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In vitro PROPAGATION OF SOME CITRUS ROOTSTOCKS. 1- EFFECT OF EXPLANT SOURCE AND GROWING MEDIA GROWTH REGULATORS CONTENT.

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ABSTRACT

This investigation was carried out during 2013 to 2015 seasons in tissue culture laboratory of Horticulture Department, Agriculture Faculty, Mansoura University, to make comparssion between shoot tips and epicotyls explants *in vitro* propagation of five Citrus rootstocks namely, Sour orange (*Citrus aurantium*, L.), Volkamer lemon (*Citrus volkmeriana*, Ten.). Cleopatra mandarin (*Citrus reshni*, Blanco), Rangpur lime and Troyer citrange . Our results showed that, epicotyl of Rangur rootstock, Sour orange and Volkamariana which cultured on MS medium containing BAP+Kin (BAP at 1 mg/L, Kin at 1.5 mg/L) gave distinct significant increase of length of shoots/plantlet from epicotyl explants comparing with another tested treatment in this study. Also, explants of epicotyl that took from Volkamariana, Sour orange and Rangpur on MS medium supplemented with IBA+NAA(IBA at 2 mg/L, NAA at 2mg/L) *in vitro* had significant increase of length of roots/explant comparing with other two citrus rootstocks in this respect.

Keyword: Citrus, Rootstock, Epicoyl, shoot tips, Micropropagation, in vitro

INTRODUCTION

In the world and Egypt, Volkamariana, sour orange, Troyer citrange and Cleopatra mandarin and Rangpur are considered important of citrus rootstocks because of it's good vigor, weel adaption to different sandy soils and weather. Furthermore, micro-propagation offers several advantages which are not possible with normal propagation methods. It economical method and gave pathogen free seedlings. Micro-propagation is an important propagation methods that can be used for production of virus free rootstock seedlings *in vitro* (Roistaecher *et al* .1976). Micro-propagation protocol have been described for a number of Citrus species, explants source and supplemented with growth regulators in vitro either at shooting or rooting period during formation of seedlings (Barlass and Skene (1982), Edriss and Burger (1984) and Duran-vila *et al*., (1989).

The aim of this investigation is to study in vitro propagation of some Citrus rootstocks using different explant source and growing media growth regulators content to reach the suitable Citrus rootstocks can propagate using micro-propagation technique.

MATERIALS AND METHODS

This investigation was carried out during 2013 to 2015 seasons in tissue culture laboratory of Horticulture Department, Agriculture Faculty,

Mansoura University, to study compassion between shoot tips and epicotyls explants in vitro micropropagation of five Citrus rootstocks namely, Sour orange (*Citrus aurantium*, L.), Volkamer lemon (*Citrus volkmeriana*, Ten.). Cleopatra mandarin (*Citrus. reshni,* Blanco), Rangpur lime and Troyer citrange .

Ripe fruits representing the 5 tested rootstocks were obtained from fruiting trees in February and March of the studied years. The seeds taken out, washed under running tap water for remove all pulp and juice residuals, air dried then stored in refrigerator at 4º C. The stored seeds were sterilized in the laboratory treated with pestfung (Rizolex-T50%) 1g/kg (w/w) before culturing process. It was done by washing seeds carefully under running tap water with 3 drops of tween - 20followed by immersing in ethanol at 70% concentration for one min. and washed 3 times with sterile distilled water. Once again they were soaked into 47.7% commercial Clorox solution for 10 minutes. Such seeds were washed 3 times with sterile distilled water in a laminar air flow(hood) to remove the residuals and to be ready for culturing.

Seeds were germinated in glass jars 370 mm (120 x 60mm) contained Murashige and Skoog medium (MS., 1962) at full strength and hormone free. Cultures were incubated in the growth chamber at 26 ± 2º C and exposed to 16 hr. Light / day photoperiod (Sen and Dhawan, 2009)

The explants source for the tested rootstocks were epicotyls prepared from germinated seeds and apical portion of shoots (shoot tips at 1 cm in length each) excised from newly growing vigoursly shoots (12 cm long) resulted from 40-day-old seedlings previously *in vitro* grown from the rootstock seeds as mentioned above. Scalpel blade and forceps were used for this excision in sterile Petri dishes under a septic condition.

The culture media for in vitro shoot organogenesis were consisted of the basic salts and vitamins of Murashige and skoog (1962) culture medium (MS) at full strength (basal medium) supplemented with 6-benzylaminopurine (BAP) at 1.50 mg/L, kinetin (Kin.) at 1.00 mg/L and naphthalene acetic acid (NAA) at 0.50 mg/L. Such media also combined with 30 g/L sucrose (as carbon and energy source) and bacto agar at 7 g/L was added for medium solidification. The pH of these media was adjusted to be 5.8 before adding agar, using NaoH and Hcl, and circulated into clean glass jars 200 mm (85x52 mm) each limited 30 ml of nutrient media.The cultured jar were capped with polypropylene closures and placed in growth chamber room at 26 ± 2^{o}C. and under fluorescent light receiving 16 hrs alimentation followed by 8 hrs dark period. Four weeks later culturing date, average number of shoots/explants, average length of proliferated shoots /explant average and number of leaves /explant were recorded for each studied rootstock explant.

The concerned experiments were achieved 2 auxins, naphthalene acetic acid (NAA) at 2mg/L, Indole-3-butyric acid (IBA) at 2mg/L and (Kin) at 0.5 mg/L. They added to the "MS 1962" basal medium at full strength either solely or in combination in term to determine the optimum culture condition for in vitro rooting of regenerated shoots of both, Sour orange (*Citrus aurantium*, L.), Volkamer lemon (*Citrus volkmeriana*, Ten.). Cleopatra Mandarin(*C. reshni,* Blanco), Rangpur limeand Troyer citrange rootstocks.

The basic salts and vitamins of Murashige and skoog (1962) (MS) at full strength were used for rooting media. At the growing period, healthy regenerated shoots were excited and transferred individually under a septic condition and cultured vertically in glass jars (9× 4.5cm) each contained 30 ml basal medium amended with sucrose at 30 g/L and 7g/L bacto agar. The rooting media pH were adjusted to 5.8 before the addition of agar. Such basal medium were supplemented with Naphthalene acetic acid (NAA) and Indole-3-butyric acid (IBA) added either solely at the concentrations of 2.00mg/L each or in combinations at2.00 mg/L each along with the addition of Kinetin (Kin) at a fixed concentration 0.50 mg/L. The cultured jars were closed with covers of high temperature resistant plastic, autoclaved at 121°C for 20 min, then left to cool and harden for five days before being used.

Regenerated shoot micro-cuttings were cultured individually in glass culture jars each contained 30 ml of rooting media. The cultured jars incubated in the 25±1ºC and exposed to photoperiod high light intensity for 16 hrs (1500 lux) and 8 hrs darkness. Data recorded after 5 weeks from culturing, date were average number of roots, root length (cm) /plantlet.

Experiments design and Statistical analysis

Experiments was designed in factorial design with compete randomized block. The obtained data were subjected to analysis of variance (ANOVA) by using "Genstat 11.1" (2008). The mean comparisons were performed by the least significant difference value (LSD) at 5% level probability according to Gomez and Gomez. (1984)

RESULTS AND DISCUSSION

1. Effect of specific and interaction effect of treatments on number of shoots/explant , length of shoot/explant and number of leaves/plantlet in vitro.

- Specific effect.

Data presented in table (1) cleared that, tested citrus rootstocks *in vitro* gave significant variations among them, plantlet of Cleopatra mandarin rootstock had significant increase in number of shoots formation (5.27) 30 day from culturing of epicotyls or shoot tips on (M S) *in vitro*, followed by Volkamariana (5.13) and Sour orange (4.83) and Rangpur (4.80). Troyer citrange presented significantly decrease in number of shoots formation in this respect.

Regarding with used two explants of studied citrus rootstocks on MS *in vitro* for number of shoots formation, epicotyls gave high value (5.21) comparing with shoot tips (4.65).

As for supplanted growth regulators on number of shoots formation of tested citrus rootstocks, combination between BAP+Kin led to significantly increase of number of shoots per explants (7.08) that cultured on (MS) followed by BAP (4.46) and Kin (3.26

- Interaction effect:

In relations to citrus rootstocks, explants and supplemented with growth regulators on number of shoots formation /explant in vitro .Data in

Table(2) showed that, epicotyl explants of Volkamariana, Rangpur and Sour orange cultured on (MS) supplemented with BAP+Kin *in vitro* had distinct significantly increase on number of shoots formation/epicotyl in term of 10.20, 9.80 and 9.20 shoot, respectively, companing to another citrus rootstocks, explants and supplemented with growth regulators in this respect.

Data related to length of shoots(cm) in table(2) indecated that sour orange rootstock (0.61cm) gave significant increase followed by Volkamariana rootstock (0.56cm), Rangpur (0.49cm) than Troyer citrange (0.39cm) and Cleopatra mandarin (0.37cm) culturing on (MS) in vitro conditions.

With regard to explants of tested citrus rootstock data introduced that explants of epicotyls that cultured on MS supplemented with growth regulators *in vitro* on length of shoot formation/explants gave great value(3.08) then shoot tips(2.82) in this respect.

In relations with growth regulators, data in Table (2) cleared that BAP+Kin significantly on length of shoots/ explants culturing on MS *in vitro* and Kin came in the second order. BAP had significant decrease in this respect. In respect of combination of citrus rootstocks, explants and growth regulators on MS for length of shoot formation/explants *in vitro* conditions, data indicated that epicotyl of Rangpur rootstock which cultured on MS supplemented with BAP+Kin (0.88) led to significantly increase of length of shoot epicotyls of sour orange (0.78) sour orange and Volkamariana (0.68) in descending order as compare with explants of than rootstocks. In respect of combination of citrus rootstocks, type of explant and used growth regulators that added to MS medium *in vitro* conditions data indicated that epicotyl explant of citrus rootstocks combired with growth regulators in shooting stage especially BAP+Kin gave significant effect on length of shoots/plantlet formation from the explants compared with shoot tips under the same treatments in this resect.

Also, results showed that, epicotyl of Rangur rootstock, sour orange and Volkamariana which cultured on MS medium containing BAP+Kin gave distinct significant increase of length of shoots/plantlet from epicotyl explants comparing with another tested treatment in this study.

The results were compatible with the results obtained from Carlos *et al.,* (2012) who studied the influence of various basal medium and plant growth regulators on the different micro-propagation of nodal explants of alemow, sour orange and Cleopatra mandarin citrus rootstocks. They found that BA significantly improve on direct shoot initiation of them, Savita *et al*.,(2010) investigated the effect of explants type and different plant growth regulators on calls induction and plantlet regeneration in rough lemon (*citrus jambiri* Lush) they showed that number of shoot per culture was better nodal segment on MS medium supplemented with BA (3mg/L) and NAA (0.5mg/L). Fur the more Yin *et al.,* (2014) studied the *in vitro* regeneration through direct shoot organogenesis in Honey orange (*citrus tangerina*), and indicated BA and NAA were propn plant growth regulators for the induction of direct shoots from the cotyledonary rode and epicotyls explants, in addetion to Sen and Dhawan(2009) on Troyer citrange, found that BA (1.11µM) with Kin (1.16µM)

were optional for both shoot elongation (3.43cm) and multiplication rate (3.86).

Bordon *et al*., (2000) conducted that there several report demonstration the synergistic effect of BA with NAA on multiplication of epicotyls segments of citrus rootstock. More over (Germana et al., 2011) observed that BAP highly in the formation of advenitions shoots, while kinetin (Kin) regeneration of explants of citrus.

In connection with number of leaves/explants of used citrus rootstock in the study, data in Table (2) revealed that significant increase of number of leaves /explants which cultured on MS *in vitro* were obtained from sour orange (8.60) and Volkamariana (8.37) but Rangpur(7.27) rootstock which occupied the second rank of significantly increase among citrus rootstock in the study both of Cleopatra mandarin and Troyer citrange gave significant decrease of number of leaves/explants *in vitro* gnohtions in term of 6.67 and 6.43 leaves/explant, respectively.

Table (1): Effect of different treatments on number of shoots, shoot length and number of leaves during *in vitro* propagation.

	Number of shoots Per explant	**Length of shoots(cm)**	**Number of leaves Per shoot**
Citrus rootstock			
Cleopatra mandarin	5.27	0.37	6.67
Rangpur lime	4.80	0.49	7.27
Sour orange	4.83	0.61	8.60
Troyer citrange	4.63	0.39	6.43
Volkamariana	5.13	0.56	8.37
LSD at 5%	0.45	0.04	0.50
Explant source			
Epicotyl	5.21	0.54	7.45
Shoot tips	4.65	0.43	7.48
F.test	*	*	NS
Media growth regulators			
BAP	4.46	0.34	6.74
Kin	3.26	0.51	6.26
BAP+Kin	7.08	0.60	9.40
LSD at 5%	0.35	0.03	0.39

BAP=6-benzylaminopurine
Kin= kinetin

As for explan type, data cleared that there was rot variation between epicoyl and shoot tips on number of leaves/explants in the experiment.

For growth regulators in the study, data showed that explant which cultured on MS and supplemented with BAP+Kin led to significant increase on number of leaves/explants (9.40) as compared with another tested growth regulators such as BAP (6.74) or Kin (6.26) in the study.With regard to citrus rootstock, explants of them and supplemented with growth regulators together on MS media *in vitro*, data indicated that epicotyls of Volkamariana,

both epicotyls and shoot tips Sour orange and epicotyls of Rangpur on MS media supplemented with BAP+Kin as growth regulators gave significant increase on number of leaves/explants in the study. While epicotyls of Cleopatra mandarin which cultured on MS media supplemented with Kin or BAP alone gave significant decrease in the study.

Table (2): Interaction effect of treatments on number of shoots, shoot length and number of leaves during *in vitro* propagation.

Citrus rootstock	Explant source	Media growth regulators	Number of shoots Per explant	Length of shoots(cm)	Number of leaves Per shoot
Cleopatra mandarin	Epicotyl	BAP	5.20	0.24	5.60
		Kin	4.80	0.46	4.80
		BAP+Kin	6.60	0.50	9.00
	Shoot tips	BAP	4.80	0.30	7.20
		Kin	4.00	0.32	5.20
		BAP+Kin	6.20	0.42	8.20
Rangpur lime	Epicotyl	BAP	3.60	0.32	5.00
		Kin	2.60	0.64	6.80
		BAP+Kin	9.80	0.88	10.60
	Shoot tips	BAP	4.20	0.28	7.60
		Kin	3.60	0.38	6.40
		BAP+Kin	5.00	0.44	7.20
Sour orange	Epicotyl	BAP	3.00	0.56	6.40
		Kin	2.60	0.68	6.60
		BAP+Kin	9.20	0.78	11.40
	Shoot tips	BAP	4.80	0.40	9.20
		Kin	2.80	0.64	6.80
		BAP+Kin	6.60	0.62	11.20
Troyer citrange	Epicotyl	BAP	4.80	0.26	6.20
		Kin	3.00	0.38	5.60
		BAP+Kin	6.00	0.62	8.20
	Shoot tips	BAP	5.20	0.28	5.80
		Kin	3.20	0.36	5.40
		BAP+Kin	5.60	0.46	7.40
Volkamariana	Epicotyl	BAP	4.20	0.44	5.80
		Kin	2.60	0.68	8.00
		BAP+Kin	10.20	0.72	11.80
	Shoot tips	BAP	4.80	0.32	8.60
		Kin	3.40	0.60	7.00
		BAP+Kin	5.60	0.60	9.00
LSD at 5%			1.10	0.11	1.23

2. Effect of treatment on number of root/plantlet and root length in vitro.

- Specific effect:

With respect to number of adventition roots /explant for tested citrus rootstock which cultured on MS medium containing growth regulators of rooting stage in vitro, data in Table(3) revealed that there were not significant variations (differences) among used citrus rootstocks on number of roots/explant in the study (investigation), however explants of volkamariana

gave best value for number of roots /explants (3.43) followed by sour orange and Rangpur in term of (3.33) and (3.23) roots/explants comparing to Cleopatra mandarin and Troyer citrange(3.07) and(3.07) roots/explants respectively.

As for type of explants it is obvious from Table (3) that the epicotyl explant had superiority on number of roots/explants in vitro (3.47) than shoot tips which gave (2.99) root/explant in the study.

Concerning to growth regulators which used as supplementary for MS medium at tooting stage to encourage formation of roots/explant, data in Table (3) showed that significant increase on number of adventition roots/explants were observed on MS media supplemented with IBA+NAA (4.16), while the results of IBA and NAA were close to each other in the study with significant between them on number of adventition roots/explants in this respect.

- Interaction effect:

The combination of studied citrus rootstocks, part of explants and supplemented with growth regulators at rooting stage (period) which related to number of roots/explants in this study, data in Table(4) showed that epicotyls of volkamariana, sour orange, Cleopatra mandarin, Troyer citrange and Rangpur had significant increase of number of roots/explants that cultured on MS medium supplemented with IBA+NAA *in vitro* condition in term of 5.20, 5.00, 4.80, 4.20 and 4.20 roots/explant respectively, also, shoot tips of volkamariana plus IBA+NAA had 4.40 number of roots/explants in this study. On the other hand the other explants of tested citrus rootstocks which cultured on MS medium supplemented with IBA or NAA alone had greater valuoe for number of adventition roots/explants comparing with IBA in this study.

Concerning to signal treatment of studied taken explants of citrus rootstock and in presence of growth regulators at rooting period *in vitro*, data presented in Table (4) indicated that explants of volkamariana gave significant increase for length of roots/explant which cultured on MS medium in vitro comparing with another test rootstocks in this study. Also the results related to explant of Cleopatra mandarin, Troyer citrange, sour orange and Rangpur were closed each other on length of roots/explants in this investigation. As for part of explants of studied citrus rootstocks, data revealed that explant epicotyl gave superior value (3.08) than shoot tips (2.82) for length of roots/ explant in this study.

With regard to used growth regulators at rooting induced on length of roots/explant, the results cleared that IBA+NAA as supplemented with MS medium *in vitro* condition significantly increased of length of roots/explant comparing to IBA or NAA only, also, IBA alone gave significant increase of length of roots/explant in this study comparing with NAA in this respect of this study.

With related to tested citrus rootstocks, taken explants of them and growth regulators at rooting initiation which cultured on MS medium *in vitro*, data in Table (4) showed that explants of epicotyl that took from volkamariana, sour orange and Rangpur on MS medium supplemented with

IBA+NAA in vitro had significant increase of length of roots/explant comparing with most citrus rootstocks, explants plus growth regulators of IBA or NAA alone. Also explants of shoot tips of volkamariana plus IBA+NAA gave significant increase on length of roots/explant as compared with most results of another used citrus rootstock in this study.

The results were in line with the presence of auxins, including IBA or NAA, in the culture medium is necessary to promote rooting in citrus *in vitro* cultures (Carimi and De Pasquale, 2003) and Carlos *et al.,* (2012) in mature alemow explants, found that (observed) both concentration of IBA (1and 3mg L^{-1}) and IAA (0 and 1mg L^{-1}) such as significantly affected on rooting % of tested (citrus rootstock) alemow, sour orange and Cleopatra mandarin. More over Ghorbel *et al.,* (1998) and Bordon *et al.,* (2000) obtained the best results with NAA when rooting epicotyls explants of *C. macrophylla* and In Cleopatra mandarin, rooting percentages were significantly affected by IBA and the interaction IBA×IAA(Carlos *et al.,* 2012).

Perez-Tornero *et al.,* (2010) for lemon, obtained the highest root number in a medium contaning IBA and IAA 1mg L^{-1}, Mendes *et al.,* (2011) reported that combination of NAA and IBA increased the root length in epicotyls explants of different cultivars of sweet orange and sour orange.

Table (3): Effect of treatments on number of roots and root length during *in vitro* propagation.

	Number of roots	Length of roots(cm)
	Mean	Mean
Citrus rootstock		
Cleopatra mandarin	3.07	2.93
Rangpur lime	3.23	2.67
Sour orange	3.33	2.86
Troyer citrange	3.07	2.88
Volkamariana	3.43	3.41
LSD at 5%	0.41	0.33
Explants source		
Epicotyl	3.47	3.08
Shoot tips	2.99	2.82
F.test	*	*
Media growth regulators		
IBA	2.72	2.75
NAA	2.80	2.28
NAA+IBA	4.16	3.83
LSD at 5%	0.32	0.26

IBA= Indole-3-butyric acid
NAA= naphthalene acetic acid

In the final discussion The difference in number of shoots obtained through plantlets formation (induction) among the rootstock might be due to the differences in their genetic makeup (Sharma *et al.,* 2009).

Table (4): Interaction effect of treatments on number of roots and root length during *in vitro* propagation.

Citrus rootstocks	Explants source	Media growth regulators	Number of roots	Length of roots(cm)
Cleopatra mandarin	Epicotyl	IBA	2.40	3.40
		NAA	3.20	2.22
		IBA+NAA	4.80	3.80
	Shoot tips	IBA	2.40	3.20
		NAA	2.60	1.94
		IBA+NAA	3.00	3.00
Rangpur lime	Epicotyl	IBA	2.60	2.40
		NAA	3.60	2.50
		IBA+NAA	4.20	4.00
	Shoot tips	IBA	2.60	2.50
		NAA	2.80	1.82
		IBA+NAA	3.60	2.80
Sour orange	Epicotyl	IBA	2.80	3.00
		NAA	3.00	2.24
		IBA+NAA	5.00	4.00
	Shoot tips	IBA	3.00	2.80
		NAA	2.60	1.94
		IBA+NAA	3.60	3.20
Troyer citrange	Epicotyl	IBA	2.60	2.30
		NAA	2.80	2.60
		IBA+NAA	4.20	3.80
	Shoot tips	IBA	2.60	2.40
		NAA	2.60	2.40
		IBA+NAA	3.60	3.80
Volkamariana	Epicotyl	IBA	3.00	2.30
		NAA	2.60	2.40
		IBA+NAA	5.20	5.20
	Shoot tips	IBA	3.20	3.20
		NAA	2.20	2.70
		IBA+NAA	4.40	4.66
LSD at 5%			1.01	0.82

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الاكثار المختبري لبعض اصول الموالح (الحمضيات)
تاثيرمصدر الجزء المفصول ومحتوى منظمات النمو في بيئة النمو.

محمد صلاح سيف البرعى , محسن فهمى محمد مصطفى , امير ناجى محمد شعلان و عامر عجيل عبد
قسم البساتين – كلية الزراعة – جامعة المنصورة

اجريت هذه الدراسة خلال الموسم ٢٠١٣-٢٠١٥ في مختبر زراعة الانسجة في قسم البساتين كلية الزراعة جامعة المنصورة. للمقارنة بين منفصلين نباتيين هما القمة النامية والسويقة الجنينية العليا لخمسة اصول من الموالحظ (الحمضيات) وهي ,النارنج (البرتقال الحامض),الفولكاماريانا,واليوسفي كليوباترا,الرانجبور والترويز سترانج.

اظهرت النتائج ان السويقة الجنينية العليا للرانجبور, البرتقال الحامض والفولكامارينا المنزرعة على بيئة تحتوي على البنزايل امينو بيورين (١ ملغ/لتر) مع الكاينتين (١.٥ ملغ/لتر) اعطت نتائج جيدة بالنسبة لطول الافرخ مقارنة بالمعاملات الاخرى في هذه الدراسة , وكذلك السويقة الجنينية العليا للفولكاماريانا, البرتقال الحامض والرنجبور المنزرعة على بيئة تحتوي على الاندول حمض بيوترك (٢ ملغ/لتر) مع النفثالين حمض الخليك (٢ ملغ/لتر) اعطت نتائج جيدة مقارنة بالاصول الاخرى في هذه الدراسة.