In Vitro Propagation of Nemaguard Peach (*Prunus persica* l.) Rootstock

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ABSTRACT: This study was carried out at the Plant Tissue Culture Laboratory in Faculty of Environmental Agricultural Sciences (FEAS), Arish University, Egypt during 2012 through 2015. The aim of this study was using tissue culture technique for micropropagation of peach (*Prunus persica* L.*)* plant. Nodes and shoot tips were cultured on MS, NN, WPM and B5 media containing macro and micro elements as well as vitamins, supplemented with 100 mgl^{-1}myo-inositol and 30 % sucrose. Shoot tip cultured on the MS medium had the best combination for the establishment stage of mother plants. Multiple shoot tips were obtained on MS medium supplemented with 1.00 mgl^{-1} BA and 0.05 mgl^{-1} NAA. The highest elongation of shoots was with 1.0 mgl^{-1} GA_3.The highest rooting of shoots was with full strength MS medium with 2.00 mgl^{-1} IBA. Hardening the rooted shoots was done in a greenhouse in pots containing mixture of peatmoss, vermiculite and sand at the rate1:1:1. Plantlets were successfully acclimated with 93 % survive.

Keywords: *Prunus persica* L., Nemaguard rootstock, micropropagation, cytokinins, auxin, adenine sulphate, GA_3.

INTRODUCTION

Peach trees are considered as one of the most popular stone fruits, commercially produced in Mediterranean and, to a lesser extent, in continental climatic conditions. They are many promising cultivars in Egypt (El-Kosary *et al.,* 2013). North Sinai, one of the focus points of peach cultivation, is a semi-arid region with a total precipitation of about 200 mm rain water /year concentrated chiefly in January, February and March (Ahmed *et al.,* 2001). Average production in this region declined from 3.14 ton/feddan in 2010 to 1.64 ton/feddan in 2017 according to Ministry of Agriculture, A.R.E., (2017). However, tissue culture techniques created a large number of applications in the last decades, such as: massive micropropagation of commercial plants (Sulusoglu and Cavusoglu, 2013); germplasm conservation (Isac *et al.,* 2010). Micropropagation is the process of vegetative growth and multiplication from plant tissues or seeds. It is carried out in aseptic and favorable conditions on growth media, using various plant tissue culture techniques (Nitish and Reddy, 2011).

In vitro propagation is probably the most extended application of plant tissue culture in plants (Thorpe, 2007). There are different ways that a scientist could approach firstly to obtain large numbers of plants *in vitro* (Boxus, 1979). Theoretically, the most efficient method would be to stimulate embryogenesis directly from callus, via embryogenesis, one could obtain thousands of plants in very little time from continuous cultures (Kochba *et al*., 1978). Consequently, to stimulate adventitious bud formation directly from plant organs such as leaves and stems. This could also be used to produce large numbers of plants (Skirvin, 1981). Besides, involves the stimulation of axillary bud growth. On using shoot cultures, the expansion of the dormant axis is stimulated because each of these buds would become a single shoot that could be rooted and grown to a whole plant. Clonally propagation of fruit trees would help to increase the availability and commercialization of selected genotype carrying the desired traits, production of high quality fruit trees (Espinosa *et al.,* 2006). Therefore, the aim of the study is to investigate the factors needed for establishing an effective protocol for propagation of Prunus *persica* L.

MATERIALS AND METHODS

This study was carried out in Prof. Dr. Abdelfatah Helmy Belal laboratory for Plant Tissue Culture in Faculty of Environmental Agricultural Sciences (FEAS), Arish University, El-Arish, North Sinai Governorate, Egypt, during the period from 2014 to 2016 to establish a protocol for plantlets formation from peach rootstocks (*Prunus persica* L.) by using micro-propagation techniques.

Active growing of new shoots was excised from Five year old trees Nemaguard cv peach rootstock from Rafah region, North Sinai Governorate, Egypt. Shoots were collected in the active growth period (March to July) and were brought to the laboratory in plastic bags. Shoot tip and node cutting were rinsed and washed thoroughly under running tap water to remove other superficial contamination with soap for 60 min, then washed with sterilized-distilled water, placed in 500 ml beaker containing 400 ml sterilized-distilled water for 48 hrs at 21±2˚C. The explants were soaked in 20% commercial bleach of Clorox solution (5.25 % NaOCl) for 20 minutes, followed by washing 3-4 times in sterile-distilled water to remove all traces of the disinfectant. Sterilization steps were done under aseptic condition in Laminar Flow System using sterilized instruments. The growth parameters i.e. Number of leaves or roots / explant and shoot or root length/explant were evaluated after 6 weeks.

Establishment Stage

Medium Types: MS, W.P.M, B5 and N.N media without any hormone were examined to select the best one that induces the highest explant development (Table 1). All media were adjusted to pH 5.7 - 5.8 using either 0.10 N NaOH or 0.10 N HCL before agar. The medium was cooked and distributed in to glass jars (60 mm diameter and 120 mm height) each jar contained about 50 ml

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media. Finally jars were sterilized in autoclave at 121°C for 20 min.

Explant Types: Shoot tips and one-node cuttings of Nemaguard rootstock were tested to select the best explant type which encouraged the highest development. Shoot tips were excised from terminal portion with 0.5-1.0 cm long containing apical meristem and 2-3 leaf primordial. One–node was prepared by dividing the rest of the shoot into 1.0-1.5 cm segments.

Proliferation stage

BA and NAA concentrations: Different benzyl adenine (BA) concentrations i.e. 0, 0.5, 1.0, 1.5, 2.0 and 2.5 mgl^{-1} in combination with 0.05, 0.1, 0.2, 0.3 mgl^{-1} NAA were tested to investigate the most suitable concentration induced the highest multiplication.

Rooting stage

Root formation phase

Medium strength and auxin type: The elongated shoots were taken and cultured on full, half and quarter MS strengths of basal medium supplemented either with 2.0 mgl^{-1} indole-3-butyric acid (IBA) or 2.00 mgl^{-1}naphthalene acetic acid (NAA) to determine which combination of medium strength and auxin type enhanced the best root formation.

IBA concentrations: Shoots were cultured on full, half and quarter MS medium with different concentrations of IBA (0, 0.5, 1.0, 1.5 and 2.0 mgl^{-1}) to investigate the suitable concentration for encouraging the highest root formation.

Acclimatization Stage

Healthy and well rooted plantlets selected *in vitro* were taken away from the jars and washed thoroughly with running water to get rid of medium residues. Then dipped in antifungal solution (vitavax) at rate of 0.2% for 15 minutes. The roots were washed by sterilized distilled water and planted in black polyethylene pots 8 cm in diameter filled with three mixtures as follow: 1) 1:1:1 (v: v: v) peat moss, vermiculite, and sand; 2) 1:1:1 (v: v: v) peat moss, vermiculite, and perlite; and 3) 1:1 (v: v) peat moss, and vermiculite, then covered with white transparent bags then left under laboratory temperature till the formation of the first new leaf. Small holes were made widening these holes each week gradually and continuously for four weeks until the plantlets become suitable for transferring to bigger pots of 30 cm diameter and transferred to the greenhouse then transferred to field conditions.

Statistical analysis

This experiment was designed in a completely randomized design (CRD) with one or two factors and three replicates per treatment. Data were tested using the analysis of variance (ANOVA) by the General Linear Models (GLMS) procedures using SAS (SAS, 2004). Means comparisons were done using Duncans multiple range test (Duncan, 1995) at 5% level.

Table (1): Chemical constitutes of different media i.e. MS, WPM, B5 and NN media

Chemical components	Culture media			
	MS	WPM	B5	NN
Macro elements ($mg.l^{-1}$)				
NH_4No_3	1650.00	400.00	-	720
KNo_3	1900.00	-	2500.00	950
$CaCl_2.2H_2O$	440.00	96.00	150.00	166
$MgSO_4.7H_2O$	370.00	370.00	250.00	185
KH_2PO_4	170.00	170.00	-	68.0
$(NH_4)2SO_4$	-	-	134.00	-
$NaH_2Po_4.H_2O$	-	-	150.00	-
$Ca(NO_3)_2.4H_2O$	-	556.00	-	-
K_2So4	-	999.00	-	-
Micro elements ($mg.l^{-1}$)				
$NH_4H_2PO_4$	-	-	-	-
H_3BO_3	6.20	6.20	3.00	10
$MnCl_24H_2O$	-	-	-	-
$MnSO_4.H_2O$	16.90	22.30	10.0	25
$ZnSO_47H_2O$	8.60	8.60	2.00	10
Kl	0.83	-	0.75	-
$Na_2MoO_4.2H_2O$	0.25	0.25	0.25	0.25
$CuSO_4.5H_2O$	0.025	0.25	0.025	0.025
$CoCl_2.6H_2O$	0.025	-	2500.025	-
$FeSO_4.7H_2O$	27.80	27.80	27.80	27.6
$Na_2EDTA(2H_2O)$	37.30	27.30	37.30	37.30
Organic components ($mg.l^{-1}$)				
Myo-Inositol	-	100	100	-
Biotin	-	-	-	0.05
Nicotinic acid	0.50	0.50	1.00	5.0
Thiamine.HCL	0.10	1.00	10.0	0.50
Pyridoxine.HCL	0.50	0.50	1.00	0.50
Glycine	2.00	2.0	-	2.00
Sucrose (g/l)	30.00	30.00	30.00	30.00

Where: M.S= Murashige and Skoog medium, W.P.M = Woody Plant Medium, B5= Gamborg medium, N.N= Nitsch and Nitsch medium

RESULTS AND DISCUSSION

Establishment stage

Effect of medium types: Results of the *in vitro* studies of *P. persica* L. "Nemaguard" peach rootstock presented in Figure (1) showed different responses to the types of media. The best plant length, leaves number, shoot numbers and number of leaves per shoot were occurred on MS medium (3, 23.2, 4.5 and 10.7 cm), respectively. Moreover the largest number of shoots was obtained on MS medium, while the lowest number on WPM and B5 media. Shoots growing on the MS medium formed the significantly longest axillary shoots while the significantly shorter shoots were obtained on the B5 medium. These results agree with results obtained by (Ahmed *et al.*, 2003; Kassim *et al.*, 2010).

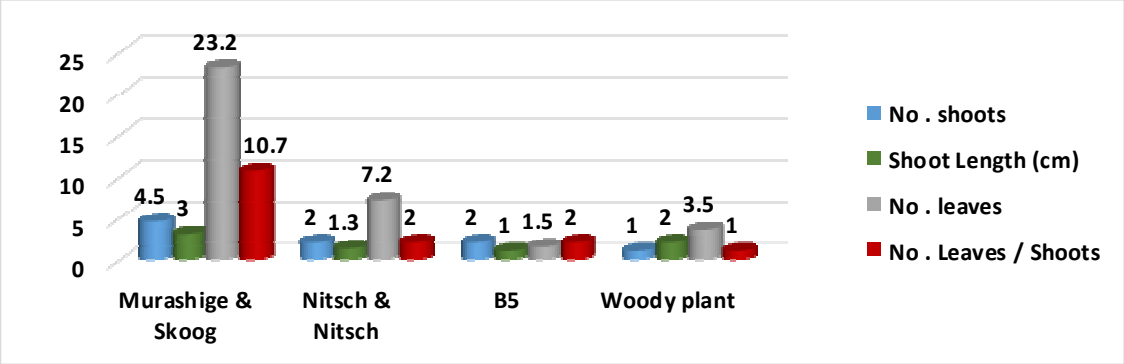

Fig (1): Effect of medium types on number of shoot, shoot length, number of leaves and number of leaves /shoots of Nemaguard peach rootstock *(Prunus persica* L.).

Effect of explant types: It was noticed from Figure (2) that culturing of shoot tip on MS medium increased number of shoots, shoot length, number of leaves, and number of leaves /shoots as compared with one-node cuttings explant (2.2 cm, 11.7 and 5.7) respectively. The superiority of shoot tip with MS medium was obtained by (Xhulaj *et al.,* 2015 and Kassaye, 2017) who found that the best shoot tip explant development was occurred on MS medium.

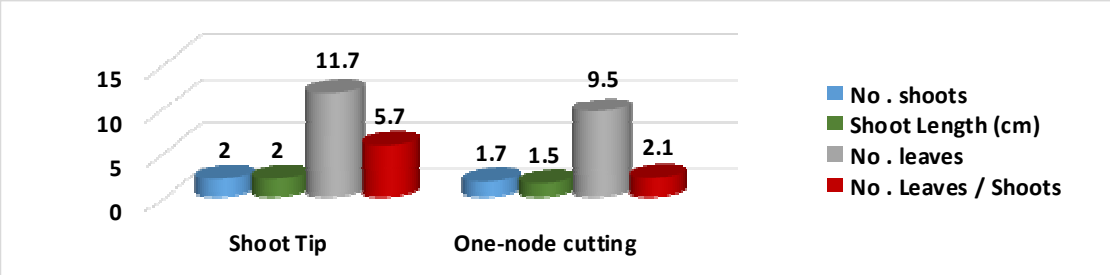

Fig (2): Effect of explant types on number of shoots, shoot length, number of leaves and number of leaves /shoots of Nemaguard peach (*Prunus persica* L.).

Proliferation Stage

Effect of BA and NAA concentrations

It was clear from Figure (3) that both No. of shoots (0.4) and No. of leaves (15.6) were significantly maximized when the proliferation medium supplemented with 1.0 mgl^{-1} BAP. However, shoot length (6.5cm) was significantly increased as medium free of cytokinin was used as a control. In addition, Figure (3) also reflects the effect of different NAA concentrations on multiplication rate. Multiplication was significantly increased by using 0.05 mgl^{-1}NAA in the medium in comparison with the other concentrations .Besides, all used NAA concentrations under study enhanced significantly the No. of leaves (10.3) in relation to the control .On the contrary, shoot length (6.5cm) the highest in the control compared with the other concentrations. Referring to the results of combination of cytokinin and auxin concentrations, it is appear from Figure (3) that adding of 1.0 mgl^{-1} BAP in combination with 0.05 mgl^{-1}NAA to the culture medium succeeded in inducing the best significant No. of shoots as compared with all other combinations. Moreover, all combinations under study showed more or less significant differences as No. of leaves. However, shoot length was significantly maximized in the control as it was free of either

cytokinin (BAP) or auxin (NAA) in comparison with all combinations under study.

It can be recommended to supplement the culture medium with 1.0 mgl^{-1}BAP in combination with 0.05 mgl^{-1} NAA for maximizing multiplication rate as compared with BA and NAA on number of shoots, shoot length and number of leaves at multiplication stage of peach (2.3, 3.8cm and 21), respectively. Results in Figure (3) illustrated that the best shoot number and number of leaves parameters were obtained with 1.0 mgl^{-1} BA and 0.05 mgl^{-1} NAA. These results were in harmony with the findings of Priyakumari and Sheela (2005) who found that maximum proliferation rate of "Peach Blossom" was observed on MS medium supplemented with 4 mgl^{-1}BA and 0.5 mgl^{-1} NAA . Moreover, Shehata *et al.* (2013) mentioned that the highest average shoot length obtained with0.5 mgl^{-1} BA plus 0.1 mgl^{-1} IBA on MS medium. In addition, many authors had reported that a combination of BAP or BA plus IAA on different media (LP, MS, and WPM) were required for shoot multiplication (Teixeira *et al.,* 2004; Yang−Ning *et al.,* 2004 and Dejampour *et al.,* 2011).

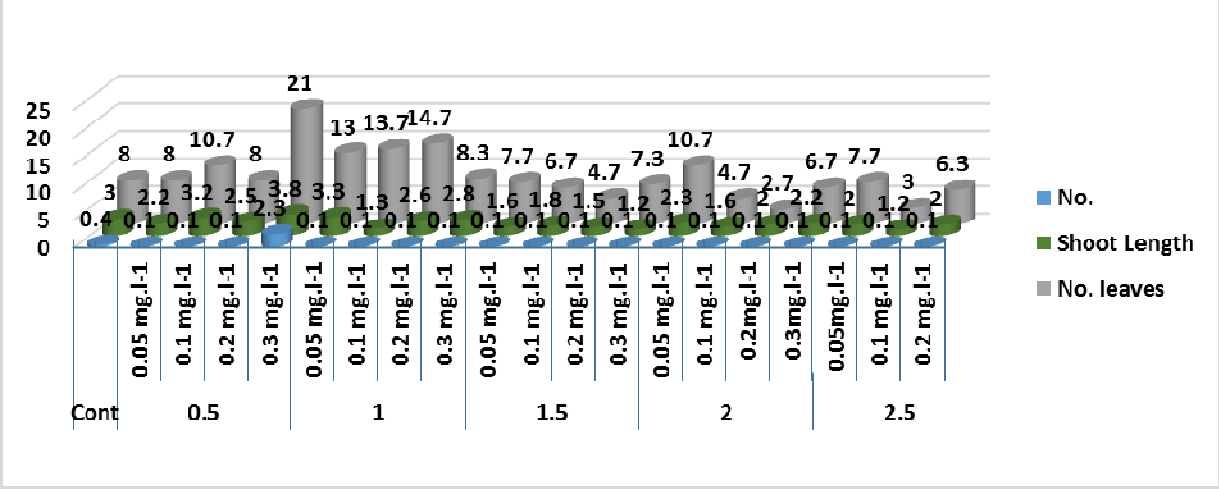

Fig (3): Interaction effect between BA and NAA on number of shoots, shoot length and number of leaves of Nemaguard peach rootstock (*Prunus persica* L.).

Root formation phase

Effect of medium strength: Data in Figure (4) and photo (1) showed that full MS medium strength improved all parameters under study, number of main roots, root length and plant length of Nemaguard peach rootstock (P. presica L). The data clearly show that the highest records for all number of main roots (1.1) and root length (0.8cm), respectively. The finding of the current study was in consistent with the reports of Fotopoulos and Sotiropoulos (2005) who observed excellent rooting of shoots with MS medium (full strength) and (half strength).

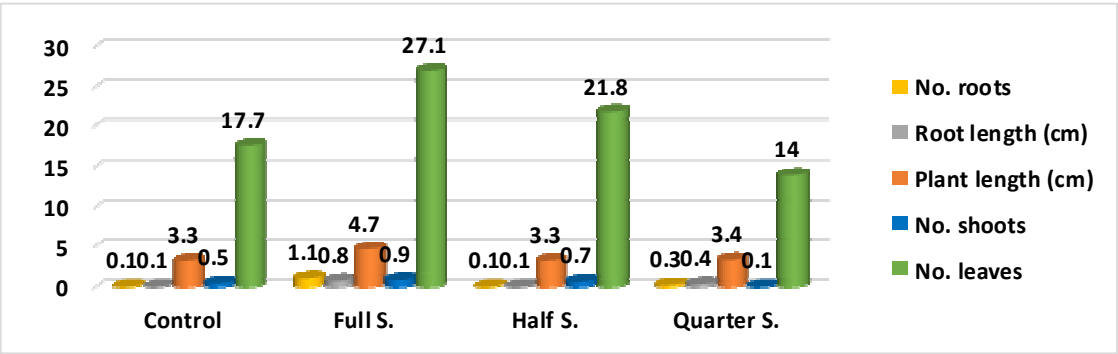

Fig (4): Effect of medium strength on number of main roots, root length, number of shoots, shoot length and number of leaves of Nemaguard peach rootstock (*Prunus persica* L.)

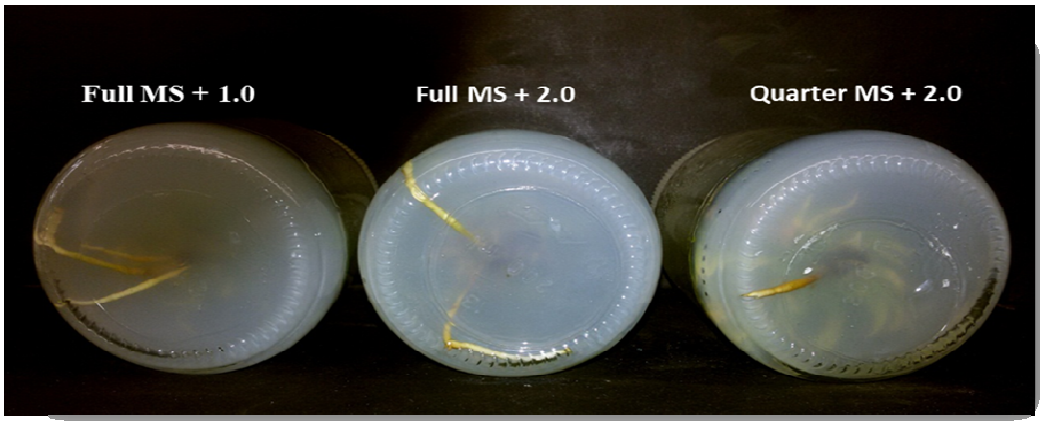

Photo (1): Effect of medium strength and IBA concentrations on rooting of Nemaguard peach rootstock (*Prunus persica* L.)

Effect of auxins type: Results in Figure (5) and Photo (2) indicated that the indole-3 butyric acid (IBA) was significantly surpassed naphthalene acetic acid (NAA) in increasing all parameters under study (number of main roots, root length and plant length) of Nemaguard peach rootstock when full strength medium supplemented with (2.00 mgl^{-1} IBA). The data clearly showed that the highest records were for number of main roots (2.3) and root length (0.18 and 6.5cm). This may be due to the fact that IBA produced healthier lengthy roots and hence absorbed more nutrients and water which had resulted in higher number of leaves produced by the plant. The results in general agreement with the finding of Hassan (2004) who found that IBA was the most effective auxin in enhancing root formation with 1.00 mgl^{-1} to MS medium of apple rootstock.

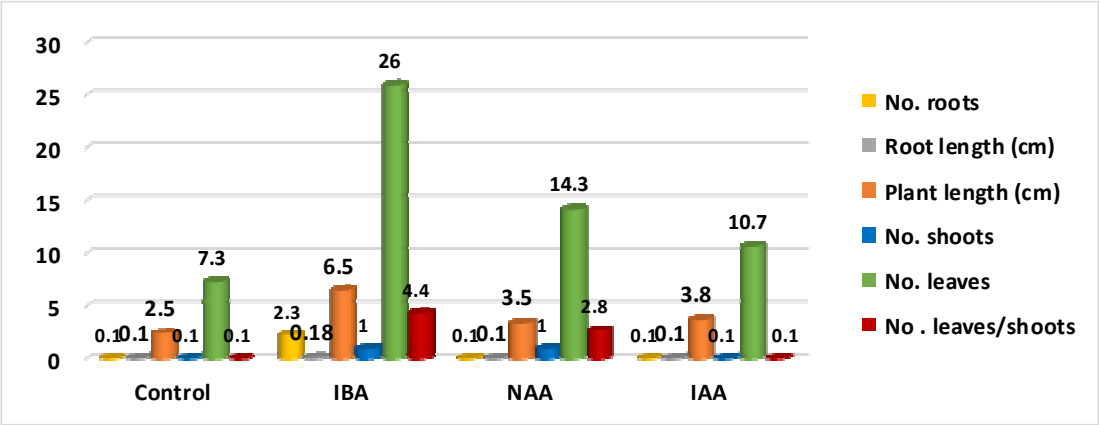

Fig (5): Effect of auxins type on number of roots, root length, number of shoots, shoot length, number of leaves and number of leaves/shoots of Nemaguard peach rootstock (*P. persica* L.)

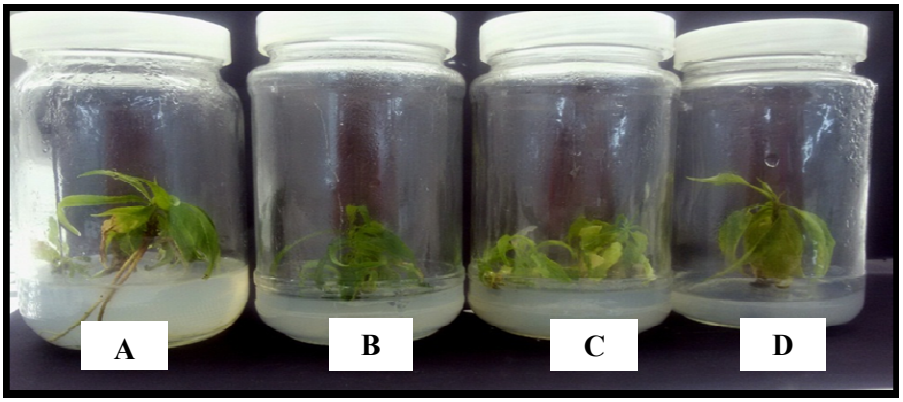

Photo (2): Effect of auxins type on root formation of Nemaguard peach rootstock (*P. persica* L.), Where: A = IBA B = NAA, C = IAA and D = control (zero)

Effect of IBA concentrations:

Data presented in Figure (6) IBA showed that induced a strong rooting response and promoted efficient root induction. The best concentration of IBA was (2 mgl^{-1}) on all parameters. The highest values for number of main roots, root length and plant length were 1.333, 0.916 cm, 4.333 cm) respectively. The above results agreed with Dejampour *et al.* (2011). They found that IBA comparatively more effective than other two auxins (IAA and NAA). Also, many authors have reported IBA as the best auxin for rooting of peach rootstock. In addition, IBA was superior for almond Hybrid Mayor (Cos *et al.,* 2004), peach rootstock (Al-Salihy *et al.,* 2004) and Washington navel and Red khalili (EL-Hadidy, 2000), apple orine cv. (Gamage *et al.,* 2000). Almond cultivars (Channuntapipat *et al.,* 2003). Similar results were obtained by Marcelo *et al.* (2003) who evaluated that the effect of different IBA on the *In vitro* rooting of *Prunus* rootstocks capdeboscq and GF677, and the selection vp411 and vp417 for the *In vitro* rooting stage, the level of 1.0 mgl^{-1} of IBA for the rootstocks capdeboscq, Gf677 and vp411 and the level of 2-0 $mgl^{-1}$of IBA for the vp417. The same trend obtained by Priyak Umari and Sheela, (2005) who claimed that IBA (2 mgl^{-1}) produced the earliest rooting (7day) and the longest root (5 cm) in vitro. In contrast, Touqeer *et al.* (2003) found that the best root system was developed on half MS strength supplemented with 3.0 mgl^{-1} IBA. Higher level of IBA (4.0 mgl^{-1}) induced callus.

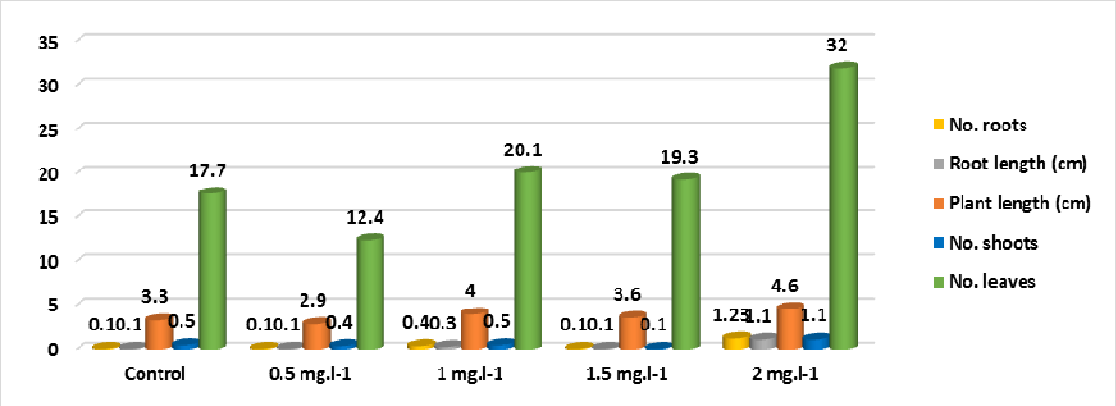

Fig (6): Effect of IBA concentrations on number of roots, root length, number of shoots, shoot length and number of leaves of peach rootstock (*P. persica* L.).

Acclimatization stage

In vitro rooted plantlets were acclimatized by transferring to black polyethylene pots 8 cm in diameter filled with three mixtures as follow: 1) 1:1:1 (v: v: v) peat moss, vermiculite, and sand; 2) with 1:1:1 (v: v: v) peat moss, vermiculite, and perlite; and 3) 1:1 (v: v) peat moss, and vermiculite. The results illustrated that Plantlets were successfully acclimated with 93 % survive in mixture number1, which was extremely appropriate for Acclimatization stage unlike others mixtures as shown in Figure (7). However, Kamali *et al.* (2001 b) mentioned that propagated plants via tissue culture of f677 rootstock were transferred to the soil consisting of either 40% peat or 60% sand mixture. Marina *et al.* (2009) suggest that the improved acclimatization procedure for up 4 weeks increased the survival to 45%.

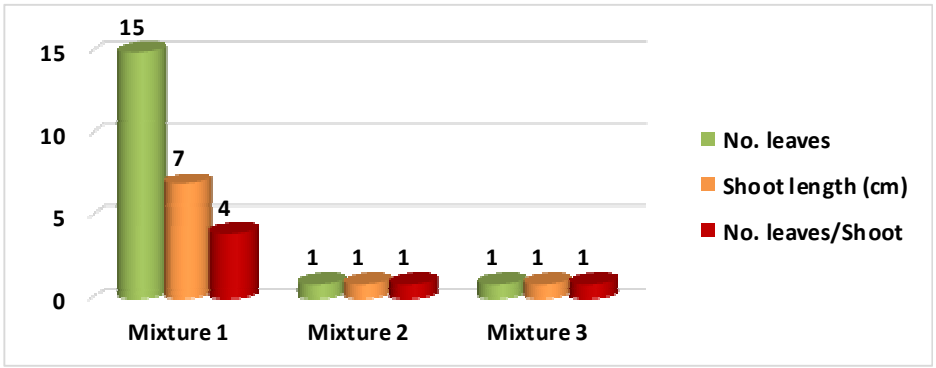

Fig (7): Effect of different mixtures for acclimatization stage on number of leaves, shoot length, and number of leaves/shoot of Nemaguard peach rootstock (*P. persica* L.).

Photo (3): The best mixture 1:1:1 (v: v: v) peat moss, vermiculite, and sand for acclimatization stage of Nemaguard peach rootstock (*P. persica* L.).

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الأكثار الدقيق لأصل الخوخ نيماجارد (*Prunns persica* L.)

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أجريت الدراسة في معمل المرحوم الاستاذ الدكتور/ عبد الفتاح حلمي بلال لزراعة الانسجة النباتية بكلية العلوم الزراعية البيئية ، جامعة العريش ، مصر خلال الفترة من 2012 إلى 2015. كان الهدف من هذه الدراسة هو استخدام تقنية زراعة الأنسجة من أجل الإكثار الدقيق للخوخ (*Prunus perisca* L.). تم أخذ القمم النامية و العقلة ذات البرعم الواحد لاصل النيماجارد وتم تعريضها لماء جارى مستمر وصابون لمدة ساعة ثم غمرها فى محلول كلوركس بنسبة 20% لمدة 20 دقيقة وغسلها بماء مقطر معقم خمس مرات ثم تم زراعتها بعد ذلك تحت ظروف معقمة على بيئات MS ، NN ، WPM ، B5 التي تحتوي على العناصر الكلية والجزئية وكذلك الفيتامينات و قد ظهر أن أفضل هذه البيئات هى بيئة مواراشيج و سكوج و هى البيئة التى تم عليها اجراء مرحلة التضاعف العددي لأفضل جزء نباتى و هو القمة النامية و كذلك استخدام حامض الجبريليك لاستطالة النبات و من ثم اجراء التجذير باستخدام انواع مختلفة من الاوكسينات و اختيار أفضلها وكان أفضلها اندول حامض البيوتريك 2 مللى ، أما مرحلة الأقلمة فقد تمت باستخدام مخلوط من البيتموس : والفرموكوليت: الرمل 1:1:1.