In Vitro Clonal Propagation of *Citrus grandis* L.

Han Su Yin Thet[1], Ohn Maung[2]

Abstract

Micropropagation of *Citrus grandis* L. was carried out in the tissue culture laboratory, Vegetable and Fruit Research and Development Center. The best quality pummelo fruits were collected from healthy mother plants of VFRDC. Aseptic seeds were cultured on Murashige and Skoog medium. The vigorous pummelo shoots were cultured on Ms medium supplemented with BAP and Kinetin. Optimum shoot formation was obtained from MS medium supplemented with 1 mg L^{-1} BAP and 1 mgL^{-1} IAA, the highest shoot multiplication and shoot elongation from 1 mg L^{-1} BAP and the maximum number of roots and the longest of root length from 1 mg L^{-1} Kinetin and 1 mg L^{-1} NAA. Regenerated plants with strong and healthy roots were successfully transferred to hardening.

Keywords: Micropropagation, shoot length, number of roots, plant growth regulators

Introduction

Citrus grandis L. belongs to family *Rutaceae* and a genus at about 16 species of evergreen aromatic shrub and trees mostly with thorny branches. The synonym of *Citrus grandis* L. is Citrus maxima. It is grown in many tropical countries, particularly in Southeast Asia and temperate regions of the world. The largest Citrus in the world, the pummelo can reach 12" in diameter. Skin is yellow with white or pinkish colored flesh. The pummelo grows best in warmer climates with lots of rainfall. The pulp segments are either pallid or pink and shell out easily from the thick rind. Pummelo contains vitamins, mineral, potassium, crucial trace mineral like iron and source of vitamin C. Which support for Hepatoprotective, anticancer, antiplatelet, antidiabetic and strengthen immune system and digestive health. Plant tissue culture is the technique of growing plant cell tissue and organs in a culture prepared nutrient medium solid or liquid under aseptic condition. The process of micropropagation can be divided into four stages; (1) Initiation stage (2) multiplication stage (3) rooting state (4) acclimatization stage. Plant growth regulators (auxins and cytokinin) which have different regulatory effects on growth and development in whole plant.

Materials and Methods

Collection of seeds

The specimens used in this research were collected during the fruiting periods extending from VFRDC. The experiments were carried out in the tissue culture laboratory from VFRDC.

Surface sterilization

Pummelo seeds were removed from fruit and washed with tap water and with 0.5 mgL^{-1} of homai fungicide and few amount of detergent solution for 30 mins. And then the seeds were socked in 150 mgL^{-1} of Rifampicin and 2 to 3 drop of soap solution for 10 mins and rinsed with sterile distilled water at tree times. Then it was dipped in 70 % ethanol and 2 to 3 drops of soap solution for 5 minutes and washed with sterile distilled water simply.

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Experiment (1) *In vitro* propagation of *Citrus grandis* L. by using seed culture

Murashige and Skoog supplemented with benzylaminopurine (BAP)(0.5, 1, 1.5 mgL^{-1}) and indol acetic acid (IAA) (1, 2 mgL^{-1}) for shoot formation. There are six treatments and each treatment with five replicates in this experiment. One seed was cultured in each culture vessel.

Figure 1. Preparation of seed

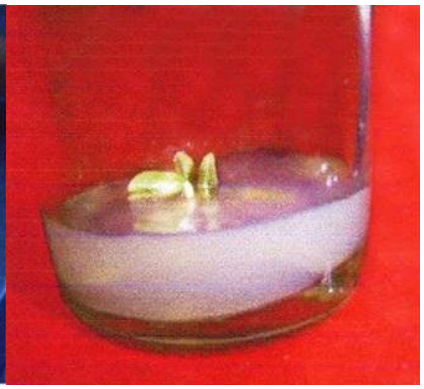

Figure 2. Seed culture in bottle

Experiment (2) Effect of plant grow regulator on shoot multiplication of *Citrus grandis* L. *in vitro*

After two months, pummelo nodes with leaves were cultured in different concentration of cytokinin (BAP and kinetin) for shoot multiplication. In this experiment, a completely randomized design was used, with seen treatments and each treatment contains 5 replications. Each replication consists of one node.

Treatments

T1 = MS only
T2 = MS + 0.5 mgL^{-1} BAP
T3 = MS + 1 mgL^{-1} BAP
T4 = MS + 2 mgL^{-1} BAP
T5 = MS + 0.5 mgL^{-1} Kinetin
T6 = MS + 1 mgL^{-1} Kinetin
T7 = MS + 2 mgL^{-1} Kinetin

Experiment(3) Effect of plant growth regulators on roots formation from plantlet of *Citrus grandis* L.

The plantlets cultured on ½ MS medium which containing different concentration of kinetic and NAA for roots formation. Each treatment has five replications and each replication was cultured on one plantlets.

Treatments

T1 = ½ MS + Kinetin (0.5) + NAA (0.5) mgL^{-1}
T2 = ½ MS + Kinetin (0.5) + NAA (1) mgL^{-1}
T3 = ½ MS + Kinetin (0.5) + NAA (1.5) mgL^{-1}
T4 = ½ MS + Kinetin (1) + NAA (0.5) mgL^{-1}
T5 = ½ MS + Kinetin (1) + NAA (1) mgL^{-1}
T6 = ½ MS + Kinetin (1) + NAA (1.5) mgL^{-1}

Inoculation and incubation condition

These inoculated bottles were adjusted to pH 5.2 with 1NNaOH or HCL before autoclaving at 121 ˚C and 1.05 kg cm^{-2} for 20 minutes. The temperature of incubation room (28± 2℃), light intensity (1000 _ 1200 lux) and relative humidity (30-50%).

Data collection and statistical analysis

All experiments were set up in complete randomized design for shoot formation, shoot multiplication and roots formation. Data were recorded in every week. Collected data were calculated to statistical analysis according to IRRISTAT software.

Results

Experiment (1) *In Vitro* propagation of *Citrus grandis* L. by using seed culture

The cut of seeds were cultured in MS medium supplemented with IAA (1, 2)mgL^{-1}and BAP (0.5, 1, 1.5)mgL^{-1}. After one week culture, radical starts formation was found in all treatments. Shoot formation was started within 4 weeks after culture. The highest shoot formation was found BAP 1mgL^{-1} + IAA 1mgL^{-1} after two months culture.

Table (1) Effects of different concentration and combination of BAP with IAA for shoot formation

Treatments	No. of shoots per seed	Mean shoot length (cm)
T0 MS	1	2.3
T1 MS + BAP(0.5) + IAA (1)	1	4.8
T2 MS + BAP(0.5) + IAA (2)	2	4.3
T3 MS + BAP(1) + IAA (1)	5	6.1
T4 MS + BAP(1) + IAA (2)	2	2.4
T5 MS + BAP(1.5) + IAA (1)	3	3.5
T6 MS + BAP(1.5) + IAA (2)	1	3.1
CV %	21.6	4.3
F- test	**	**
5% LSD	0.59	0.22

**=highly significant,

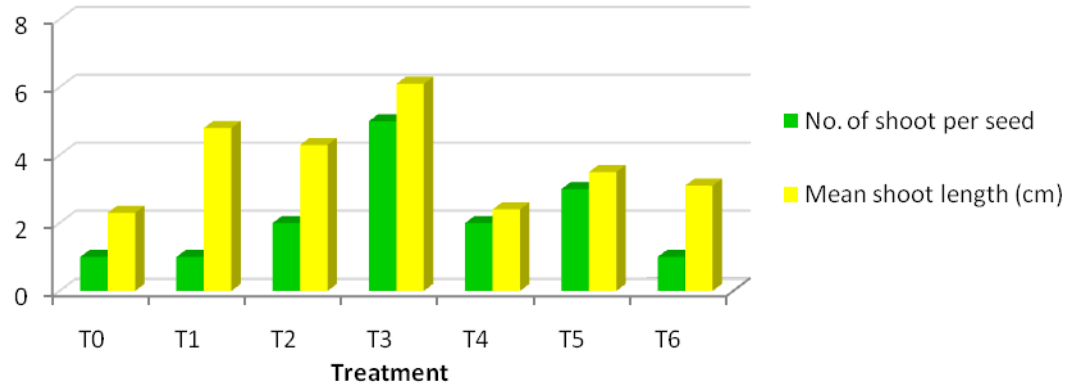

Figure 1. Effects of different concentration of BAP and IAA for shoot formation

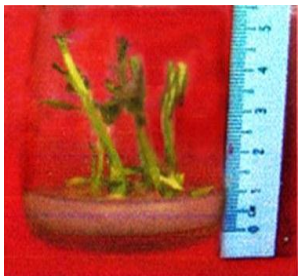

Figure 3. Highest shoot formationfrom MS and BAP(1) and IAA

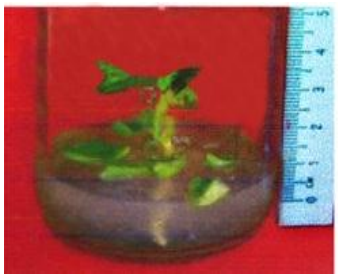

Figure 4.Lowest shoot formationfrom MS only

Experiment (2) Effect of plant growth regulator on shoot multiplication of *Citrus grandis* L. *in Vitro*

Nodal explants, about 0.3 mm were cultured on MS only and MS medium supplemented with (0.5, 1, 2) mgL^{-1} BAP and (0.5, 1, 2)mgL^{-1} Kinetin for shoot multiplication. Among them, the concentration of MS and BAP (1 mgL^{-1}) was the most effecting for providing numbers of shoots and shoot length during two months.

Table (2) Mean value of number of shoots and shoots elongation in plant grow regulator

Treatments	Mean number of shoots	Mean shoot length (cm)
T1 control	1	1.3
T2 MS + BAP(0.5) mgL^{-1}	2	1.6
T3 MS + BAP(1) mgL^{-1}	6	3.4
T4 MS + BAP(2) mgL^{-1}	3	1.4
T5 MS + Kinetin (0.5) mgL^{-1}	2	1.7
T6MS + Kinetin (1) mgL^{-1}	3	1.37
T7MS + Kinetin (2) mgL^{-1}	2	1.54
CV %	15.3	19.6
F- test	**	ns
5% LSD	1.03	0.38

**=highly significant, ns = non-significant

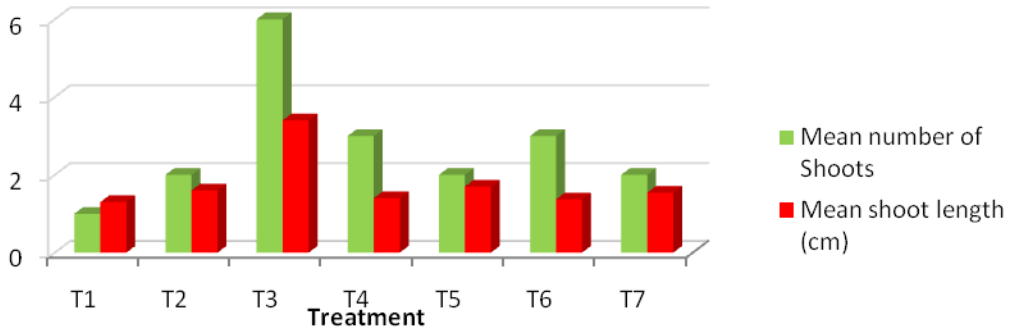

Figure 2. Mean value of number of shoots and shoots elongation in plant grow

Figure 5. Initiation stage

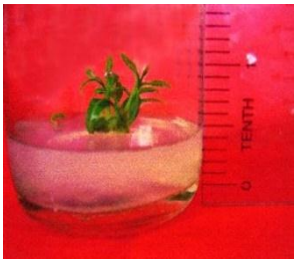

Figure 6. Highest shoot formation from MS and BAP(1) mgL^{-1}

Experiment (3) Effect of plant growth regulators on root formation from plantlets of*Citrus grandis* L

The explants of *Citrus grandis* L. were cultured to produce rooting in ½ MS medium within combination of Kinetin and NAA at different concentration. The roots were initiated from the explants within 30 days. Among treatments, the highest number of root (4.3) and the longest length of roots (3.34 cm) were observed in (1 mgL^{-1}) Kinetin and (1 mgL^{-1}) NAA within two months.

Table (3) Media components root formation of *Citrus grandis* L.

Treatments	Mean number of root	Mean root length (cm)
T1 K (0.5) + NAA (0.5)	0	0
T2 K (0.5) + NAA (1)	0	0
T3 K (0.5) + NAA (1.5)	0	0
T4 K (1) + NAA (0.5)	1	2.3
T5 K (1) + NAA (1)	4.3	3.34
T6K (1) + NAA (1.5)	0	0
CV %	1.3	31.2
F- test	**	**
5% LSD	0.118	0.368

**= highly significant

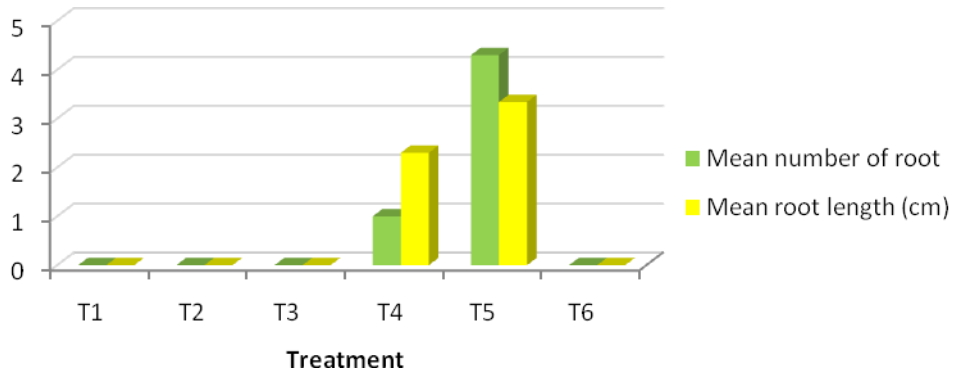

Figure 3. Media components root formation of *Citrus grandis* L.

Figure 7. Highest no. of rootfromKinetin(1) and NAA (1)

Figure 8. Second no. of root from Kinetin(1) and

Hardening and Maintenance

After eight months, the rooted of pummelo plantlets (longer than 2 cm) *in Vitro* were transferred in plastic bags which contain sand, rise husk and compost (1:1:2). Before transferring to the plastic bags, the plantlets were washed with water to remove the agar. Then, they are dipped in the solution (5 mlL^{-1} fertistep and 2-3 drops atonic and 0.5 gL^{-1}homai fungicide) to reduce the transpiration shock and free from disease attached. When these plantlets planted in plastic bags, these were covered with clear plastic bags. The harden pummelo plantlets were placed under the normal temperature 35 ˚C and humidity 60%. After two weeks, 70% the healthy and vigorous pummelo plantlets can be found in plastic bags.

Figure 9. The plantlets were transferred to the plastic bags for hardening stage

The pummelo aseptic seeds were cultured in the different concentrations and combination of the plant growth regulators and MS medium. The cut of pummelo seeds sere sensitively changed originally white into the greenish color within 2 weeks culture. Similarly, radical starts formation was found in all treatments. Shoot formation was started within 4 weeks after culture. The highest shoots formation was observed from BAP 1 mgL^{-1} + IAA 1 mgL^{-1} after four months culture. Begum *et al* (2003) found that shoots formation from seeds was remarkably influence by type and concentration of auxin and cytokinin used Earle and Langhans (1973) revealed that the high levels of both hormones caused the best shoot formation.BAP (1 mgL-1) and IAA (1 mgL-1) was more effective on producing and more improve quality shoots than other combination of treatments. (0.3 mm) shoots were cultured on different concentration of BAP and Kinetin for shoot multiplication for eight weeks. After two weeks, many shoots produced of the cutting edges. The concentration of BAP (1 mgL^{-1}) was the most effecting for providing numbers of shoots and shoot length. Therefore, BAP 1mgL^{-1} is the best for shoot multiplication of *Citrus grandis* L. cultured on medium. The similar results has been obtained by Weliton Artonio Bastos de Almeida (2002) who stated that BAP concentration above 1 mgL^{-1} caused on

entagenic effect with the higher BAP concentration causing the lowest number of shoots per explant. Pathasarathy (2002) said that cytokinin enhance axillary shoots, which help prolific shoot multiplication *in vitro*.

Rooting was induced from the base of shoots in half MS media containing the different concentration of kinetin and NAA. Among treatments, NAA (1 mgL^{-1}) + Kinetin (1 mgL^{-1}) give the best result with the highest number of roots per explant (4.3) and the longest length of roots (3.34 cm) for rooting. Similar results were also obtained by Chin et.al, (1990) and Begum *et.al*, (2003) in their experiment where they used ½ MS + Kinetin (1) + NAA (1) mgL^{-1} for root induction in pummelo. So the combination of Kinetin and NAA were tested for rooting in *Citrus grandis* L.After two months the rooting culture, the plantlets of *Citrus grandis* L. were hardened in plastic bag. The harden pummelo plantlets were placed under the green house. After two weeks, 70% the vigorous pummelo plantlets can be found in plastic bags.

Acknowledgements

I wish to express my deepest gratitude to Dr. Aye Pe, Professor and Head, Department of Botany, University of Yangon, for his permission. I am thankful to Vegetable and Fruit Research and Development Center (VFRDC) for allow me to use the departmental facilities to do this research work. I am thankful to Department of Botany, Dagon University and Chonbuk National University Korea for active participation me to present my research paper in 3th Myanmar-Korea Conference on "Useful Plant".

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