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Optimization of Sucrose, Plant Hormones and Photoperiod for in vitro Propagation of Lemon (c. limon) and Macrophylla (c. macrophylla) using Shoot Tip

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Abstract
Although citrus is an important fruit in Ethiopia, its production is limited by different factors like shortage of planting material and disease transmission. This study was conducted to investigate the combined effect of growth regulators and sucrose concentration for *in vitro* propagation of citrus using shoot tip. Seeds were extracted from mature fruits and cultured on hormone free MS media in the light and dark condition for initiation. Initiated shoots were cultured in MS medium supplemented with different concentrations of BAP and Sucrose. The treatments arranged in 2x4x4 factorial experiment laid out in CRD with three replications. Macro-shoots were then transferred onto 1/2 MS medium supplemented with different combination of NAA (0, 1, 2, 3, 4, 5 mg/l) for rooting. Statistical analysis revealed that there was highly significantly difference ($P<0.01$) among all treatments in shoot initiation, shoot multiplication and rooting experiments. Hundred percent shoot initiation was obtained in light condition for lemon. Maximum number of shoots per explant (10 ±0.55) and (7 ±0.0) was obtained from MS medium containing 2.5 mg/l BAP combined with 30 g/l sucrose for lemon and macrophylla respectively. The highest number of roots per shoot (20.3±0.6) obtained at 1 mg/l NAA for lemon. Among the rooted plantlets used for acclimatization, 65% of them survived. According to the result obtained in this study, shoot initiation in light condition, MS medium supplemented with 2.5 mg/l BAP combined with 30 g/l sucrose for shoot multiplication and 1/2 MS medium supplemented with 1 mg/l NAA for rooting were recommended for *in vitro* propagation of lemon and macrophylla.
Keywords/Phrases: BAP, NAA, germination, shoot multiplication, rooting

1. Introduction

Citrus is a common term and genus (*Citrus*) of flowering plants in the family, Rutaceae. All citrus species have 2n = 18, with very similar karyotypic morphology and size, although a single seedling of *C. sinensis* cv. Berna was triploid (Guerra *et al.* 1997). Total production of *Citrus* on the global level were 131 million tons of fruit harvested over 8.7 million hectares and are primarily utilized for juice making and fresh fruit consumption (Faostat 2013). Citrus (*Citrus* spp.) is one of the most economically important fruit crops grown by small holders and commercial farmers in Ethiopia (Seifu 2003; Tessega *et al.* 2006). The total area coverage and the annual production of citrus were estimated at 6,950 ha and 77,087 tons, respectively (Faostat 2013). In Ethiopia, citrus production has expanded with privates and government farms. Upper Awash Agro-industry is the largest enterprise, which produces different types of citrus.

Citrus fruits are considered as the number one fruits of the world due to their high nutritional value, great production potential and preparation of large number of fruit products from them. Citrus species are cultivated in most tropical and subtropical regions of the world. They are very attractive due to their distinctive fruits, colors, and attractive smell, unique from other plants. Containing high amounts of vitamin C, they can be consumed raw or extracted for production of highly nutritious beverages. Citrus species can also be used as traditional medicine, whereby the smell of citrus leaves and fruits can overcome headache and nausea (Azim *et al.* 2013). They are widely used to prevent flu and colds and support the immune system (Dhanawade *et al.* 2011). Citrus fruits also used for patients susceptible to health problems such as gastritis, fever and arterial sclerosis. The juice of lemon used in the pharmaceutical industry since it contains a high quantity of citric acid and essential oils (Bansode and Chavan 2012). There are also reports about positive effects of lemon fruits against cancer of gastrointestinal and upper respiratory tracts (Foschi *et al.* 2010).

Citrus can be propagated by both sexual and asexual methods; generally, rootstocks are propagated through seeds, while most of the commercial varieties are propagated through various asexual methods. Conventional methods for citrus propagation are based on bud wood selection and grafting for scion varieties. Rooted cuttings, or more frequently nucellar seed propagation is used for preparation of rootstocks (Bajaj 1988). The importance of the citrus industry and the continuous introduction of new improved genotypes emphasize the use of modern methods used to rapidly propagate new and promising plant material (Gmitter Jr and Moore 1986).

Conventional vegetative propagation of citrus plant is time consuming and mainly dependent on season and availability of plant material which restricts the faster adoption and replacement of new varieties (Rathore *et al.* 2007). Currently, in Ethiopia citrus production is declining (quality and quantity) due to different factors.

Disease is one of the serious problems on citrus production in Ethiopia. For example, *Pseudocercospora* leaf and fruit spot disease of citrus (PLFS) which is identified in Jimma area (Yesuf 2007; Dagnew *et al.* 2014). Jimma University is now working to get disease resistant varieties of citrus and started nursery establishment for this purpose. In the conventional method, production of disease resistance planting material takes a long period.

Shortage of planting material is another serious problem in Ethiopia and it is the main issue of this research. Propagation of citrus trees is done mostly by bud grafting, i.e., the insertion of buds of a desired variety onto a stock grown from seed of another variety**.** Ethiopia planned to cover 86,875 ha of land by citrus in 2020 (EIA 2012). The planting material required to cover this area is 43,437,500 cuttings, which is difficult to meet this need by conventional methods of propagation. *In vitro* propagation is a technique that can solve such problems. It can produce planting material on a relatively large scale compared to conventional methods (Savita *et al.* 2010).

In vitro culture eliminates infections and is faster than conventional propagation methods (Savita *et al.* 2010). Tissue culture and micropropagation protocols have been described for a number of citrusspecies and explant sources (Bajaj 1988; Fatuma 2006; Sharma *et al.* 2009; Pérez-Tornero *et al.* 2010).

Siwach *et al*. (2012) studied i*n vitro* shoot regeneration on Kinnow mandarin (*Citrus reticulate* Blanco) through shoot tip explants obtained from *in vitro* germinated seedlings. The medium supplemented with 2.5 mg/L BAP and 30 g/L sucrose supported maximum shoot proliferation (2.45 shoots per explant).

Explants of *C.limon*were cultured on 16 different media supplemented with various combinations of plant growth regulators, both auxins and cytokinins, such as BAP, NAA, 2,4-D and kinetin. The best shoot induction was obtained when the leaf explants were cultured on Murashige & Tucker media supplemented with 3 mg/L BAP alone (Kasprzyk-Pawelec *et al.* 2015).

BAP is the most commonly used cytokinin in tissue culture for the genus citrus, but the optimum concentration for maximum proliferation varies among species. For instance, shoot tips of *C.mitis require* 4.44-µM BAP, *C.grand is* requires 1.8-µM BAP and *C. depressa, C. jambhiri*and *C. reshni*require 4.44-µM BAP for maximum shoot proliferation (Sharma *et al.* 2009).

Several researchers have used different genotypes and plant growth hormones for *in vitro* propagation of citrus but there is no enough information on such work done in Ethiopia. Therefore, the objective of *this study was to investigate the combined effect of growth regulators and sucrose concentration for in vitro propagation of two citrus* species (lemon *(C. limon)* and macrophylla *(C. macrophylla))*.

2. Materials and methods

2.1. Plant material and description

The genotypes used for this study were Lemon and Macrophylla. These genotypes were selected based on their high demand at the local market, less commercial production, high disease resistance and macrophylla is the best rootstock. Macrophylla was obtained from Upper Awash Horizon Plantation and that of Lemon from Jimma local market.

Lemons are belonging to the species *C. limon* (L.) Burm. f. They have ~lsuallya high acidity, although acidless cultivars also exist (Dugo and Di Giacomo 2003). It is grown outside of the subtropical areas were limes are rhe substitute. Main cultivars include 'Eureka', 'Lisbon', 'Verna' and 'Femminello'. For this experiment Verna type was used. Macropylla is a hybrid between the Citron and biasong (*C. micrantha*) (Khan 2007). It is used as a rootstock for other citrus and it also good initial production compared to other rootstocks (Broadbent *et al.* 1980). More vigorous growth has been observed by grafting the second time on to *C. macrophylla* instead of on to *C. aurantium (Khan 2007)*

2.2. Media preparation

MS basal medium (Murashige and Skoog 1962) was used for shoot initiation, shoot multiplication and ½ MS medium was used for rooting. After melting the prepared media by using hot plate magnetic stirrer, the medium was dispensed into each culture jar and autoclaved at 121^{o}C and 15 psi for 15 minutes.

2.3. Surface sterilization and preparation of explants

Seeds were extracted from matured fruits of macrophylla and lemon. The seeds were collected in autoclaved beaker and covered by paraffilm to protect from microorganisms contact and dried at room temperature for three days. The seeds were washed under tap water for 15 minutes to remove mucus and sugar present on the seed coat under aseptic condition. Then the seeds were soaked in sterilized double distilled water for 30 minutes. This was followed by peeling of seed coat under aseptic conditions in laminar airflow cabinet. Peeled seeds were immersed in 70% ethanol for 1 minute and rinsing three times with double distilled water. Subsequently, the seeds were surface sterilized with 50% sodium hypochlorite for 20 minute and thoroughly rinsed with sterilized double distilled water for three times to remove the traces of sodium hypochlorite .

2.4. culture condition

All cultures were kept under 16h/8h light and dark photoperiod at optimum photosynthetic flux provided by cool fluorescent lamps and maintained at 25 ± 2°C.

2.5. Experimental Design and Treatments

In this study, three different experiments were carried out using Completely Randomized Design (CRD) with three replications for shoot multiplication and for rooting experiments whereas six replications were used for the shoot initiation experiment.

Experiment 1: Effect of photoperiod on shoot initiation: The extracted seeds were cultured on MS medium which was prepared without growth hormones and kept under light (on the shelf with white fluorescent) and dark (dark room covered with black curtains) conditions. Numbers of seeds per petridish were five, with six replications. The treatments were dark and light.

Experiment 2: The combined effects of BAP, sucrose and genotypes on shoot multiplication: The shoots tips initiated at experiment one were separated and the edge were cut and then cultured on MS media supplemented with four different concentrations of BAP (0.0, 2.5, 5, 7.5 mg/L) and four different (0, 15, 30, 45 g/L) concentrations of sucrose. The treatments arranged in a 2x4x4 factorial arrangement in Completely Randomized Design (CRD); for genotypes, concentrations of BAP and concentrations of sucrose respectively with three replications.

Experiment 3: **The effect of NAA on rooting of micro-shoots:** In this experiment, ½ MS basal medium with different concentrations of NAA (0.0, 1.0, 2.0, 3.0, 4.0, 5.0 mg/L) were used. The treatments were arranged in a 2x6 factorial experiment laid in Completely Randomize Design (CRD); for genotypes and NAA concentration levels respectively with three replications.

Acclimatization: Eighty plantlets with well-developed shoots and roots were taken out of the culture jars and washed with tap water in order to remove any adhering medium and soaked with anti-fungal to prevent fungus infection. The plantlets were planted into plastic pots containing autoclaved garden soil mixed with compost in the ratio of 1:1. Hardening of potted plantlets was accomplished in culture room set at 26±2 °C, 16h light by covering with plastic cover to maintain high humidity. In the first week of transfer, plantlets were watered with autoclaved water regularly and after that watering frequency was reduced. The plastic cover was removed initially for a short duration 30 minutes daily for about one week. After 15 days gradually, the daily exposure time was increased by 30 min for each day. Plastic cover was completely removed after 20 days. After one month, plantlets were transferred into greenhouse.

2.6. Data collection and Analysis

Percentage and date of shoot initiation data were recorded in the 1st experiment. The data collected in 2nd experiment were shoot number, shoot length and leaf number. Din the 3rd experiment root number and root length were collected. Survive plantlets percentage was recorded during acclimatization. The collected data were analyzed using SAS (Version 9.2) software (Institute Inc, 2008). The collected data were subjected to two way and three way analysis of variance (ANOVA) according to the CRD model for factorial experiments and means were compared using Duncan's Multiple Range Tests (DMRT) at 1%.

3. Results and discussion

3.1. Effect of photoperiod on shoot initiation

The analysis of variance indicated that the effect of photoperiod was highly significantly different ($P<0.01$) on initiation date and percentage. Seeds cultured in complete dark conditions showed 10.0±11.1 initiation at the fourth day for lemon, whereas lemon seeds cultured under light condition showed 53.33±20.6 initiation at the third day (Figure 1). For macrophylla, seeds cultured under complete dark condition showed 10.0±11.1 initiation at fifth day whereas macrophylla seeds cultured under light condition showed 43.33±11.05 at the third day. At the seventh day, shoot initiation percentage was higher 100±0.0 for lemon than macrophylla 90±16.7 under light conditions.

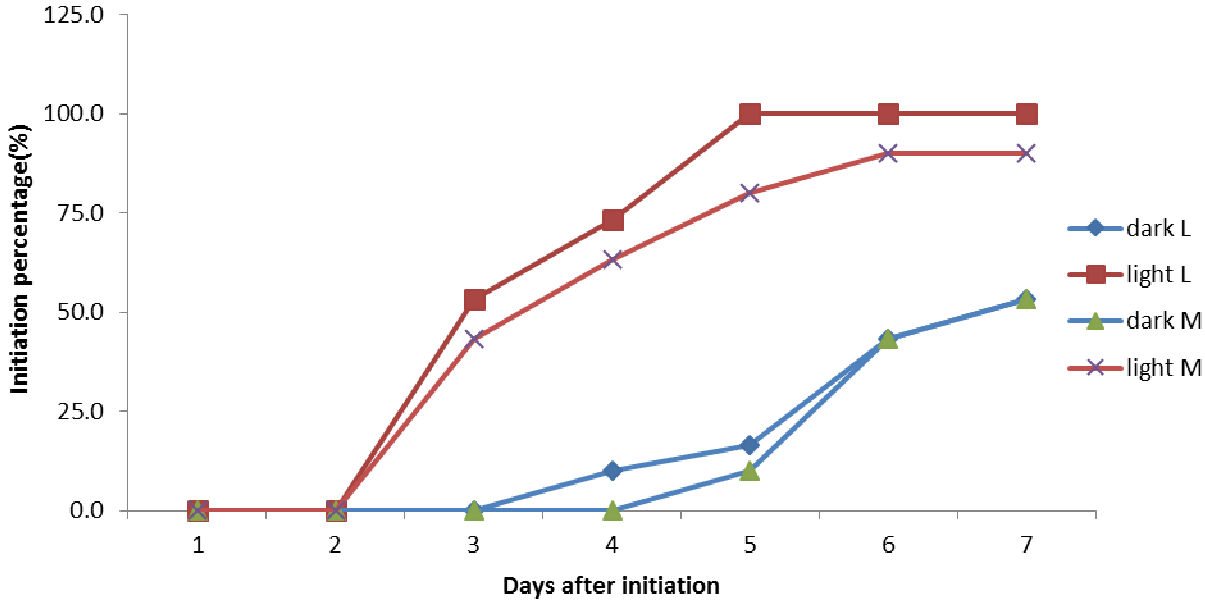

Figure 1: Effects Photoperiod on *in vitro* shoot initiation of lemon and Macrophylla.
Dark L= lemon cultured under dark condition; Light L=lemon cultured under light condition; Dark M=macrophylla cultured under dark condition; Light M=macrophylla cultured under light condition.

The initiation percentage was the same for both genotypes at complete dark condition 53.33±10.32 at the seventh day but a little different on the light condition for Lemon 100±0.0 and 90±16.7 for macrophylla in the same day.

Initiation started on the third day for both genotypes under light condition but it was delayed to an average of 4 and 5 days for lemon and macrophylla in the dark condition, respectively. This is in agreement with Kasprzyk-Pawelec *et al.* (2015), who found the best result in seed cultured in a chamber with a 16/8 photoperiod (16 hours of light, 8 hours of darkness, 170 µ molm/s^2 light intensity) and also with Sharma *et al,* (Sharma *et al.* 2009) who reported under 16 h photoperiod culture conditions compared to dark condition. This is perhaps because citrus species need warm conditions than cold environment. Contrary to Siwach *et al.(2012)* who found 93.33 ± 4.63 seed germination from seeds cultured under complete dark condition with a mean of 10.23 days and 83.33 ± 6.92 seed germination from seeds cultured under a photoperiod of 16 hours with mean of 16.48 days . This difference is may be because of the seed regeneration media they have used. The media were MS media supplemented with different concentrations of KN, GA_3 and IBA. Citrus seeds have been reported to germinate at a range of temperatures (11-35°C) but 30°C is recommended as a best temperature, with germination usually completed by 4-7 weeks (Soetisna *et al.* 1985).

3.2. The combined effect of BAP, sucrose and genotype on *in vitro* shoot multiplication

The analysis of variance indicated that the interaction effects of BAP, sucrose and genotype was highly significantly different ($P<0.01$) for shoot number, shoot length and leaf number . The best results for shoot number 10.0±0.0 and shoot length 8.0±0.1 were obtained at 2.5 mg/L BAP combined with 30 g/L sucrose for lemon (Table 1). The minimum shoot number obtained1.0±0.0 for macrophylla at hormone free media. When the BAP and sucrose level increased above 2.5 mg/L and 30 g/L respectively, the shoot number and shoot length were decreased in both genotypes. In the same way, a decrease in BAP and sucrose from these levels (2.5 mg/L & 30 g/L) showed a decrease in shoot number and shoot length in both genotypes.

Increasing the concentration level of sucrose from 0 g/L to 45 g /L, keeping BAP at 0.0 mg/L has increased shoot number, shoot length and leaf number. At sugar levels (0, 15, 30, 45 g/L), shoot number has increased from 2.67±0.57 to 6.0±0.0 for lemon. The same sugar levels (0, 15, 30, 45 g/L) increased shoot length from 3.96±0.05 to 5.3±0.0 for lemon. For leaf number, these sugar levels (0, 15, 30, 45 g/L) increased it from 5.8±0.15 to 16.8±0.25 for lemon. For macrophylla, similar levels of sugar increased shoot number from 1.0±00 to 4.0±0.0. Similarly, these sugar levels increased shoot length from 1.9±0.06 to 2.6±0.0. Leaf number also increased from 2.4±0.17 to 8.67±0.57 with the same levels of sugar (0, 15, 30, 45 g/L).

Table1: The Effect of BAP, Sucrose and Genotype on i*n vitro* shoot multiplication.

	BAP	Sucrose	Shoot No. ±SD	Shoot length ±SD	Leaf No. ±SD
Lemon	0	0	2.6^{i} ±0.6	3.96^{h}±0.1	5.8 ^{g} ±0.15
	0	15	3.0^{ih}±0.0	4.9^{g} ±0.1	9.2^{d}±1.2
	0	30	4.0 ^{g} ±0.0	5.2 ^{f} ±0.0	11.3^{c} ±0.35
	0	45	6.0 ^{e} ±0.0	5.3 ^{f} ±0.0	16.8 ^{a} ±0.2
	2.5	0	6.0^{e} ±0.0	5.9^{e}±0.15	1.7^{onp} ±0.1
	2.5	15	7.0^{d} ±0.0	7.5 ^{b} ±0.0	2.0^{moln} ± 0.1
	2.5	30	10.0^{a} ±0.0	8.0 ^{a} ±0.1	2.5^{mjlik} ±0.1
	2.5	45	9.0^{b} ±0.0	7.0 ^{c} ±0.1	12.6 ^{b}±0.2
	5	0	8.0 ^{c} ±0.0	6.8 ^{c} ±0.0	4.7 ^{h} ±0.0
	5	15	8.0 ^{c} ±0.0	6.5 ^{d} ±0.0	5.9 ^{g} ± 0.1
	5	30	7.0 ^{d} ±0.0	5.06^{gf} ±0.1	6.4 ^{g} ±0.1
	5	45	4.0 ^{g} ±0.0	4.0^{g} ±0.1	7.6^{f}±0.1
	7.5	0	7.0^{d} ±0.0	3.6 ^{i} ±0.0	5.96 ^{g} ±0.1
	7.5	15	6.0^{e} ±0.0	5.3 ^{f} ±0.0	8.1^{ef} ±0.0
	7.5	30	6.0^{e} ±0.0	6.9^{c} ±0.0	8.8^{ed} ±0.1
	7.5	45	3.0^{ih} ±0.0	2.4 ^{nm}±0.0	8.2^{ef} ±0.1
Macrophylla	0	0	1.0^{k}±0.0	1.9^{qp} ±0.1	2.4 ^{mjlnk}±0.2
	0	15	3.0^{ih} ±0.1	2.5 ^{lm} ±0.0	2.9^{jik} ±0.2
	0	30	3.0^{ih} ±0.0	2.53 ^{lm}±0.4	4.1 ^{h} ±0.2
	0	45	4.0 ^{g} ±0.0	2.6^{lkm} ±0.0	8.7^{ed} ±0.6
	2.5	0	4.0 ^{g} ±0.0	1.3 ^{t}±0.0	2.96^{jik} ±0.12
	2.5	15	4.0^{g}±0.0	1.5^{srt} ±0.0	1.3 ^{op} ±0.1
	2.5	30	7.0 ^{d} ±0.0	3.03 ^{j}±0.1	2.2^{mlnk} ± 0.1
	2.5	45	6.0 ^{e} ±0.0	2.8 ^{k} ±0.0	2.3^{mlnk} ±0.05
	5	0	5.0^{f} ±0.0	2.0 ^{op} ±0.1	0.93^{p} ±0.1
	5	15	4.0 ^{g} ±0.0	2.2 ^{on} ±0.0	2.2^{mlnk} ±0.6
	5	30	4.0 ^{g} ±0.0	2.7^{lk} ±0.0	3.3 ^{i} ±0.0
	5	45	3.0^{ih} ±0.0	1.6^{sr} ±0.0	1.83^{mon} ±0.2
	7.5	0	2.0 ^{j} ±0.0	1.9^{qp}±0.1	1.8^{mon} ± 0.3
	7.5	15	3.3 ^{h} ±0.6	1.7^{qr} ±0.0	2.2^{mlnk} ±0.2
	7.5	30	3.0^{ih} ±0.0	1.4st ±0.0	2.7^{jlik} ±0.0
	7.5	45	2.0 ^{j} ±0.0	1.0 ^{u} ±0.0	3.13^{ji} ±0.3

Means followed by different alphabets denote significant differences within column at 1%.

When the BAP level increased from 0 mg/L to 2.5 mg/L, 5 mg/L the shoot number increased from 2.67±0.5, 6.0±0.0 and 8.0±0.0 respectively but it showed a decrease at 7.5 mg/L with the mean value of 7.0 ±0.0 for lemon. With the same BAP levels, the shoot number for macrophylla increased from 1.0±0.0, 4.0±0.0 and 5.0±0.0 respectively but it showed a decrease at 7.5 mg/L with the mean value of 2.0±0.0 for macrophylla. This result is in agreement with (Fatima 2006) who reported similar results with BAP at different concentrations for the proliferation of citrus shoots and indicated that shoot induction from citrus cultivars was directly proportional to the increase in BAP levels, although the highest concentrations reduced shoot induction.

The shoot length shows continuous increase from 5.93±0.15 to 8.0±0.1 in lemon and in macrophylla from 1.3±0.0 to 3.03±0.06 at BAP 2.5 mg/L and sucrose levels of 0, 15 and 30 g/L respectively. The longest shoot length 8.0±0.1 found at 2.5 mg/L BAP and 30 g/L sucrose for lemon and minimum shoot length 1.0±0.0 was recorded at BAP 7.5 mg/L and sucrose at 45 g/L for macrophylla.

The reduction in shoot length at high concentration of BAP might be due to the toxic effects of ethylene, produced at high cytokine in concentration. This result is similar with Thomas and Blakesley (1987) who reported that the production of ethylene by the excessive cytokinins application causing the inhibition of internode elongation and regeneration of tobacco disc. Although cytokinins are necessary to stimulate cell division and promote shoot proliferation, cautions, needed as high levels of cytokinin may inhibit elongation, increasing number of short shoots (Kadota and Niimi 2003).

The leaf number showed maximum 16.75±0.25 at BAP 0 mg/L and sucrose at 45g/L for lemon and for macrophylla. This is in agreement with Eng *et al*., (Eng *et al.* 2014), who found highest mean number of leaves (5.41 leaves) with medium devoid of BAP on *kinnow* mandarin. At a low concentration of BAP (0.25 mg/L), more leaves were recorded.

The number of shoots or buds obtained per explant has been found to vary with the genotype (Carimi and De Pasquale 2003). In the current study, maximum shoot number 10.0±0.0 observed for lemon seeds were cultured on MS supplemented 2.5 mg/L BAP combined with sucrose 30 g/L (Figure 2). This is similar to that of Perez-Torner*oet al*., (Pérez-Tornero *et al.* 2010) who found similar results in three variables; shoot number, shoot length and leaf number in macrophylla and the highest percentage of shoot multiplication was obtained when shoots were cultured on MS media supplemented with 2.0 mg/L of BAP and 30 g/L sucrose in musambi

and lemon. The shoot number and leaf length also showed the maximum number on lemon.

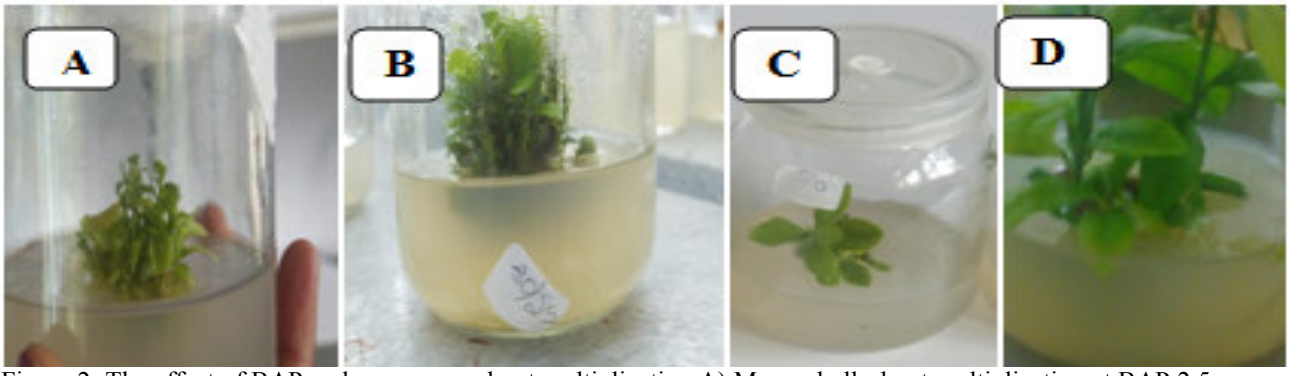

Figure 2: The effect of BAP and sucrose on shoot multiplication A) Macrophyllashoot multiplication at BAP 2.5 mg/L and 30 g/L sucrose after 45 days B) Lemon shoot multiplication at BAP 2.5 mg/L and 30 g/L sucrose after 45 days C) Macrophylla shoot multiplication at BAP 0 mg/L and sucrose 0 g/L after 45 days D) Lemon shoot multiplication at BAP 0 mg/L and sucrose 0g/L after 45 days.

The reduction in number of shoots at concentrations above 2.5 mg/L of BAP may be due to the inhibitory effect of higher concentration of BAP. These results are in agreement with those of Kotsias and Roussos (2001); these authors observed that in explants of lemon seedlings the greatest shoot length was obtained with 2 mg/L.

According to Moreira-Dias *et al.* (2001), the BAP level is variety dependent that BAP at 1.0 mg/L was the best concentration for Natal, Valencia and Hamlin, with averages of 1.59, 1.76 and 2.43 shoots per explant, respectively. For Rangpur lime, 3.0 mg /L of BAP gave the best result. Troyer citrange gave an average of 10.4 shoots per explant, from which only 7.2 developed into plantlets. Each of these authors used a different combination of cultivar, explant type and induction medium.

3.3. The effect of NAA on *in vitro* rooting of micro-shoots

The analysis of variance indicated the interaction effects of NAA and genotypes were significantly different ($P<0.01$) for root number and root length. The highest mean root numbers per shoot for lemon and macrophylla were 20.3±0.57 and 3.7±0.57 respectively and the highest mean root length were 8.3±0.15 and 4.23±0.32 for lemon and macrophylla respectively obtained at 1 mg/L NAA (Table 2). The second highest mean root numbers 13.7±0.57 and 3.13±0.2 and the root length 4.9±0.1 and 4.9±0.1 for lemon and macrophylla respectively were obtained at 2 mg/L NAA.

Increasing NAA from 0 mg/L to 1 mg/L showed a significant increase in the number of roots per shoot from 1.0±0.0 to 20.3±0.57 in lemon (Figure 3) and from 1.0±0.0 to 3.66±0.57 in macrophylla and mean root length from 3.66±0.57 to 8.3±0.15 in lemon and 1.2±0.15 to 4.23±0.32 in macrophylla. Increasing NAA concentration from 1 mg/L to 2 mg/L showed a significant decrease in the number of roots per shoot from 20.3±0.57 to 13.7±0.57 in lemon and from 3.66±0.57 to 3.13±0.23 in macrophylla and mean root length from 8.3±0.15 to 8.3±0.15 in lemon and from 4.23±0.32 to 3.26±0.25 in macrophylla.

Table 2: The effect of NAA and genotype on rooting of lemon and macrophylla.

Genotype	NAA(mg/l)	Root number ±SD	Root length ±SD
Lemon	0.0	1.0^{e}±0.0	3.0^{k}±0.1
	1.0	20.3^{a}±0.57	8.3^{a}±0.15
	2.0	13.7^{b}±0.57	4.9^{b}±0.1
	3.0	13.0^{b}±1.0	4.5^{cb}±0.5
	4.0	7.3^{c}±0.58	3.4^{d}±0.1
	5.0	1.0^{e}±0.0	1.4^{g}±0.1
Macrophylla	0.0	1.0^{d}±0.0	1.23^{c}±0.15
	1.0	3.7^{d}±0.58	4.23^{d}±0.32
	2.0	3.13^{ed}±0.23	3.26^{fg}±0.25
	3.0	2.83^{d}±0.28	2.23^{feg}±0.2
	4.0	2.13^{d}±0.23	2.03^{fe}±0.1
	5.0	1.0^{e}±0.0	1.7^{e}±0.15
Means		5.8	3.3
CV(%)		7.85	6.58

Means followed by different alphabets denote significant differences within column at 1%.

Generally, the root parameters showed continuous decreasing when NAA level increased beyond 1 mg/L. This is may be due to the inhibitory effects of high concentration of auxins. These results agree with those of Bordónet *al.* (2000), who obtained maximum 2.5 roots per explant, in a medium with 1 mg/L NAA. Kim *et al. (2002)* reported that MS media supplemented with 1.5 mg /L NAA was most effective for root induction in Yooza mandarin. From the two genotypes, Lemon showed the highest performance compared with macrophylla. In all higher concentration of auxins, lower root number and root length were recorded. In this study, the optimum concentration was found to be 1 mg/L NAA. Mukhtar *et al*., (2005) reported that the results regarding root formation percentage under different concentrations of NAA revealed that highest rooting percentage in musambi was obtained at concentration of 1.5 mg /L NAA.

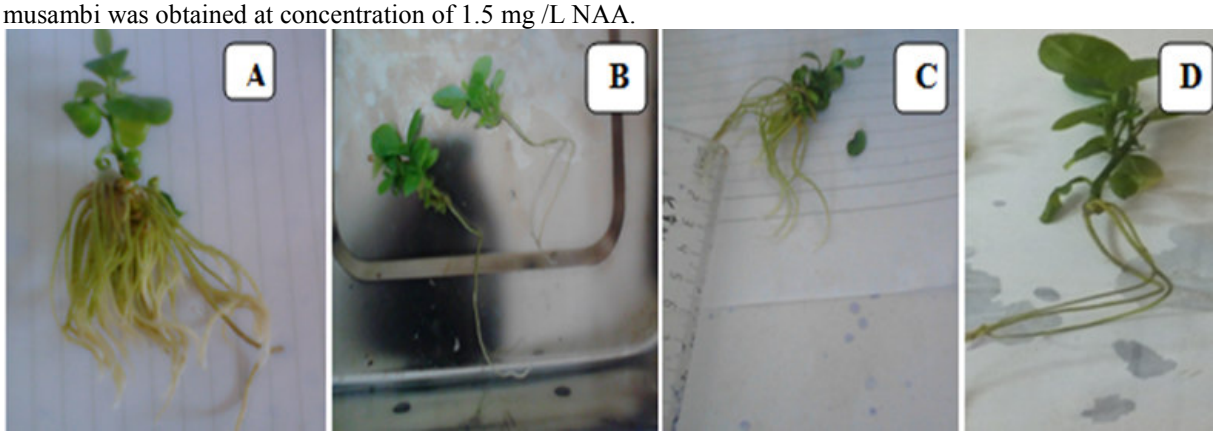

Figure 3: The effect of NAA on *in vitro* rooting A) Lemon rooting at NAA 1 mg/L after 30 days B) Lemon rooting at hormone free media after 30 days C) Lemon rooting at NAA 2 mg/l after 30 days D) Macrophylla rooting at hormone free media after 30 days.

3.4. Acclimatization

Plantlets or *in vitro* grown seedlings were transferred from the jars into pots. Among the acclimatized plantlets, 65% survived which is similar to the results of Singh *et al.(1994)* who found 60 % success with plants transplanted in potting mixture of garden soil and kept in humid chamber initially for three weeks. It is known that plants suffer from a high rate of water loss immediately after transplanting due to the high size of intercellular spaces, the slowness of stomatal response to water stress and the poor connection between the adventitious roots and the vascular system of stem. Acclimatization of plantlets or the seedlings for three weeks under plastic pots was an essential prerequisite for successful transfer from jars to pots. During this time, the plantlets or seedlings undergo morphological and physiological adaptations enabling them to develop sufficient water control.

4. Conclusion and recommendation

In shoot multiplication experiment plant growth regulators, sucrose and genotypes were highly significantly affected for shoot multiplication rooting. Light condition was found to be optimal for shoot initiation. Shoot initiation under light condition was started at the third day for both lemon and macrophylla. However, under dark condition shoot initiation was started on the fourth and fifth day for both lemon and macrophylla respectively under dark condition. Shoot initiation percentage was higher 100±0.0 for lemon and 90±16.73 for macrophylla under light conditions at the seventh day as compared to 53.33±10.32 for lemon and the same 53.33±10.32 for macrophylla under dark condition at the seventh day.

BAP at a concentration of 2.5 mg/L combined with 30 g/L sucrose was found to be optimal concentration for shoot multiplication. NAA at a concentration of 1 mg/L found to be optimal for rooting. Both maximum shoot number and shoot length were recorded at 2.5 mg/L BAP combined with 30 g/L sucrose and the maximum leaf number was recorded at 0 mg/L BAP combined with 45 g/L sucrose for lemon. The maximum root number (20.3±0.57) and mean root length (8.3±0.15) was recorded in the concentration of 1 mg/L NAA. Increasing NAA concentration from 0 mg/L to 1 mg/L shows a dramatic change in both root number and root length.

Hence, shoot initiation under light condition could be recommended for shoot initiation. MS medium supplemented with 2.5 mg/L BAP combined 30 g/L sucrose could be recommended for shoot multiplication and ½ MS medium supplemented with 1 mg/L NAA could be recommended for *in vitro* rooting of lemon and macrophylla.

As the future line of work, further protocol optimization may be required for mass propagation of citrus as this study is limited to two genotypes. Half MS media supplemented with NAA between 0 mg/L to 1 mg/L may be better to be tried for best rooting and also sucrose concentration for leaf number needs further study to

get the optimum concentration level.

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