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EFFECT OF SOME NATURAL MATERIALS ON *in vitro* MICROPROPAGATION of "NAVEL ORANGE "

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ABSTRACT

The main goal of this work was studying the effects of adding some natural materials to the culture media instead of the growth regulators on the growth of navel orange plantlets . Natural materials with variant concentrations were used (coconut milk , jojoba oil , orange juice and two types of humic acid *viz*., powder and liquid. Explants cultured on MS medium supplemented with 15% orange juice showed the highest percentage of contamination (74.10%), while explants cultured on medium with 250mg/l humic acid (powder) showed the lowest percentage of contamination (7.40%) . Explants cultured on medium with 1% , 3% or 6% jojoba oil and the control (1) medium (MS basal medium with 2mg/l BAP+ 1mg/l kinetin +1mg/l NAA) did not show any contamination.

Explants cultured on a medium with 0.25ppm humic acid (liquid), medium with 250 mg/l humic acid (powder), medium with 5% coconut milk; and the control (1) medium showed the highest percentage of sprouting (87.50 , 83.80 , 83.30 and 85.20%, respectively) while , all explants cultured on medium with 750mg/l humic acid (powder) or medium with 0.75ppm humic acid (liquid) failed to sprout (zero %). Sprouting explants cultured on medium with 10% orange juice , medium with 5 or 10 % coconut milk and medium with 0.25ppm humic acid (liquid)resulted in the highest shoot number / explant (6.92 , 6.52, 6.07 and 5.90 shoots / explant, respectively) . Explants cultured on medium with 750mg/l humic acid (powder) or 0.75ppm humic acid (liquid) failed to produce any shoots . Sprouting explants cultured on a medium with 10% orange juice or control (1) medium showed the highest shoot length (1.60 and 1.57 cm, respectively) and the highest leaves number (4.73 and 5.40 leaves/shoot, respectively).*In vitro* shoot explants cultured on rooting medium with 750mg/l humic acid (powder) produced the highest number of roots/shoot (7.93 roots/shoot) followed significantly by 7.00 roots/shoot rooting medium with 500 mg/l humic acid (powder), while *in vitro* shoot explants cultured on rooting medium with 5% coconut milk, 250 mg/l humic acid (powder) , 10% orange juice , control(1) medium , 0.5ppm humic acid (liquid) and 500mg/l humic acid (powder) produced the longest roots (1.60 , 1.53 , 1.50 , 1.50 , 1.43 and 1.40 cm, respectively**).**

Key words: *culture media, micropropagation , natural materials, navel orange.*

1. INTRODUCTION

Orange is considered the first citrus crop in Egypt, in respect to the exporting, as 61% of the total citrus production in Egypt (Agriculture Statistics , 2007 , Ministry of Agriculture , Egypt) Micropropagation is an important asexual method that can be used for producing virus-free plants (Roistacher *et al*., 1976). Tissue culture and micropropagation protocols have been described for a number of citrus species and explant sources (Barlass and Skene ,1982). *In vitro* plant regeneration of citrus species has been demonstrated through somatic embryogenesis (Vardi *et al*., 1982; De Pasquale *et al*., 1994) and organogenesis (Moore,1986; Sauton *et al.*,1982;) and Barlass and Skene, 1982 from various citrus explants .One of the major problems of *in vitro* plant cultivation is the high level of somacolnal variation, that may be due to an interaction between the explant , the growth regulators and long periods of subculture (Corneanu *et al*., 2002) .Several experiments were carried out to develop protocols for the *in vitro* propagation of citrus using several explants . Murashige and Skoog 1969 (MS) medium supplemented with various concentrations of natural materials has been used to determine the rate of shoot proliferation and rooting (Einest, 1978).

The objective of this study was to develop a tissue culture protocol for navel oranges using nodal explants from mature trees to explore the effect of adding some natural materials in the culture media *i.e.*, jojoba oil , coconut milk, humic acid and orange juice instead of the growth regulators on micropropagation of navel oranges for avoiding somaclonal variation .

2. MATERIALS AND METHODS

This work was carried out at the Tissue Culture Laboratory, Horticulture Research Institute , Agricultural Research Center, Giza, Egypt . The experiment was carried out during the period of 2006 to 2009 ,to investigate the effect of some natural components added to MS media on the rate of growth (shooting and rooting), which replaced growth regulators. The additions used included the natural components coconut milk , humic acid (liquid and powder) , orange juice and jojoba oil .

2.1.Preparation of explants

Actively growing new shoot (10 cm long) were taken from mature trees, about 10 year old of navel orange grown in the orchard of the Horticulture Research Institute , Giza , at the beginning of March . The collected shoots were washed carefully under running tap water for one hour , and then sterilized in 70% ethanol for 3-5 min. , followed by 2% (w/v), sodium hypochlorite for 15 min. containing one drop of Tween-20, and then rinsed three times in sterile distilled water for 4 min. for each one. Sterilization was carried out according to the procedure reported by Al-Kayri and Al-Baharany (1982) and Al- Baharany (2002). Sterilized shoots were stripped of their leaves and then cut into segments (1cm long) and each segment contained one lateral bud .

2.2.Basal medium composition

Throughout the study , MS basal medium (Murashige and Skoog,1962) was used .The media were solidified with 7-8 g/l agar and supplemented with sucrose at 30 g/l . The pH of the medium was adjusted to 5.8 with 0.5 M potassium hydroxide (KOH) , dispensed in 250 ml jar, each jar contained 30 ml of the culture medium . The culture jars were sealed with caps of polyvinyl propylene , and autoclaved at 121 ºC for 20 minutes .

The natural materials were used as substitutes for the growth regulators according to the experimental stages as mentioned below . Natural materials were sterilized by the filtration throughout the special sterilized microbial filters .

2.3.Shoot multiplication medium

To examine the response of node explants to natural additions and growth regulators , the sterilized node explants were cultured on media supplemented with the following concentrations of the studied materials :-

1. Jojoba oil (1% ,3% and 6%) ,
2. Coconut milk(2.5% ,5% and 10%) ,
3. Orange juice(5% and 10% 15%)
4. Two types of humic acid (actosol ®) as mentioned in Table (1)
   - powder(250mg/l ,500mg/l , and 750mg/l)
   - liquid(0.25ppm ,0.5ppm and 0.75ppm)

Table (1): Main characteristic of actosol ® used in the investigation.

Components	Value	Components	Value
Humic acid (%)	2.9	P(%)	10.00
Organic matter/total	42.51	K(%)	10.00
Total HA/total solid	165.80	Ca(%)	0.06
Organic carbon (%)	24.64	Mg(%)	0.05
C/N ratio	2.46	B (mg/l.)	70.00
pH	8.10	Fe(mg/l.)	900.00

Different treatments were compared with control (1) medium (MS basal medium with the additions of 2mg/l 6-benzylaminopurine (BAP), 1mg/l kinetin and 1 mg/l 1-naphthaleneacetic acid (NAA)) and control (2) medium (MS basal medium without any additions of growth regulators or natural additions).

Nine explants were cultured per treatment (three jars / replicate and three replicates / treatment). Cultures were incubated at 24±2 ºC under 16 h photo period of cool- white fluorescent light. The cultured explants of each treatment were subcultured on fresh medium at four-week intervals. Data were recorded at the end of the second subculture including percentages of contamination , sprouting (%) ,number of shoot , shoot length (cm) and leaf number .

2.4.Rooting medium

In vitro shoots were separated from shoot cluster and cultured on rooting media which consist of MS basal medium without any additions of growth regulators to test the effect of natural additions on root development. Rooting media had the same concentrations of natural additions and under the same light and temperature regime used for shoot multiplication. Resulting data were compared with control (1) medium which consists of MS basal medium with the addition of 1mg/l (NAA) and control (2) medium, which consists of

MS basal medium without any additions of growth regulators or natural additions . Data were recorded as rooting percentage , root number and length of root(cm) .

2.5.Statistical analysis

Data of shoot multiplication and rooting were subjected to analysis of variance (ANOVA) and means were separated where appropriate , using the least significant difference (LSD) at 5% significant as described by Snedecor and Cochran (1980).

3.RESULTS

3.1.Contamination

The data in Table(2) refer to the effect of different concentrations of coconut milk , humic acid(powder and liquid) , orange juice and jojoba oil on contamination percentage . Data revealed that adding 15%orange juice to the MS basal medium resulted in the highest percentage of contamination (74.10%) followed by the contamination percentage (51.87%) of explants cultured on medium supplemented with 10%orange juice. Explants cultured on medium with 250mg/l humic acid (powder) showed the lowest percentage of contamination (7.40%). All the explants cultured on media with 1% , 3% or 6% jojoba oil and control (1) medium (MS basal medium with 2mg/l BAP+ 1mg/l kinetin +1mg/l NAA) did not show any contamination .The contamination percentages of the other treatments were in between**.**

3.2.Sprouting

The data in Table (3) show the effect of treatments on sprouting percentage, data revealed that the explants cultured on a medium with humic acid (liquid) at 0.25ppm, medium with 2mg/l BAP+ 1mg/l kinetin +1mg/l NAA control (1), medium with humic acid (powder) at 250 mg/l and medium with coconut milk at 5% resulted in the highest significant sprouting percentage (87.50, 85.20, 83.80 and 83.30% respectively) without significant differences between them. These percentages are followed significantly by the sprouting percentage (76.67%) of explants cultured on MS basal medium supplemented by orange juice at 10%. On the other hand, all explants cultured on MS basal medium supplemented with humic acid (powder) at 750mg/l or humic acid (liquid) at 0.75ppm failed to sprout as the sprouting percentage was zero. The sprouting percentage of the other treatments were in between .

Table (2):Effect of coconut milk, humic acid, orange juice and jojoba oil on contamination percentage of navel orange explants (After 6 to 10 days of culture).

Treatment	Concentration	Contamination (%)
Coconut milk %	2.5	37.00 d
	5	37.00 d
	10	33.30 e
Humic acid (powder) mg/l	250	7.40 h
	500	14.80 f
	750	14.80 f
Humic acid (liquid) ppm	0.25	11.10 g
	0.5	7.40 h
	0.75	14.80 f
Orange juice %	5	40.70 c
	10	51.87 b
	15	74.10 a
Jojoba oil %	1	0.00 i
	3	0.00 i
	6	0.00 i
*** Control (1) medium**		0.00 i
**** Control (2) medium**		7.40 h

Means designated with the same letter in the same column are not significantly different at L.S.D 0.05 level of probability

* MS basal medium with growth regulators ** MS basal medium without growth regulators

3.2.1.Shoot number

The data presented in Table (4) show the effect of coconut milk , humic acid(powder and liquid), orange juice and jojoba oil on shoot number/ explant . Explants cultured on medium with orange juice at 10% , medium with coconut milk at 5% or 10% and medium with humic acid (liquid) at 0.25 ppm resulted in the highest significant number of shoots per explant (6.92, 6.52 ,6.07 and 5.90 shoot/explant, respectively) without significant differences between them .Explants cultured on MS basal medium supplemented with humic acid (powder) at 750mg/l or humic acid (liquid) at 0.75ppm failed to produc any shoots. The shoot number per explant of the other treatments was in between.

3.2.2.Shoot length

The results in Table (4) indicate that the highest shoot length (cm) was obtained from explants cultured on medium with orange juice at 10% or medium with 2mg/l BAP+ 1mg/l kinetin +1mg/l NAA control (1) medium, as shoot length was 1.60 cm and 1.57cm, respectively . On the other hand, all explants cultured on medium supplemented with humic acid (powder) at 750mg/l and humic acid (liquid) at 0.75ppm failed to produce any shoots .The shoot length of the other treatments were in between.

3.2.3.Leaf number

The effects of coconut milk , humic acid(powder and liquid) , orange juice and jojoba oil on leaf number are shown in Table (4) .The greatest leaf formation (5.40 and4.73 leaves / shoot) was observed by adding 2mg/l BAP+ 1mg/l kinetin +1mg/l NAA control (1) or orange juice at 10%, respectively to culture medium , followed significantly by leaf number (3.67 and 3.10 leaves / shoot) of explants cultured on medium supplemented with coconut milk at 5% or humic acid (powder) at 250mg/l, respectively . Explants cultured on medium supplemented with humic acid (powder)at 750mg/l and humic acid (liquid) at 0.75ppm failed to form any shoots .The other treatments gave slightly lower leaf number per shoot compared with the optimum treatment.

Table (3): Effect of coconut milk , humic acid , orange juice and jojoba oil on sprouting percentage of navel orange explants (After 2 weeks of culture).

Treatment	Concentration	Sprouting (%)
Coconut milk %	2.5	24.50 h
	5	83.30 a
	10	62.40 c
Humic acid (powder) mg/l	250	83.80 a
	500	22.03 h
	750	0.00 j
Humic acid (liquid) ppm	0.25	87.50 a
	0.5	24.07 h
	0.75	0.00 j
Orange juice %	5	31.10 g
	10	76.67 b
	15	44.50 e
Jojoba oil %	1	14.80 i
	3	37.00 f
	6	51.87 d
* Control (1) medium		85.20 a
** Control (2) medium		36.10 f

Means designated with the same letter in the same column are not significantly different at L.S.D 0.05 level of probability

* MS basal medium with growth regulators ** MS basal medium without growth regulators

3.3.Rooting of *in vitro* shoots

In vitro shoots from different treatments were separated and cultured on rooting media, which consist of MS basal medium with addition of different concentrations of natural components. Table (5) demonstrates the effect of coconut milk,

Table (4): Effect of coconut milk , humic acid , orange juice and jojoba oil on shoot number , shoot length and leaf number of navel orange explants (After 8 weeks of culture) .

Treatment	Concentration	Shoot number	Shoot length (cm)	Leaves number
Coconut milk %	2.5	3.17 def	0.90 efg	2.23 cdef
	5	6.52 a	1.37 c	3.67 b
	10	6.07 ab	0.99 ef	1.50 fg
Humic acid (powder) mg/l	250	4.62 c	1.33 c	3.10 bc
	500	2.17 fg	0.77 g	0.80 gh
	750	0.00 h	0.00 h	0.00 h
Humic acid (liquid) ppm	0.25	5.90 ab	1.30 c	2.67 cde
	0.5	3.33 de	1.03 ef	1.50 fg
	0.75	0.00 h	0.00 h	0.00 h
Orange juice %	5	4.17 cd	1.07 de	1.43 fg
	10	6.92 a	1.60 a	4.73 a
	15	3.50 de	0.87 fg	1.40 fg
Jojoba oil %	1	1.83 g	1.40 bc	2.83 bcd
	3	3.42 de	1.23 cd	2.27 cdef
	6	1.40 g	1.03 ef	1.70 efg
*** Control (1) medium**		5.08 bc	1.57 ab	5.40 a
**** Control (2) medium**		2.92 ef	1.23 cd	1.87 def

Means designated with the same letter in the same column are not significantly different at L.S.D 0.05 level of probability

* MS basal medium with growth regulators ** MS basal medium without growth regulators

Table (5): Effect of coconut milk , humic acid , orange juice and jojoba oil on rooting formation of *in vitro* shoot explants navel orange explants(After 4 weeks of culture) .

Treatment	Concentration	Root number	Root length (cm)
Coconut milk %	2.5	1.70 hi	0.93 cd
	5	4.87 e	1.60 a
	10	2.10 gh	1.10 cd
Humic acid (powder) mg/l	250	6.13 cd	1.53 a
	500	7.00 b	1.40 ab
	750	7.93 a	1.07 cd
Humic acid (liquid) ppm	0.25	6.00 cd	0.95 cd
	0.5	4.87 e	1.43 ab
	0.75	6.33 c	1.00 cd
Orange juice %	5	1.63 hi	0.93 cd
	10	5.87 cd	1.50 a
	15	2.40 g	0.60 e
Jojoba oil %	1	1.23 i	1.16 bc
	3	3.43 f	1.09 cd
	6	5.63 d	0.83 de
***Control(1) medium**		5.63 d	1.50 a
****Control(2)medium**		2.47 g	0.93 cd

Means designated with the same letter in the same column are not significantly different at L.S.D 0.05 level of probability

* MS basal medium with growth regulators ** MS basal medium without growth regulators

humic acid , orange juice and jojoba oil on rooting of *in vitro* shoots. The highest percentage of rooting approximately 7.93 roots /shoot ,was produced by adding humic acid (powder)at 750mg/l to the culture medium followed significantly by the rooting percentage (7.00 %) of explants cultured on medium supplemented with humic acid (powder)at 500 mg/l, while the lowest percentage of rooting was observed by adding jojoba oil at 1% to culture medium(1.23 roots/shoot).The rooting percentages of the other treatments were in between.

3.3.1.Root length

The results in Table (5) indicate that the longest roots observed by adding coconut milk at 5% ,humic acid (powder) at 250mg/l, 1mg/l NAA control (1) medium, orange juice at 10%, humic acid (liquid) at 0.5 ppm and humic acid (powder) at 500mg/l to culture medium , (1.60, 1.53, 1.50, 1.50, 1.43 and 1.40 cm respectively) without significant differences between them. On the other hand, adding orange juice at 15% to culture medium resulted in the lowest root length (0.60 cm). The root length of the other treatments was in between.

4.DISCUSSION

The work presented in this investigation provides a simple protocol for the micropropagation of navel orange using nodal explants from mature trees to investigate the effect of some natural materials on growth development of navel orange lateral buds. No contamination was observed from explants by using jojoba oil in culture medium at different concentrations. Using humic acid (powder or liquid) in culture medium at different concentrations showed the lowest contamination percentage. while, using orange juice in the culture medium at different concentrations showed the highest contamination percentage. Nodes cultured on a medium with high concentrations of humic acid (powder or liquid) can not able to form any shoots while, shoots can be easily derived from node cultures on medium containing other concentrations of humic acid (powder or liquid), coconut milk , orange juice and jojoba oil at different concentrations activation effects . All proliferated shoots can be easily rooted on rooting media consisted of different concentrations of natural materials activation effects .

These results are in agreement with many investigations where the initiation period of the shoot was significantly shortened in the presence of humic acid,and root initiation was significantly induced, especially when humic acid was used in a liquid medium with tropical crops (Goenadi and Sudharama., 1995). Beside, humic acid increased the peroxidase activity in the *in vitro* culture of Carnation cv.Red charry (Corneanu *et al*., 2002) . Using a combination of potassein N (K-N) as a foliar spray at 2 ml/L plus humic acid at 20mL as a soil drench monthly improved vegetative growth and promoted the nutrients uptake of date palm cv. Sakkoty plantlets (Abdel-Galeil , 2010) .

In vitro growth of explant cultures from citrus cultivars was stimulated by addition of orange juice to a basal medium (Einest, 1978) .

Regarding culture establishment, the best results were achieved when 50ml/l coconut water and 2.22 μM BAP were added to the medium. For the *in vitro* multiplication stage the highest proliferation rates with an average of 3.4 new explants after 30 days were achieved with the coconut water concentration at 50 ml/l (Peixe *et al*., 2007) .

Jojoba oil ,*Candida tennis* and microelements mixture showed pronounced effect on bud sprouting and hastened the bud emerging on flame seedless grapevine (Abd El-Moity *et al*., 2006) .

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تأثير إضافة بعض المواد الطبيعية على مراحل الإكثار الدقيق للبرتقال أبو سرة معملياً

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ملخص

يهدف هذا البحث إلى دراسة تأثير إضافة بعض المركبات الطبيعية إلى بيئة الزراعة بدلاً من منظمات النمو على نمو النباتات. تم استخدام بعض المركبات الطبيعية بتركيزات مختلفة وهى لبن جوز الهند ،زيت الجوجوبا ،عصير البرتقال ، ونوعين من حامض الهيوميك (بودرة – سائل) .

وقد أظهرت النتائج أن أعلى نسبة تلوث(74.10%) قد تحققت مع العزلات المنزرعة على بيئة مضاف إليها عصير البرتقال بتركيز 15% ،بينما تحققت اقل نسبة تلوث (7.40%) مع معاملة مسحوق حامض الهيومك بتركيز 250 مليجرام/لتر. لم يظهر بالعزلات التي تم زراعتها على بيئة تحتوي على كلا من زيت الجوجوبا بتركيزاته المختلفة (1% و 3% و6%) بجانت الكنترول (1) (2مليجرام /لتر 6-بنزيل امينو بيورين + 1مليجرام كينيتين + 1مليجرام نفثالين حامض الخليك) لم يظهر بها اي تلوث .

أظهرت العزلات المنزرعة على بيئات تحتوي على حامض هيوميك (سائل) بتركيز 0.25جزء فى المليون أو مسحوق حامض هيوميك بتركيزات 250 مليجرام/لتر اولبن جوز الهند بتركيز 5% او الكنترول (1) أظهرت أعلى نسبة من التفريع (87.50 و 83.80 و 83.30 و 85.20 % ،على التوالي)

كانت العزلات المنزرعة على بيئة تحتوي على أما مسحوق حامض هيوميك بتركيز 750 مليجرام/لتر او حامض هيوميك (سائل) بتركيز 0.75 ppm كانت غير قادرة على تكوين أية فروع (صفر %).

حققت المعاملة عصير البرتقال بتركيز 10% ، معاملة لبن جوز الهند بتركيزات 5 او 10 % ، معاملة حامض هيوميك (سائل) بتركيز 0.25 جزء فى المليون أعلى نسبة تفريع (6.92 ، 6.52 ، 6.07 و 5.90 فرع / عزلة على التوالي) وعلى العكس لم تحقق كلا من معاملات مسحوق حامض هيوميك بتركيز 750 مليجرام / لتر او حامض هيوميك (سائل) بتركيز 0.75جزء فى المليون اى فروع خلال هذه المرحلة. حققت كلا من معاملتي عصير البرتقال بتركيز 10% والكنترول (1) أعلى طول للفروع وهر 1.60 و 1.57 سم على التوالي أما عدد الأوراق فكانت أفضل

معاملة هى عصير البرتقال بتركيز 10% والكنترول (1) حيث كان عدد 4.73 5.40 ورقة / فرع على التوالي في حين لم تحقق كلا من معاملة مسحوق حامض هيوميك بتركيز 750 مليجرام او معاملة حامض الهيوميك (السائل) بتركيز0.75 جزء فى المليون أية أوراق في هذه المرحلة (صفر ورقة/ فرع).

في مرحلة التجذير كانت أعلى نسبة تجذير في البيئة التي تحتوي على مسحوق حامض الهيوميك بتركيز 750مليجرام / لتر حيث حققت نسبة تجذير 7.93 جذر /فرع تتبعها معنويا نفس المعاملة ولكن بتركيز 500 مليجرام/لتر حيث حققت نسبة تجذير 7 جذر / فرع بينما تحققت أعلى طول للجذور في المعاملات لبن جوز الهند بتركيز 5% ثم مسحوق حامض الهيوميك بتركيز 250مليجرام /لتر ثم عصير البرتقال بتركيز 10% ثم الكنترول (1) ثم حامض الهيوميك (سائل) 0.5 ppmجزء فى المليون مسحوق حامض الهيوميك بتركيز 500مليجرام وهى (1.60 و 1.53 1.50 و 1.50 1.43 و 1.40 سم) ، على التوالي .