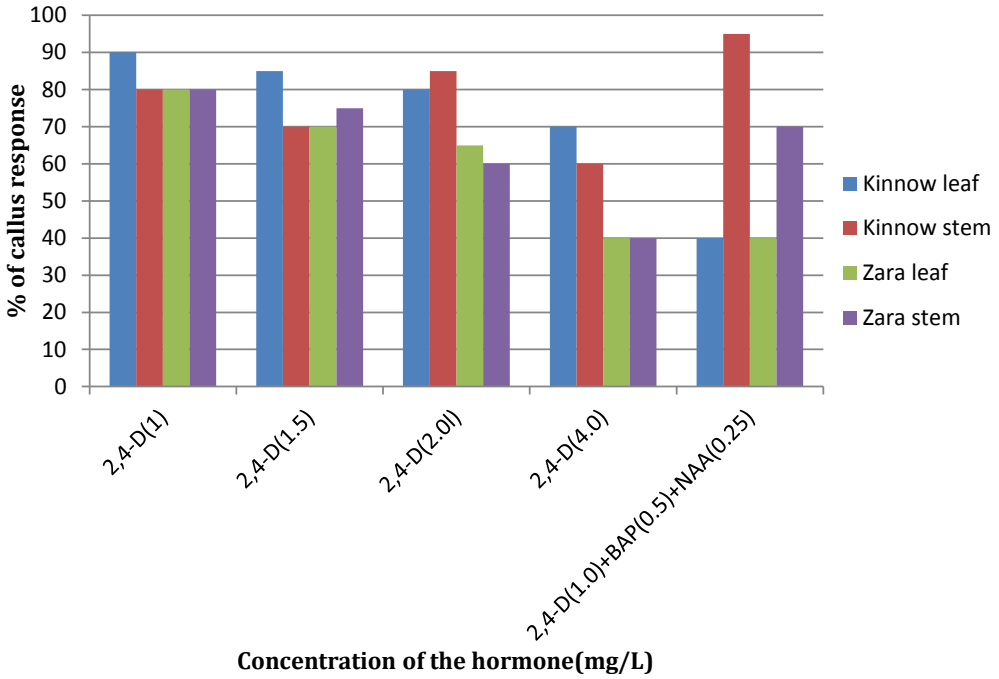


**Figure 7.** Effects of different hormonal combination in callus induction from leaf and stem of Kinnow mandarin and citron (callus response shown in percentage after 45 days of observation on a total 20 replication per hormonal combination).

(1 mg/L) was sufficient to induce callus in 96% of cultures from nodal segments whereas for leaf segments higher concentration of 2,4-D (4 mg/L) was to be used to achieve 98% callus induction (Savita et al., 2010). Different experiment shows that callus induction depended on explant type as well as concentration and type of plant growth regulator used, for example *Albizzia lebbek* (Lakshmana Rao and De, 1987); *Lonicera japonica* (Georges et al., 1993) and *Holarrhena antidysenterica* (Raha et al., 2003). In this experiment, approximately 40% of the callus of citron was nodular compact, while 60% was smooth and compact. The color of the callus produced was whitish and yellowish white (Table 3). For leaf, maximum callus were nodular and thus no of calli was countable and but for stem callus were smooth. By comparison with kinnow mandarin, hormonal combination of 2,4-D with BAP and NAA is less efficient for callus induction in citron (Figure 7).

In case of callus induction from leaf of kinnow mandarin 2,4-D (1.0 mg/L) shows (90%) best result and 2,4-D (1.0 mg/L)+BAP (0.5 mg/L)+NAA (0.25 mg/L) shows best (95%) for stem. In case of callus induction of Citron 2, 4-D (1.0 mg/L) shows (80%) best result for both leaf and stem. Thus, we can consider media with hormonal

combination of 2,4-D (1.0 mg/L) for efficient callus induction from leaf and stem of both citrus cultivar.

**REFERENCES**


Altaf N, Khan AR (2009). "*In vitro* culture of kinnow explants." Pak. J. Bot. 41(2):597-602.

Azim F, Rahmam MM, Prodhan HS, Shikdar US, Zobayer N, Ashrafuzzaman M (2011). "Development of efficient callus initiation of Malta (*Citrus sinensis*) through tissue culture." Int. J. Agri. Res. Innov. Tech. 1 (1 and 2):64-68. http://dx.doi.org/10.3329/ijarit.v1i1-2.13935.

Barlass M, Skene KGM (1982). *In vitro* plantlet formation from Citrus species and hybrids. Scientia Horticul. 17(4):333-341. doi: 10.1016/0304-4238(82)90114-5.

Bhatti A, Abbasi NA, Hafiz IA (2007). Response of explant sources of Kinnow mandarin towards regeneration. Proceedings "Prospects of Horticultural Industry in Pakistan", March 28-30, 2007. University of Agriculture, Faisalabad, Pakistan. (Ed.): pp. 362.

Chaturvedi HC, Mitra G (1974). Clonal propagation of Citrus from somatic callus cultures. Hort. Sci. 9:118-120.

Duran Vila N, Ortega V, Navarro L (1989). Morphogenesis and tissue cultures of three Citrus species. Plant Cell Tiss. Org. Cult. 16:123-133. DOI: 10.1007/BF00036520.

Edriss MH, Burger DW (1984). *In vitro* propagation of troyer citrange from epicotyls segments. Scientiat Hort. 23:159-162.

Gill MIS, Singh Z, Dhillon BS, Gosal SS (1994). Somatic embryogenesis and plantlet regeneration on calluses derived from seedling explant of Kinnow mandarin (Citrus nobilis x Citrus deliciosa). J. Hort. Sci. 69(2):231-236.

Grinblat H (1972). Differentiation of citrus stem *in vitro*. J. Am. Soc. Hort. Sci. 97:599-603.

Jaskani MJ, Khan IA, Khan MM (2005). Fruit set, seed development and embryo germination in interploid crosses of Citrus. Scientia Horticul. 107(1): 51-57. DOI: 10.1016/j.scienta.2005.06.011.

Jeong BR, Fujiwara K, Kozai T (1995). Environmental control and photoautotrophic micropropagation. Horticultural Review (ed. Janick, J.), 17:125-172.John Wiley and Sons, Inc.

Johnston JC (1968). Growing seedlings from seed. In Propagation of citrus (eds.), Opitz K.W., Plate R.G., Frolich E.F., pp. 1-6. Division of Agricultural Sciences, University of California, California.

Jumin HB, Nito N (1995). Embryogenic protoplast cultures of orange Jessamine (*Murraya paniculata*) and their regeneration on plant flowering *in vitro*. Plant Cell Tiss. Org. Cult. 41:227-279. DOI: 10.1007/BF00045092.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures, Physiol. Plant. 15:473-497. DOI: 10.1111/j.1399-3054.1962.tb08052.x.

Praveen S, Pawar V, Ahlawat YS (2003). Somatic embryogenesis and plant regeneration in Kinnow mandarin. J. Plant Biochem. Biotech. 12: 163-165. DOI: 10.1007/BF03263180.

Savita V, Virk GS, Avinash N (2010). Effect of Explant Type and Different Plant Growth Regulators on Callus Induction and Plantlet Regeneration in Citrus jambhiri Lush. Environ. We Int. J. Sci. Tech. 5: 97-106

Singh B, Sharma S, Rani G, Virk GS, Zaidi AA, Nagpal A (2006). *In vitro* flowering in embryogenic cultures of Kinnow mandarin (*Citrus nobilis Lour x C. deliciosa Tenora*). Afr, J. Biotech. 5: 1470-1474. DOI: 10.5897/AJB06.245.