

PRODUCTION OF SALT-TOLERANT SOUR ORANGE PLANTS (*Citrus aurantium* L.) BY USING TISSUE CULTURE TECHNIQUE:

IV-MICROPROPAGATION OF SALT-TOLERANT PLANTLETS

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ABSTRACT

This study was conducted through 1996-1999 years in the Department of Pomology, Faculty of Agriculture, Alexandria University. This work involved the development of an efficient protocol for *in vitro* propagation of salt tolerant plantlets of sour orange from shoot tips excised from the fully grown embryoids (obtained by somatic embryogenesis).

The main results can be summarized in the following points:

- 1-On the MS (1962) medium containing 1mg l^{-1} BAP, 0.5mg l^{-1} kinetin and 0.5mg l^{-1} NAA, the percentage of cultures with multiple shoots was significantly the highest (80%). The average number of proliferated shoots was significantly the highest (6.798). The average length of proliferated shoot was significantly the highest (2.64 cm).
- 2- The MS (1962) medium containing 0.25mg l^{-1} BAP + 0.5mg l^{-1} NAA + 0.5mg l^{-1} IBA resulted significantly the highest rooting percentage of proliferated shoots (80.00%). Also, on the same medium, the average number of roots per proliferated shoot was significantly the highest (4.84). The average length of root per proliferated shoot was significantly the highest (1.6cm).
- 3- Eighty percent of the salt – tolerant plants of sour orange were successfully transplanted in soil. These plants survived, grew vigorously and showed no damage at 10g l^{-1} NaCl compared to the control plants (derived from NaCl sensitive callus) which showed severe leaf burn, progressive wilting and decreased growth at 2g l^{-1} NaCl.

INTRODUCTION

Citrus is ranking superior over the top list of fruit crops in Egypt. The total area devoted for *Citrus* cultivation reached 336822 feddans producing about 2150256 tons of fruits according to the statistics of the Ministry of Agriculture, Cairo, 1998.

It is well known that salinity of irrigation water is the main problem that face the agriculture sector, since it reduces the crop of many species. This conclusion is documented by Maas and Hoffman (1977). Reduction of plant growth under salt stress is usually attributed to osmotic stress due to a lowering of external water potential (Maas and Nieman, 1978), or to specific ions effect on metabolic processes in the cell (Greenway and Munns, 1980). The two effects are not mutually exclusive. Thus, ion regulation and osmoregulation are subjects of intensive research into possible mechanisms of salt tolerance (Maas and Nieman, 1978).

Citrus plants are among the most sensitive fruit crops to salinity (Furr *et al.*, 1963). Therefore, it is so important to develop plants that can grow and thrive well under variable salt levels of irrigation water (Ben-Hayyim and Kochba, 1983). Concerning this point, Soost and Roose (1996) stated that

Citrus rootstocks, adapted to difficult soil situations, such as high salt levels, are needed.

Objectives of the present study

1. Developing of an efficient and reliable protocol for micropropagation of salt-tolerant obtained plantlets of sour orange.
2. Transplanting of salt-tolerant plants of sour orange to soil.
3. Finally, production of uniformity salt-tolerant sour orange plants.

MATERIALS AND METHODS

The present investigation was carried out during four successive years (1996-1999), in the Department of Pomology, Faculty of Agriculture, Alexandria University, in order to study the possibility of using tissue culture technique for rapid and economical micropropagation of the regenerated plantlets of sour orange from salt-tolerant callus (by somatic embryogenesis).

The objective of this study was to determine the optimal culture conditions for *in vitro* plant regeneration of sour orange using shoot tip explants taken from *in vitro* obtained salt-tolerant plantlets (by embryogenesis). These experiments examined the effect of plant growth regulators at the multiplication and rooting stages.

1. Plant Material

Shoot tip explants were excised from 6 weeks-old sour orange plantlets derived from salt-tolerant callus (by somatic embryogenesis) under aseptic conditions.

2. In Vitro Shoot Multiplication

2.1 Culture Media

The effects of various combinations and concentrations of growth regulators on shoot multiplication from shoot tip explants of salt-tolerant plantlets (derived from the fully grown embryoids) of sour orange were tested.

Shoot tips (2 to 3 mm long) were excised from the fully grown embryoids under aseptic conditions and inoculated vertically into culture tubes (20 x 150 mm) containing 10 ml (each) of nutrient media, one shoot tip per culture tube. To study the effect of Benzylaminopurine (BAP) at 0.5 and 1 mg l⁻¹, Kinetin (KIN) at 0.5 and 1 mg l⁻¹, and α -naphthalene acetic acid (NAA) at 0.5 mg l⁻¹ on shoot proliferation, they were supplemented solely or in various combinations and concentrations in basal Murashige and Skoog (1962) (MS) medium containing 3% sucrose and 0.8% agar. The medium was distributed into culture test tubes and autoclaved at 121^o C for 20 min. The pH of the media was 5.7 before autoclaving. The proliferated shoots were transferred to fresh medium after four weeks for another subculture.

2.2 Culture Conditions

The cultures were maintained at $25 \pm 2^\circ\text{C}$, under fluorescent light on a 16/8hrs light/dark cycle, for one month.

2.3 Statistical Analysis

Percentages of cultures with multiple shoots (by shoot multiplication index), average number and length of new shoots proliferated from the shoot tip explant (shoots more than 5mm in length) after one subculture (4 weeks) were recorded. At least 15 explants of shoot tips were used per treatment. The shoot multiplication percentage and index calculated as follows:

$$\text{Shoot multiplication percentage} = \frac{\text{No. of cultured tubes with multipleshoots}}{\text{Total no. of cultured tubes}} \times 100$$

Shoot multiplication index = number of proliferated shoots per shoot tip explant: \geq two shoots

Each treatment was represented by five replicates with three explants per each replicate in a completely randomized design and the statistical procedures were applied according to Steel and Torrie (1980).

3. In Vitro Rooting of Proliferated Shoots

This experiment was carried out on salt-tolerant proliferated shoots of sour orange after one subculture (4 weeks).

3.1 Culture Media

Proliferated shoots longer than 8 mm were excised and transferred under aseptical conditions and inoculated vertically into culture tubes (20 X 150mm) containing 10ml (each) of Murashige and Skoog (1962) (MS) basal medium amended with 2% sucrose and 0.8% agar. The MS medium was used alone or supplemented with 0.25 mg l^{-1} BAP, 0.5 and 1 mg l^{-1} NAA, 0.5 and 1 mg l^{-1} IBA. One proliferated shoot (derived from shoot tip explants) cultured in culture tube. The pH of the media was 5.7 before autoclaving.

3.2 Culture Conditions

The cultures were maintained for one month in a 16/8-hrs light/dark cycle at $25 \pm 2^\circ \text{C}$ with fluorescent light.

3.3 Statistical Analysis

Percentages of rooted shoots (by rooting index), average number and length of roots per proliferated shoot were recorded after four weeks of culture. The rooting percentage and index calculated as follows:

$$\text{Rootingpercentage} = \frac{\text{No. of cultured tubes with rooted shoots}}{\text{Total no. of cultured tubes}} \times 100$$

Rooting index = number of the formed roots per proliferated shoot: \geq two roots.

Each treatment consisted of five replicates, three proliferated shoots (one shoot per tube) for each replicate (three test tubes) in a completely randomized design and the statistical procedures were applied according to Steel and Torrie (1980).

4. Transplanting of Salt Tolerant Plants To Soil

The obtained salt tolerant plantlets of sour orange were potted (when reaching 5 cm in height) in a sterilized mixture of 1vermiculite: 1 peatmoss for one month. Irrigation was carried out every four days with saline water (10gl^{-1} NaCl) with the addition of appropriate volume of nutrient $\frac{1}{2}$ MS medium without sucrose. The plantlets were then transferred to plastic pots containing a mixture of 1 sand : 1 soil and acclimatized rapidly for 3 months under intermittent mist in greenhouse. Observations on survival and growth were recorded.

RESULTS AND DISCUSSION

1. *In Vitro* Shoot Multiplication

1.1 Percentage of Cultures with Multiple Shoots

Data concerning the effect of various media formulations on the percentage of cultures with multiple shoots (proliferated from shoot tip cultures of salt tolerant plantlets derived from fully grown embryoids) of sour orange, are shown in Table (1).

The obtained results indicated that, the percentage of cultures with multiple shoots was significantly higher (80.00%) on the MS medium containing $1\text{mg}\text{l}^{-1}$ BAP, $0.5\text{mg}\text{l}^{-1}$ kinetin and $0.5\text{mg}\text{l}^{-1}$ NAA (M.2) than that of the other media. On the contrary, the percentage of cultures with multiple shoots was significantly lower (40.00%) on the MS medium containing $0.5\text{mg}\text{l}^{-1}$ BAP, $0.5\text{mg}\text{l}^{-1}$ kinetin and $0.5\text{mg}\text{l}^{-1}$ NAA (M.1) in comparison with the other media.

Keeping the kinetin concentration constant ($0.5\text{mg}\text{l}^{-1}$) and the NAA concentration constant ($0.5\text{mg}\text{l}^{-1}$) in the MS medium, as the BAP concentration was increased from $0.5\text{mg}\text{l}^{-1}$ to $1\text{mg}\text{l}^{-1}$, the percentage of cultures with multiple shoots was significantly increased from 40.00% to 80.00% (M.1 to M. 2).

On the other hand, keeping the kinetin concentration constant ($1\text{mg}\text{l}^{-1}$) and the NAA concentration constant ($0.5\text{mg}\text{l}^{-1}$) in the MS medium, as the BAP concentration was increased from $0.5\text{mg}\text{l}^{-1}$ to $1\text{mg}\text{l}^{-1}$, the percentage of cultures with multiple shoots was decreased from 53.33% to 46.67% (M.3 to M.4). Generally, no multiple shoots occurred (0.00%) on the MS medium without growth substances (M.0).

Table (1): Effect of five medium formulations^z on the percentage of cultures with multiple shoots (proliferated from shoot tip cultures of salt tolerant plantlets) of sour orange.

Medium code	Basal medium	(mg l ⁻¹)			% Cultures with multiple proliferated shoots ^y
		BAP	Kin	NAA	
M.0	MS	0.0	0.0	0.0	00.00 ^x C*
M.1	MS	0.5	0.5	0.5	40.00 B
M.2	MS	1.0	0.5	0.5	80.00 A
M.3	MS	0.5	1.0	0.5	53.33 B
M.4	MS	1.0	1.0	0.5	46.67 B
L.S.D. _{0.05}					20.629

^z There were 15 shoot tip explants (one per culture tube) per treatment (medium formulation).

^y Values refer to the percentage of explants that produced multiple shoots.

^x Zero values indicate absence of multiple shoots.

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

1.2 Average Number of Proliferated Shoots

The obtained results in Table (2) indicated that, the average number of proliferated shoots per explant (shoot tip) was significantly the highest (6.798) on the MS medium containing 1mg l⁻¹ BAP, 0.5mg l⁻¹ kinetin and 0.5mg l⁻¹ NAA (M.2). On the contrary, the average number of proliferated shoots was significantly the lowest (2.198) on the MS medium containing 0.5mg l⁻¹ BAP, 0.5mg l⁻¹ kinetin and 0.5mg l⁻¹ NAA (M.1).

Keeping the kinetin concentration constant (0.5mg l⁻¹) and the NAA concentration constant (0.5mg l⁻¹) in the MS medium, as the BAP concentration was increased from 0.5mg l⁻¹ to 1mg l⁻¹, the average number of proliferated shoots was significantly increased from 2.198 to 6.798 (M.1 to M.2).

Table (2): Effect of five medium formulations^z on the average number of proliferated shoots per shoot tip explant of salt tolerant plantlets of sour orange.

Medium code	Basal medium	(mg l ⁻¹)			Average number of proliferated shoots/ shoot tip explant
		BAP	Kin	NAA	
M.0	MS	0.0	0.0	0.0	0.000 D*
M.1	MS	0.5	0.5	0.5	2.198 C
M.2	MS	1.0	0.5	0.5	6.798 A
M.3	MS	0.5	1.0	0.5	3.396 B
M.4	MS	1.0	1.0	0.5	3.198 B
L.S.D. _{0.05}					0.483

^zThere were 15 shoot tip explants (one per culture tube) per treatment (medium formulation).

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

On the other hand, keeping the kinetin concentration constant (1mg^l⁻¹) and the NAA concentration constant (0.5mg^l⁻¹) in the MS medium, as the BAP concentration was increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the average number of proliferated shoots was decreased from 3.396 to 3.198 (M.3 to M.4). Generally, no proliferated shoots occurred on the MS medium without growth substances (M.0).

1.3 Average Length of Proliferated Shoot

The obtained results in Table (3) indicated that, the average length of proliferated shoot was significantly the highest (2.64cm) on the MS medium containing 1mg^l⁻¹ BAP, 0.5mg^l⁻¹ kinetin and 0.5mg^l⁻¹ NAA (M.2). On the contrary, the average length of proliferated shoot was significantly the lowest (1.04cm) on the MS medium containing 0.5mg^l⁻¹ BAP, 0.5mg^l⁻¹ kinetin and 0.5mg^l⁻¹ NAA (M.1).

Keeping the kinetin concentration constant (0.5mg^l⁻¹) and the NAA concentration constant (0.5mg^l⁻¹) in the MS medium, as the BAP concentration was increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the average length of proliferated shoot was significantly increased from 1.04 cm to 2.64 cm (M.1 to M.2).

On the other hand, keeping the kinetin concentration constant (1mg^l⁻¹) and the NAA concentration constant (0.5mg^l⁻¹) in the MS medium, as the BAP concentration was increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the average length of proliferated shoot was decreased from 1.20 cm to 1.06 cm (M.3 to M.4).

Table (3): Effect of five medium formulations² on the average length of proliferated shoot per shoot tip of salt tolerant plantlets of sour orange.

Medium code	Basal medium	(mg ^l ⁻¹)			Average length of proliferated shoot/ shoot tip (cm)
		BAP	Kin	NAA	
M.0	MS	0.0	0.0	0.0	0.000 C*
M.1	MS	0.5	0.5	0.5	1.040 B
M.2	MS	1.0	0.5	0.5	2.640 A
M.3	MS	0.5	1.0	0.5	1.200 B
M.4	MS	1.0	1.0	0.5	1.060 B
L.S.D. _{0.05}					0.176

²There were 15 shoot tip explants (one per culture tube) per treatment (medium formulation).

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

Generally, no proliferated shoots occurred on the MS medium without growth substances (M.0).

In conclusion, a combination of 1.0 mg^l⁻¹ BAP, 0.5 mg^l⁻¹ kinetin, and 0.5 mg^l⁻¹ NAA (M.2) gave the highest percentage of shoot proliferation in shoot tips of salt tolerant plantlets of sour orange (Table 1). After 4 weeks of culture, the average number of multiple shoots (proliferated shoots per shoot

tip explant), and average length of proliferated shoot were significantly higher in M.2 medium compared to other tested media (Tables 2 and 3).

The obtained results indicated that the combination of BAP, NAA, and kinetin appeared to be essential for multiple-shoot formation in shoot tip explants of salt tolerant plantlets of sour orange.

These results were in complete agreement with those reported by Singh *et al.*, (1994). They obtained the multiple shoots from shoot tips of *Citrus reticulata* Blanco cv. "khasi mandarin" and *C. limon* Burm. f. cv. "Assam lemon" when were cultured on the Murashige and Skoog (1962) medium, supplemented with 1.0 mg^l⁻¹ BAP, 0.5 mg^l⁻¹ kinetin, and 0.5 mg^l⁻¹ NAA.

On the other hand, these findings partially agreed with those obtained by Barlass and Skene (1982). Who reported that the development of adventitious buds from shoot apex of *C. reticulata* cv. Cleopatra mandarin was achieved in the MS medium with BAP (0.5 mg^l⁻¹). The same authors (1986) reported that, the media used for micropropagation of *Citrus* have largely involved the basal salts of Murashige and Skoog (1962) for shoot regeneration. They mentioned that an essential growth component for shoot regeneration of *Citrus in vitro* is cytokinin, benzyladenine. In the meantime, Starrantino and Caruso (1988) reported that the shoot proliferation in troyer, carrizo and trifoliolate oranges were obtained when shoot apices were cultured on the MS (1962) medium with growth regulators. They also mentioned that the best results for the citranges were produced with BA at 1 mg^l⁻¹, and IBA at 0.5 mg^l⁻¹; the best results for trifoliolate orange were produced with BA at 0.5 mg^l⁻¹, and IBA at 0.25 mg^l⁻¹. In addition, Nagao *et al.*, (1994) cultured the apical buds of *Poncirus trifoliata* on the MS (1962) medium supplemented with 1.0 mg^l⁻¹ BA and 0.1 mg^l⁻¹ NAA. They found that the best responses were obtained on medium supplemented with 30-45mg^l⁻¹ sucrose. In more recent experiments, Baruah *et al.*, (1996) found that the BAP was superior to kinetin for shoot proliferation (derived from shoot tips) in *C. assamensis*, *C. latipes* and *C. indica*. They reported that the best shoot proliferation was observed in the presence of BAP at 0.5mg^l⁻¹.

2. In Vitro Rooting of Proliferated Shoots

2.1 Rooting Percentage of Proliferated Shoots

Data concerning the effect of various media formulations on the rooting percentage of proliferated shoots (regenerated from shoot tip cultures of salt tolerant plantlets derived from fully grown embryoids) of sour orange, are shown in Table (4).

The obtained results indicated that, the rooting percentage of proliferated shoots was significantly the highest (80.00%) on the MS medium containing 0.25mg^l⁻¹ BAP, 0.5mg^l⁻¹NAA and 0.5mg^l⁻¹ IBA (R.M.1). On the contrary, the rooting percentage of proliferated shoots was significantly the lowest (53.33%) on the MS medium containing 0.25mg^l⁻¹ BAP, 1mg^l⁻¹ NAA and 1mg^l⁻¹ IBA (R.M.2).

Keeping the BAP concentration constant (0.25mg^l⁻¹) in the MS (1962) medium, as the NAA and IBA concentrations were increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the rooting percentage of proliferated shoots was significantly

decreased from 80.00% to 53.33% (R.M.1 to R.M.2). Generally, no rooted shoots occurred on the MS medium without growth substances (R.M.0).

In conclusion, the addition of plant growth regulators (BAP, NAA and IBA) to the basal MS (1962) medium is necessary for plant proliferation. However, in the MS medium lacking plant growth regulators, no proliferation occurred. The MS medium containing BAP (0.25 mg l⁻¹) + NAA (0.5 mg l⁻¹) + IBA (0.5 mg l⁻¹) resulted the highest rooting percentage of proliferated shoots (80.00 %).

Table (4): Effect of three medium formulations^z on the rooting percentage of proliferated shoots (derived from shoot tip cultures of salt tolerant plantlets) of sour orange.

Medium code	Basal medium	(mg l ⁻¹)			Proliferated shoots formed roots ^y (Rooting %)
		BAP	NAA	IBA	
R.M.0	MS	0.00	0.00	0.00	0.00 C*
R.M.1	MS	0.25	0.50	0.50	80.00 A
R.M.2	MS	0.25	1.00	1.00	53.33 B
L.S.D. _{0.05}					26.629

R.M.= Rooting medium.

^zThere were 15 proliferated shoot explants (one per culture tube) per treatment (medium formulation).

^y Values refer to the percentage of proliferated shoots that produced roots.

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

The MS medium lacking plant growth regulators, particularly auxines, failed to induce rooting in the *in vitro* proliferated shoots (Table 4). Supplementation of basal medium with BAP, NAA and IBA induced rooting. Likewise, the NAA/ IBA solely or in combination with another growth regulator, induced rooting in *C. aurantifolia* and *C. sinensis* (Raj Bhansali and Arya, 1978), *C. sinensis* and *C. limonia* (Barlass and Skene, 1982), and "Kinnow" mandarin (Gill *et al.*, 1994). The *in vitro* regenerated shoots rooted on the MS medium containing 0.25 mg l⁻¹ BAP, 0.5 mg l⁻¹ NAA + 0.5 mg l⁻¹ IBA gave the best plantlet survival *in vivo*. The latter may have been due to the greater root number per regenerated shoot on this medium. These results were in accordance with those reported by Singh *et al.*, (1994). They found that root induction on regenerated shoot (of *C. reticulata* and *C. limon*) was observed on the MS medium supplemented with 0.25mg l⁻¹ BAP, 0.5 mg l⁻¹ NAA and 0.5 mg l⁻¹ IBA.

2.2 Average Number of Roots

The obtained results in Table (5) indicated that, the average number of roots per proliferated shoot was significantly the highest (4.84) on the MS medium containing 0.25mg l⁻¹ BAP, 0.5mg l⁻¹ NAA and 0.5mg l⁻¹ IBA (R.M.1). On the contrary, the average number of roots per proliferated shoot was significantly the lowest (2.56) on the MS medium containing 0.25mg l⁻¹ BAP, 1mg l⁻¹ NAA and 1mg l⁻¹ IBA (R.M.2).

Keeping the BAP concentration constant (0.25mg^l⁻¹) in the MS medium, as the NAA and IBA concentrations were increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the average number of roots per proliferated shoot was significantly decreased from 4.84 to 2.56 (R.M.1 to R.M.2). Generally, no rooted shoots occurred on the MS medium without growth substances (R.M.0).

Table (5): Effect of three medium formulations² on the average number of roots per proliferated shoot (derived from shoot tip cultures of salt tolerant plantlets) of sour orange.

Medium code	Basal medium	(mg ^l ⁻¹)			Average number of roots/ proliferated shoot
		BAP	NAA	IBA	
R.M.0	MS	0.00	0.00	0.00	0.00 C*
R.M.1	MS	0.25	0.50	0.50	4.84 A
R.M.2	MS	0.25	1.00	1.00	2.56 B
L.S.D. _{0.05}					0.373

R.M.= Rooting medium.

²There were 15 proliferated shoot explants (one per culture tube) per treatment (medium formulation).

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

These results were in complete agreement with those reported by Gill *et al.* (1994). They found that the plantlet of "Kinnow" mandarin survival *in vitro* was directly related to the number of roots produced per regenerated shoot. They also indicated that the number of roots per shoot ranged from 1-2 on the MS medium containing 2 mg^l⁻¹ NAA alone or with 1mg^l⁻¹ IBA to 6-8 on half –strength MS medium with 0.5 mg^l⁻¹ NAA and 1mg^l⁻¹ IBA. In addition, Gill *et al.* (1995) reported that the plantlet of mandarin cv. "Local Sangtra" survival *in vitro* was directly proportional to the number of roots produced per shoot.

2.3 Average Length of Root

The obtained results in Table (6) indicated that, the average length of root per proliferated shoot was significantly the highest (1.6cm) on the MS medium containing 0.25mg^l⁻¹ BAP, 0.5mg^l⁻¹NAA and 0.5mg^l⁻¹ IBA (R.M.1). On the contrary, the average length of root per proliferated shoot was significantly the lowest (0.814cm) on the MS medium containing 0.25mg^l⁻¹ BAP, 1mg^l⁻¹ NAA and 1mg^l⁻¹ IBA (R.M.2).

Keeping the BAP concentration constant (0.25mg^l⁻¹) in the MS medium, as the NAA and IBA concentrations were increased from 0.5mg^l⁻¹ to 1mg^l⁻¹, the average length of root per proliferated shoot was significantly decreased from 1.6 cm to 0.814cm (R.M.1 to R.M.2). Generally, no rooted shoots occurred on the MS medium without growth substances (R.M.0).

Table (6): Effect of three medium formulations² on the average length of root per proliferated shoot (derived from shoot tip cultures of salt tolerant plantlets) of sour orange.

Medium code	Basal medium	(mg l ⁻¹)			Average length of root/ proliferated shoot (cm)
		BAP	NAA	IBA	
R.M.0	MS	0.00	0.00	0.00	0.000 C*
R.M.1	MS	0.25	0.50	0.50	1.600 A
R.M.2	MS	0.25	1.00	1.00	0.814 B
L.S.D. _{.0.05}					0.149

R.M.= Rooting medium.

²There were 15 proliferated shoot explants (one per culture tube) per treatment (medium formulation).

*Values followed by the same letters are not significantly different at the 0.05 level of probability.

Roots were formed and developed when excised single proliferated shoot (derived from shoot tip explant) was transferred to R.M.1 and R.M.2 media (Table 4). A combination of 0.50 mg l⁻¹ NAA, 0.50 mg l⁻¹ IBA, and 0.25 mg l⁻¹ BAP (R.M.1) was the best for root development in proliferated shoots derived from the shoot tip explant.

The average number and length of roots per proliferated shoot were found to be significantly the highest in R.M.1 medium code after 4 weeks of culture (Tables 5 and 6). Root formation usually occurred near the cut surface of the shoot explant's basal ends.

In conclusion, the addition of 0.5mg l⁻¹ NAA, 0.5mg l⁻¹ IBA and 0.25mg l⁻¹ BAP in the MS medium were effective in rooting formation of proliferated shoots derived from shoot tips than 1mg l⁻¹ NAA, 1mg l⁻¹ IBA and 0.25mg l⁻¹ BAP (Table 4).

These results were in complete agreement with those reported by Singh *et al.* (1994). They found that root induction of single shoot (derived from shoot tip culture) of *Citrus reticulata* Blanco cv. "Khasi mandarin" and *C. limon* Burm. f. cv. "Assam lemon" was observed on the MS medium, supplemented with (mg l⁻¹) 0.25 BAP, 0.5 NAA, and 0.5 IBA. These findings also agreed with those obtained by Barlass and Skene (1982). They reported that NAA at 10µM in the MS medium induced the best rooting of five *Citrus* rootstocks; carrizo citrange, (*Citrus sinensis*), trifoliolate orange, Cleopatra mandarin, Rangpur lime and Symon's sweet orange. Meanwhile, Starrantino and caruso (1988) reported that the rooting occurred when excised shoots of troyer, carrizo and trifoliolate orange were cultured on the MS (1962) medium without cytokinin or IBA, and with NAA at 1mg l⁻¹. Moreover, Pasqual and Ando (1989a,b) used IBA alone or combined with NAA and BA for rooting of *Poncirus trifoliata* and Valencia orange. The best results of rooting were obtained with 1.0mg l⁻¹ NAA plus 2.0 mg l⁻¹ IBA. In the meantime, Omura and Hidaka (1992) found that the MS (1962) medium containing 0.1µM NAA or 10 µM IBA resulted in good rooting and 90% of the plants (derived from shoot tip culture of satsuma cv. "Aoshima Unshiu" and Tangor cv. "Kiyomi") survived after potting and acclimatization under mist for one month. Moreover, Goh *et*

al., (1995) reported that roots developed when regenerated shoots of pummelo were excised and cultured on half –strength MS (1962) medium with 2.5 μM IBA. In addition, Tapati *et al.* (1995) working on *Citrus sinensis* var. “Mosambi”, found that “*in vitro*” regenerated shoots rooted in the MS (1962) half strength basal medium with NAA and IBA (each at 0.5 mg l^{-1}). However, Nagao *et al.*, (1996) found that the percentage of rooting and the number of roots on *P. trifoliata* regenerated shoots were the highest in the MS (1962) medium supplemented with 45.0 g l^{-1} sucrose and 5.0 mg l^{-1} IBA, respectively. In more recent experiments, Normah *et al.* (1997) reported that the rooting of regenerated shoots of *C. halimii* was the highest on the MS (1962) medium supplemented with 2.7 μM NAA.

On the other hand, the above mentioned results were in agreement with those obtained by Kitto and Young (1981), who reported that the proliferated shoot tips of carrizo citrange rooted when were cultured on the Murashige and Tucker’s medium (1969) containing 1 mg l^{-1} NAA and gelled with 0.5% agar. In addition, Nel (1987) reported that the root from shoot meristems of *in vitro*-grown seedlings of *Citrus aurantium* was developed after 2 months in half-strength Murashige and Tucker (1969) medium supplemented with NAA only.

3. Transplanting of Salt Tolerant Plants To Soil

The obtained salt -tolerant plants of sour orange were transferred to the greenhouse conditions after acclimatization for three months. These NaCl tolerant plants of sour orange survived and showed no damage and increased growth at 10 g l^{-1} NaCl compared to the control plants (regenerated from NaCl sensitive callus) which showed sever leaf burn, progressive wilting and decreased growth at 2.0 g l^{-1} NaCl. Finally, 80% of salt- tolerant plants of sour orange were successfully transplanted to soil.

These findings agreed with those obtained by Spiegel-Roy and Ben-Hayyim (1985), who investigated the effect of salt stress on rooted shoots from salt-tolerant selected callus cell lines of *Citrus* (*C. sinensis*) and (*C. aurantium*) after being subjected to culture medium containing up to 0.17 M NaCl for ten subcultures. They reported that 40% of the plants survived and showed no damage compared to the control plants (derived from unselected callus cell lines) which showed sever leaf burn and progressive wilting. In other work of Beloualy and Bouharmont (1992), plantlets of *Citrus* rootstock (*Poncirus trifoliata* cv. Pomeroy) showed a normal growth in the presence of NaCl at 10 g l^{-1} while those from unselected ones indicated decreased growth at 2.5 g l^{-1} NaCl. Furthermore, Omura and Hidaka (1992) found that 90% of the plants (derived from shoot tip culture of satsuma cv. “Aoshima Unshiu” and Tangor cv. “Kiyomi”) survived after potting and acclimatization under mist for one month. In addition, Bouharmont *et al.*, (1993) found that the tolerant callus cell lines of *Poncirus trifoliata* and citrange (*P. trifoliata* X *Citrus sinensis*) were selected and their salt tolerance was tested during several months. The growth of plants regenerated from these callus cell lines was improved and remained high with salt concentrations as high as 10 g l^{-1} , while that of unselected ones decreased at concentrations of 2.5 g l^{-1} . In the meantime, Gill *et al.*, (1995) reported that the *in vitro* - derived plantlets of

mandarin cv. "Local Sangtra" were successfully transplanted to soil. Another report by Tapati *et al.* (1995) stated that the plantlets of *Citrus sinensis* var. Mosambi were established in soil with survival rate of 60-70%. Recently, Normah *et al.* (1997) reported that the survival of regenerated plantlets of *C. halimii* was 83.3% after transfer to a soil mixture in the greenhouse.

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"إنتاج نباتات نارنج متحملة للملوحة باستخدام تقنية زراعة الأنسجة":

٤- الإكثار المعملى الدقيق للنباتات المتحملة للملوحة

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أجريت هذه الدراسة خلال الفترة من بداية عام ١٩٩٦ وحتى نهاية عام ١٩٩٩ بقسم الفاكهة كلية الزراعة جامعة الإسكندرية وكان الهدف من إجراء هذه الدراسة هو إيجاد طريقة فعالة للإكثار المعملى الدقيق (على بيئة صناعية) للنباتات المتحملة للملوحة وذلك باستخدام القيم النامية المفصولة من النباتات المتحصل عليها والناجمة من الأجنة الجسمية. ويمكن تلخيص النتائج الرئيسية لهذه الدراسة فى النقاط التالية:

- 1- على بيئة موراشيچ وسكوج لسنة ١٩٦٢ والمحتوية على ١ مليجرام فى اللتر بنزىل امينو بيورين + ٠,٥ مليجرام فى اللتر كينيتين + ٠,٥ مليجرام فى اللتر نفتالين حمض الخليك كانت النسبة المئوية للمزارع المحتوية على العديد من الأفرخ مرتفعة بصورة جوهريية (٨٠%) . وكان متوسط عدد الأفرخ المتكونة مرتفع بصورة جوهريية (٦,٧٩٨) . وكان متوسط طول الفرخ المتكون مرتفع بصورة جوهريية (٢,٦٤ سم) .
- 2- استخدام بيئة موراشيچ وسكوج لسنة ١٩٦٢ والمحتوية على ٠,٢٥ مليجرام فى اللتر بنزىل امينوبيورين + ٠,٥ مليجرام فى اللتر نفتالين حمض الخليك + ٠,٥ مليجرام فى اللتر إندول حمض البيوتريك أدى الى الحصول على أعلى نسبة تجذير للأفرخ الناتجة بصورة جوهريية (٨٠%) وعلى نفس البيئة تم الحصول على أعلى متوسط لعدد الجذور بالنسبة للفرخ الناتج بصورة جوهريية (٤,٨٤) . وتم الحصول أيضا على أعلى متوسط لطول الجذر بالنسبة للفرخ الناتج بصورة جوهريية (١,٦ سم) .
- 3- تم نقل ٨٠% من نباتات النارج المتحملة للملوحة وزراعتها فى التربة بنجاح. وهذه النباتات ظلت بصورة حية ونمت بقوة ولم يظهر عليها أى أعراض للضرر وذلك عند ربيها بماء يحتوى على تركيز ١٠ جرام فى اللتر كلوريد الصوديوم مقارنة بنباتات الكنترول (الناتجة من الكالس الحساس لكلوريد الصوديوم) والتي ظهر عليها احتراق للأوراق وزيادة فى ذبول الأوراق ونقص فى نمو النباتات بشكل عام وذلك عند ربيها بماء يحتوى على تركيز ٢ جرام فى اللتر كلوريد الصوديوم.