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

III- EMBRYOGENESIS INDUCTION FROM SALT-TOLERANT CALLUS

El-Gazzar, A.M.; M.B. El- Sabrout and N.S. Mustafa
Department of Pomology, Faculty of Agriculture, Alexandria University, Alexandria, Egypt.

This study was conducted through 1996-1999 years in the Department of Pomology, Faculty of Agriculture, Alexandria University. This work involved induction of somatic embryogenesis, from the salt-tolerant callus and subsequently, production of salt- tolerant plants of sour orange.

The main results can be summarized in the following points:

1- The MT (1969) medium containing 1500mgl^{-1} malt extract exhibited significantly embryogenesis percentage (60.00 %) higher than that obtained from the other tested media. The MT (1969) medium was generally more effective than MS (1962) medium in inducing somatic embryogenesis from salt-tolerant callus of sour orange.

2-The percentage of fully grown embryoids was significantly the highest (33.33%) on the MT (1969**)** medium containing 1mgl^{-1} GA_3.

INTRODUCTION

Citrus is one of the most important economic fruit crops in Egypt. The total *Citrus* cultivated area in ARE reached 336822 feddans producing 2150256 tons of fruits according to the statistics of the Ministry of Agriculture, Cairo, 1998.

Salinity is a major problem in irrigated agriculture and its role in reducing the yield of many crops is well documented (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 of great importance to obtain plants capable of growth under elevated salt levels in the irrigation water (Ben-Hayyim and Kochba, 1983).

Citrus rootstocks, adapted to difficult soil situations, such as high salt levels, are needed (Soost and Roose, 1996).

Objectives of the present study

1. Induction of somatic embryogenesis from the salt-tolerant callus of sour orange.
2. Subsequently, production of salt-tolerant plants of sour orange.

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 embryogenesis induction from salt-tolerant callus and subsequently, production of salt- tolerant plants of sour orange.

1. Embryoid Formation

1.1 Culture Media

The salt-tolerant callus of sour orange (*Citrus aurantium* L.) was used as a tissue source for induction of embryoids (somatic embryogenesis). This callus aseptically cultured on various media comprising either basal Murashige and Skoog (1962) (MS) or Murashige and Tucker (1969) (MT) salts supplemented with various concentrations of plant growth regulators including benzylaminopurine (BAP)at 1 mg l^{-1}, 5 mg l^{-1} and 10 mg l^{-1}, and α–naphthalene acetic acid (NAA) at 1 mgl^{-1}. The MS or MT medium also contained malt extract (500 mg l^{-1}, 1000 mg l^{-1} and 1500 mgl^{-1}) and Difco-Bacto-agar (8000 mgl^{-1}).

The pH of media was adjusted to 5.7 before adding agar. The media were dispensed in the culture test tubes (20X150mm) containing 10ml (each), closed with cotton and capped with aluminium foil, sterilized in an autoclave at 121°C for 20min, then left to cool and harden for 24hrs before being used.15 calluses (15 test tubes) were cultured (one callus per test tube) on each tested medium.

1.2 Culture Conditions

The cultures were incubated at 25±2°C, with 16hrs light from fluorescent lamps (2 lamps per shelf), followed by 8hrs dark periods. To maintain healthy cultures, they were subcultured every four weeks. The embryogenesis induction was evaluated after one subculture (4 weeks) by the use of embryogenesis percentage and index which calculated as follows:

$$\text{Embryogenesis } \% = \frac{\text{No. of cultured tubes with formated embryoids}}{\text{Total no. of cultured tubes}} \times 100$$

Embryogenesis index = number of the formated embryoids per callus: $\geq$ three embryoids.

1.3 Statistical Analysis

Each treatment consisted of 15 test tubes, where one callus cultured in test tube. Each three test tubes were considered a replicate. All the data of these experiments were arranged in a completely randomized design and the statistical procedures were applied according to Steel and Torrie (1980).

2. Embryoid Growth

2.1 Culture Media

Fifteen embryoids were cultured on each tested medium. The media were used: basal medium (Murashige and Skoog, 1962 and Murashige and Tucker, 1969) without growth substances or supplemented with 0.5 $mg1^{-1}$ and 1 $mg1^{-1}$ α-naphthalene acetic acid (NAA) or 0.5 $mg1^{-1}$ and 1 $mg1^{-1}$

gibberellic acid (GA_3). The aqueous solution of gibberellin was sterilized by filtration with Millipore Millex-HA (0.45μm) and subsequently added to the autoclaved medium.

The pH of media was adjusted to 5.7 before adding agar. The media were dispensed in the culture test tubes (20X150mm) containing 10ml (each), closed with cotton and capped with aluminium foil, sterilized in an autoclave at 121°C for 20min, then left to cool and harden for 24hrs before being used. Fifteen embryoids (15 test tubes) were cultured (one embryoid per test tube) on each tested medium.

2.2 Culture Conditions

The cultures were grown at 25 ± 2°C, with 16 hrs (photoperiod) light from fluorescent lamps (2 lamps per shelf), followed by 8hrs dark periods. The growth of embryoids were evaluated after four weeks (one subculture) by using the percentage of fully grown embryoids and embryoid growth index which calculated as follows:

$$\text{Percentage of fully grown embryoids} = \frac{\text{No. of cultured tubes with fully grown embryoids}}{\text{Total no. of cultured tubes}} \times 100$$

Embryoid growth index = length of fully grown embryoid : ≥ 3 cm.

2.3 Statistical Analysis

Each treatment consisted of 15 test tubes, where one embryoid cultured in test tube. Each three test tubes were considered a replicate. All the data of these experiments were arranged in a completely randomized design and the statistical procedures were applied according to Steel and Torrie (1980).

RESULTS AND DISCUSSION

1. Embryoid Formation

Data concerning the effect of various media formulations on the percentage of embryogenesis (% of calluses with embryoids) in salt- tolerant callus of sour orange, are shown in Table (1).

The obtained results indicated that, the percentage of embryogenesis was significantly the highest (60.00%) on the MT medium containing 1500 mgl^{-1} malt extract (E.M.20). On the contrary, the lowest percentage (26.67%) was resulted in MS medium containing 500 mgl^{-1} malt extract (E.M. 8).

As the malt extract concentration was increased from 500mgl^{-1} to 1500mgl^{-1} in the MT medium, the embryogenesis percentage was significantly increased from 46.67% to 60.00% (E.M. 18 to E.M. 20). However, as the malt extract concentration was increased from 500 mgl^{-1} to 1500 mgl^{-1} in the MS medium, the embryogenesis percentage was significantly increased from 26.67% to 40.00% (E.M.8 to E.M. 10).

Keeping the NAA concentration constant (1mgl^{-1}) in the MT medium, as the BAP concentration was increased from 1mgl^{-1} to 10 mgl^{-1}, the

embryogenesis percentage was significantly increased from 0.00% to 40.00% (E.M.15 to E.M.17).

Table (1): Effect of 20 medium formulations on the percentage of embryogenesis (% of calluses with embryoids) in salt-tolerant callus of sour orange.

Medium code	Basal medium	(mgl^{-1})			% Embryogenesis[z]
		BAP	NAA	Malt extract	
E.M.1	MS	0	0	0	0.00 F*
E.M.2	MS	1	0	0	0.00 F
E.M.3	MS	5	0	0	0.00 F
E.M.4	MS	10	0	0	0.00 F
E.M.5	MS	1	1	0	0.00 F
E.M.6	MS	5	1	0	0.00 F
E.M.7	MS	10	1	0	0.00 F
E.M.8	MS	0	0	500	26.67 E
E.M.9	MS	0	0	1000	33.33 DE
E.M.10	MS	0	0	1500	40.00 CD
E.M.11	MT	0	0	0	0.00 F
E.M.12	MT	1	0	0	0.00 F
E.M.13	MT	5	0	0	0.00 F
E.M.14	MT	10	0	0	0.00 F
E.M.15	MT	1	1	0	0.00 F
E.M.16	MT	5	1	0	33.33 DE
E.M.17	MT	10	1	0	40.00 CD
E.M.18	MT	0	0	500	46.67 BC
E.M.19	MT	0	0	1000	53.33 AB
E.M.20	MT	0	0	1500	60.00 A
L.S.D. 0.05					11.102

E.M. = Embryogenesis medium.

[z]There were 15 calluses (one callus 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 embryogenesis induction occurred (0.00%) on the other tested media (from E.M.1 to E.M.7 and from E.M.11 to E.M.14).

In other words, the medium code (E.M. 20) significantly exhibited higher somatic embryogenesis percentage (60.00 %) than that obtained from the other tested media. The MT medium produced most somatic embryogenesis.

Embryoids derived from salt – tolerant callus of sour orange (embryogenesis) could be induced at the percentage from 46.67% to 60.00% with 500 to 1500 mgl^{-1} malt extract.

The obtained somatic embryoids germinated like normal embryos showing both tap root and shoot. These observations were similar to those observed by Gill *et al.* (1995).

These findings were in complete agreement with those reported by Koc and Can (1992). They reported that the MT (1969) medium was

generally more effective than MS (1962) medium in inducing somatic embryogenesis of sour orange.

In our study, the levels of embryogenesis response presented in Table (1) were similar to those obtained by Vardi *et al.,* (1982) using malt extract (ME) at 1500mgl^{-1}.

In addition, Beloualy (1991) stated that the complete plants of sour orange (*C. aurantium*), were regenerated by somatic embryogenesis. Isolated embryoids were differentiated on the MT (1969) medium containing 1000 to 1500 mgl^{-1}malt extract.

These findings partially agreed with those obtained by Gill *et al.* (1991), who reported that the maximum somatic embryogenesis was found in "Local Sangtra" mandarin (86.6%) with callus subcultured on the MS (1962) medium and in "Kinnow" mandarin (40%) with callus subcultured on MT (1969) medium. In the same line, Oh *et al.* (1991) found that the somatic embryogenesis was induced in callus of *C. grandis* [*C. maxima*] cultured on MT (1969) medium supplemented with 0.01 mgl^{-1} 2,4-D, and was enhanced by addition of malt extract (up to >70 % at 700 mgl^{-1}).

On the other hand, these results disagreed with those reported by Piqueras and Hellin (1992). They selected the *Citrus limonum* [*C. limon*] callus on medium at several levels of NaCl. The addition of gibberellic acid enhanced embryogenesis in this callus. In the meantime, Gill *et al.,* (1995) reported that MS (1962) medium supplemented with NAA (10 mgl^{-1}) and kinetin (1 mgl^{-1}) were best for induction of somatic embryogenesis from callus cultures in mandarin cv. Local Sangtra.

2. Embryoid Growth

Data concerning the effect of various media formulations on the percentage of fully grown embryoids (derived from salt – tolerant callus) in sour orange, are shown in Table (2).

The obtained results indicated that, the percentage of fully grown embryoids was significantly the highest (33.33%) on the MT medium containing 1mgl^{-1} GA_3 (E.G.M.10). On the contrary, the lowest percentage (6.67%) was resulted in MT medium without growth substances (E.G.M.6) and MS medium containing 0.5mgl^{-1} NAA (E.G.M.2).

As for the addition of GA_3 in medium, a very similar trend was observed with either MT or MS medium. As the GA_3 concentration was increased in medium from 0.5 mg1^{-1} to 1.0 mg1^{-1}, the percentage of fully grown embryoids increased. Such increase in case of MT medium was ranged from 26.67% to 33.33% (E.G. M. 9 to E.G. M. 10) and with MS medium from 20.00% to 26.67% (E.G. M. 4 to E.G. M.5).

Likewise, the addition of NAA to medium, as its concentration was increased in medium from 0.5 mg1^{-1} to 1.0 mg1^{-1} , the percentage of fully grown embryoids increased. The values tabulated were from 13.33% to 20.00% (E.G.M. 7 to E.G.M. 8) for MT medium and from 6.67% to 13.33% (E.G.M.2 to E.G. M.3) for MS one.

Generally, no embryoids germination occurred (0.00%) on the MS medium without growth substances (E.G.M.1).

These findings agreed with those reported by Beloualy (1991). Who mentioned that the complete plants of sour orange, were regenerated by somatic embryogenesis. He found that gibberellin enhanced stem elongation and rooting in embryoids.

Table (2): Effect of ten medium formulations on the percentage of fully grown embryoids (derived from salt-tolerant callus) in sour orange.

Medium code	Basal medium	(mgl^{-1})		% Fully grown embryoids[z]
		NAA	GA_3	
E.G.M.1	MS	0.0	0.0	0.00 D*
E.G.M.2	MS	0.5	0.0	6.67 CD
E.G.M.3	MS	1.0	0.0	13.33 BCD
E.G.M.4	MS	0.0	0.5	20.00 ABC
E.G.M.5	MS	0.0	1.0	26.67 AB
E.G.M.6	MT	0.0	0.0	6.67 CD
E.G.M.7	MT	0.5	0.0	13.33 BCD
E.G.M.8	MT	1.0	0.0	20.00 ABC
E.G.M.9	MT	0.0	0.5	26.67 AB
E.G.M.10	MT	0.0	1.0	33.33 A
L.S.D. 0.05				19.053

E.G.M. = Embryoid growth medium.

[z] There were 15 embryoids (one embryoid per culture tube) per treatment (medium formulation).

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

The obtained results indicated that BAP repressed embryogenesis induction in sour orange. These findings agreed with those reported by Kochba and Spiegel-Roy (1977). They stated that various cytokinins depressed embryogenesis in “Shamouti” callus. In addition, Beloualy (1991) found that BAP markedly repressed induction of embryogenesis in *Citrus aurantium.*

Concerning NAA, the obtained results were similar to those of Matsumoto and Yamaguchi (1983), but these authors utilized NAA at high concentration (2 mgl^{-1}).

These results disagreed with those reported by Gill *et al*., (1994), who mentioned that MS (1962) medium supplemented with 3 mgl^{-1} BA + 0.5 $mg1^{-1}$ NAA + 500 $mg1^{-1}$ malt extract showed germination of somatic embryos of “Kinnow” mandarin.

Citrus callus cell line have been selected for salt – tolerance and regenerated into plantlets (Kochba *et al.,* 1982; Ben-Hayyim and Kochba, 1983; Ben-Hayyim *et al.,* 1985; Spiegel-Roy and Ben –Hayyim, 1985).

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إنتاج نباتات نارنج متحملة للملوحة باستخدام تقنية زراعة الأنسجة:
3- تكوين الأجنة الجسمية من الكالس المتحمل للملوحة

عبد العظيم محمود الجزار ومحمد بدر الصيروت ونبيل ثابت مصطفى
قسم الفاكهة – كلية الزراعة- جامعة الإسكندرية - الإسكندرية – مصر

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 تكوين الأجنة الجسمية من الكالس المتحمل للملوحة وبالتالى يمكن إنتاج نباتات نارنج متحملة للملوحة فيما بعد.
ويمكن تلخيص النتائج الرئيسية لهذه الدراسة فى النقاط التالية:

1- إن بيئة موراشيج وتوكر لسنة ١٩٦٩ والمحتوية على ١٥٠٠ مليجرام فى اللتر مستخلص الشعير تعطى نسبة مئوية لتكوين الأجنة الجسمية من الكالس المتحمل للملوحة مرتفعة بصورة جوهرية (٦٠%) مقارنة بباقى البيئات التى تم اختبارها. وأن بيئة موراشيج وتوكر لسنة ١٩٦٩ كانت بصفة عامة أكثر فعالية عن بيئة موراشيج وسكوج لسنة ١٩٦٢ لتكوين الأجنة الجسمية من كالس النارنج المتحمل للملوحة.
2- تم الحصول على أعلى نسبة مئوية للأجنة مكتملة النمو بصورة جوهرية (٣٣,٣٣%) وذلك على بيئة موراشيج وتوكر لسنة ١٩٦٩ والمحتوية على ١ مليجرام فى اللتر حمض الجبريلليك.