

## ***IN VITRO* PROPAGATION FOR PEACH ROOTSTOCK (NEMAGUARD)**

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A procedure for micropropagation of Nemaguard peach rootstock was developed using stem nodal segments as explants for *in vitro* establishment. Hundred percent of explants survived with high proliferation of shoots, growth percentage to survival and average shoot length was obtained on Murashige and Skoog (MS) basal medium supplemented with a combination of 0.5 mg/L 6-benzyl amino purine (BAP) plus 0.2 mg/L indole acetic acid (IAA). The maximum number of proliferated shoots (7.0 shoots/explant) was obtained on MS medium supplemented with 3.0 mg/L BAP + 0.5 mg/L 2-isopentenyladenine (2iP). Meanwhile, MS basal medium without growth regulators induced the highest shoot length compared with other treatments. However, shoot elongation decreased with the increase of BAP concentration. Ninety percent of the shoots rooted on MS medium supplemented with 3.0 mg/L indole butyric acid (IBA) to obtain complete plants with the maximum average root number/shoot (5.8). On the other hand, the maximum root length (8.5 and 7.2 cm) was obtained when IBA or naphthalene acetic acid (NAA) were supplemented to half strength MS media at 3.0 mg/L. High survival, over 90%, was obtained when the plantlets were transferred to greenhouse conditions. The Nemaguard peach rootstock can successfully micropropagated beginning with stem nodal segment without significant damage to mother plant. The *in vitro* Nemoguard peach rootstocks were planted in Saint Catherine, South Sinai, Egypt where chilling requirements for producing the rootstock seeds are available for commercial production. Hence, it is encouraged to grow Nemaguard peach rootstock under S. Catherine conditions for commercial seed production.

**Keywords:** *Prunus persica*, micropropagation, stem nodal segments, Saint Catherine

Peach tree is one of the most important deciduous fruit trees grown in Egypt, while the harvested area reached 33017 ha and produced 333487 tons (FAO, 2011). Peaches, *Prunus persica* L., which belong to the family Rotaceae, are originated in China and peach ranks second to apples among

temperate zone deciduous fruit trees from the standpoint of production and value (Childers, 1978). A peach tree is highly demanded by Egyptian consumers. There are many peach varieties growing more widely now throughout the world. Peaches are native to China and their culture dates are back to at least 4000 years (Wang, 1985). The main problem with peach trees in Egypt is the declining of the orchard in a short time due to the infection of root system with root-knot nematodes. Rootstocks play major role in modern orchards. Recently, the importance of the rootstock is recognized and the grafted cultivar influence the vegetative and generative mass and the profitability of fruit production (Racsko et al., 2004). Moreover, the rootstock determines the ecological fitness of the tree. Their effects can be recognized in the health status of critical tree phenological stages, tree kilter and tree sensitivity to pests and diseases (Holb, 2002), moreover in the efficiency of pest and diseases management programs and fruit yield (Holb et al., 2003). Nemguard peach (*Prunus persical X Prunus davidiana* Carrere) is used extensively as rootstock for peach cultivars; which is a vigorous grower and extremely disease resistant, proven resistance to root-knot nematodes, more resistant to crown gall than other rootstocks and widely used and preferred for peaches, almonds and plums.

Propagation of Nemaguard by hardwood or softwood cuttings is considering a problem (Alsalihi et al., 2004). Moreover, occurrence of the undesired segregation usually associated with the sexual propagation by seeds was also hoped to be entirely avoided. Seeds have double dormancy (external and internal dormancy). External dormancy occurs when a hard, impervious seed coat acts as a barrier to water, oxygen, and the exchange of other gases or when seed coat contains chemical inhibitors; meanwhile in internal dormancy occurs when internal conditions within the seed itself act as a barrier to germination by inhibitors (phenolics and abscisic acid). So, the seeds need different pre-germination treatments to germination as endocarp removal, stratification and gibberellic acid treatment (Davies and Duray, 2011). All nurseries use the covenantal propagation of Nemaguard by seeds, because of difficult root formation of cutting. Since there are considerable cross pollination in peach, the resulted rootstocks are not uniformity and it affect the characteristics of cultivars, which be grafted on it. So, vegetative propagation of rootstocks is a critical issue in order to replace seedling rootstocks and avoid its bad effects on produced cultivars. There are outstanding efforts made to improve rootstock root ability (Farmer and Besemann, 1974 and Robitaille and Yu, 1980) but these systems have proved to be not useful enough to become standard propagation methods, especially because its production is limited by the available number of stock plant. So, establishment of a protocol for micropropagation is very important. Whereas micropropagation by using microshoots from

Nemaguard peach mother tree as explants is a true to type propagation and most often associated with mass production (Debergh and Read, 1991).

Finally, the technique presents several advantages and could offer serious opportunities for rapid mass propagation for healthy plant materials. Growth regulators are the most important influence factors in shoot regeneration (Bhojwani and Razdan, 1996). In *Prunus* sp., some growth regulators such as benzyl aminopurine (BAP), kinetin (Kin) and 2-isopentenyladenine (2iP) have been exploited for shoot regeneration (Mant et al., 1989). Adventitious shoot formation is being significantly affected by the type and concentration of the cytokinin used in regeneration media. Cytokinins comprise a separate class of growth substances and growth regulators. They produce various effects when applied to intact plants. They particularly stimulate protein synthesis and participate in cell cycle control. The effect of cytokinins is most noticeable in tissue cultures where they are used often together with auxins, to stimulate cell division and control morphogenesis. Added to shoot culture media, these compounds overcome apical dominance and release lateral buds from dormancy (Lindsey, 1997). Also, indole-butyric acid (IBA) and naphthalene acetic acid (NAA) could improve adventitious bud development in almond (Ainsley et al., 2001). To induce this process, growth regulators (cytokinins) are added to the culture medium in order to reduce apical dominance. For peach, the most frequently used growth regulators are BAP and kinetin, with concentrations varying from a minimum of 0.5-0.6 mg/L to a maximum of 1-1.2 mg/L in relation to the genotype and type of explant (Loreti et al., 1988). Since its chilling requirements are high and not available in Egypt, and needs millions of hard currency to import the seed basis. Therefore, the main goal of this study was to establish a micropropagation protocol for Nemaguard peach rootstocks in order to produce a large scale of plants in a short period of time.

## MATERIALS AND METHODS

### 1. Plant Materials

Activity growing shoots of Nemaguard peach rootstock were collected as source of explants from seedlings grown in the greenhouse of the Tissue Culture Unit, Desert Research Center, El-Matareya, Cairo, Egypt. Stem nodal segments of about 1.5 cm in length were isolated from the shoots. The explants were washed in running tap water and then washed again thoroughly by adding few drops of tween 20 to remove the superficial dust particles as well as fungal and bacterial spores. They were then surface sterilized with 1.5% sodium hypochlorite solution for 20 min and rinsed three times with sterile double distilled water, then immersed in 250 mg/L

mercuric chloride solution for 5 min and finally rinsed six times with sterile double distilled water inside the laminar air flow chamber.

## **2. Nutrient Medium and Culture Conditions**

The basal nutrient medium containing macro and microelements applied throughout this study was Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 100 mg/L myo-inositol, 30 g/L sucrose, 2 g/L gelrite and 0.4 mg/L thiamine HCl. The pH value of the nutrient medium was adjusted at 5.7 to 5-8 with adding few drops of either 0.1 N NaOH or 0.1 N HCl, prior to the addition of gelrite. The media were dispensed into jars, each contained 50 ml of culture medium. Sterilization of the medium was achieved by autoclaving the jars containing media under pressure of 1.1 kg/ cm<sup>2</sup> and at 120°C for 20 min. Four explants were inoculated aseptically into the culture medium and placed in each jar, then incubated in a growth chamber at 25°C for 16-h/day photoperiod using cool white fluorescent lamps (3000 lux).

## **3. Establishment Stage**

Shoot tips were inoculated on MS medium supplemented with different concentrations of BAP (0.0, 0.1, 0.5 and 1.0 mg/L) either individually or in combination with indol acetic acid (IAA; 0.0, 0.1, and 0.2 mg/L). Survival percentage, growth percentage to survival and average shoot length were evaluated after six weeks from culture on the establishment medium.

## **4. Multiplication Stage**

Shoots obtained from the establishment stage were transferred to jars containing MS basal medium supplemented with BAP at concentrations of 0.0, 0.5, 1.0, 2.0, 3.0 and 4.0 mg/L and 2iP at concentration of 0.5 mg/L. Shoot proliferation was determined after six weeks of culture. Average number of new shoots formed per explant and average shoot length (cm) were recorded.

## **5. Induction of Rooting and Acclimatization**

For rooting, individual shoots 3-4 cm long were excised from the multiplication stage and cultured on half strength MS basal medium supplemented with IBA or NAA, each at concentrations of 0.0, 1.0, 2.0 and 3.0 mg/L. The percentage of rooted shoots, average number of roots formed per shoot and average root length (cm) were determined after six weeks of culture on the rooting media. Rooted shoots were removed from the culture medium and the roots were washed in sterile distilled water. The plantlets were then transferred to plastic pots containing peat moss and sand (1:1) in greenhouse (28± 2°C, 70% relative humidity). The potted plants were irrigated and initially covered with plastic bags, which were gradually

eliminated within eight weeks for completing their acclimatization and plantlets were maintained under nursery conditions. The acclimatized plantlets were transferred to be cultured under Saint Catherine conditions.

## 6. Experimental Design and Statistical Analysis of Data

Experiments were subjected to a completely randomized design. Analysis of variance (ANOVA) and Duncan's multiple range test (Duncan, 1955), as modified by Snedecor and Cochran (1998), were used to analyze the obtained data. Each experiment included at least 20 replicates and was repeated twice. The differences among mean values were estimated for significance at 5% level. The means followed by the same letter are not significantly different at  $P \leq 0.05$ .

## RESULTS AND DISCUSSION

### 1. Establishment Stage

From data represented in table (1), it could be noticed that survival percentage of Nemaguard culture, using different concentrations of BAP and IAA including the control (without plant growth regulators), ranged from 70 to 100 percent. Growth percentage ranged between 57 and 100%. However the combination of BAP and IAA at a concentration of 0.5 and 0.2 mg/L, respectively was the best treatment, since both the survival and growth percentages attained 100%. The highest average length of shoot (4.6 cm) was obtained also on the same medium and it decreased gradually with the increase in BAP concentration (Fig. 1a). The control medium gave a higher average shoot length (4.3 cm) comparing to the media supplemented with 0.1 mg/L IAA with different concentrations of BAP.

**Table (1).** Effect of BAP and IAA on the *in vitro* establishment of Nemaguard peach rootstock after six weeks of culture on MS medium.

Treatments (mg/L)		Survival (%)	Growth (%) to survival	Average shoot length (cm)
BAP	IAA			
0.0	0.0	70	57	4.3b
0.1	0.1	90	78	4.0c
0.5	0.1	80	100	3.4c
1.0	0.1	80	88	3.1d
0.1	0.2	90	89	4.5a
0.5	0.2	100	100	4.6a
1.0	0.2	80	88	4.2b

The general trend of responses of shoot growth to different auxin concentrations in the media may be interpreted by the fact that the auxin

affects mainly the length of shoots and not through any other mechanism. On the other hand, the effect of auxin on morphogenetic responses of shoots varied relatively according to concentrations of cytokinin in the media. The results are in agreement with theses of Mahdavian et al. (2011) and Edriss et al. (2014), who reported that maximum shoot length of cherry and Nemaguard rootstocks were achieved when culturing on MS medium supplemented with 0.5 mg/L of BAP followed by those cultured on 1.0 mg/L BAP.

## 2. Multiplication Stage

Effect of various combinations of BAP and 2iP on the multiplication of shoots is presented in table (2). The cytokinin free medium gave the least average number of shoots indicating strong apical dominance. BAP and 2iP stimulated the production of maxillary shoots regardless of their concentration. BAP and 2iP at concentrations of 3.0 mg/L and 0.5 mg/L, respectively, induced significantly the maximum shoots number (7 shoots/explant) than did other treatments (Fig. 1b). The results are in agreement with those of Soliman (2013), who found that maximum number of peach shoots were obtained on Woody Plant medium containing 2 mg/L BAP and 0.5 mg/L 2iP. The increases in shoot proliferation may be due to the effect of cytokinin, especially when added in appropriate concentration, where it regulates shoot proliferation, cell division and differentiation (Gross and Partiner, 1994). On MS medium without cytokinins (control), an average of 2.0 shoots per explant were recorded after six weeks of culture with the highest average shoot length (4.4 cm). It seems that BAP and 2iP could stimulate shoot growth if added to the media at low concentrations, while their high concentrations stimulate shoot multiplication.

**Table (2).** Effect of BAP and 2iP on *in vitro* shoot multiplication of Nemaguard peach rootstock after six weeks of culture on MS medium.

Treatments (mg/L)		Average no. of shoots/explant	Average shoot length (cm)
BAP	2iP		
0.0	0.0	2.0e	4.4a
0.5	0.5	5.6d	3.5b
1.0	0.5	5.6d	3.1c
2.0	0.5	6.5c	2.7d
3.0	0.5	7.0a	2.5e
4.0	0.5	6.8b	2.4e

The results indicate that BAP plays a key role in shoot propagation of Nemaguard peach rootstock. This cytokinin is an efficient growth

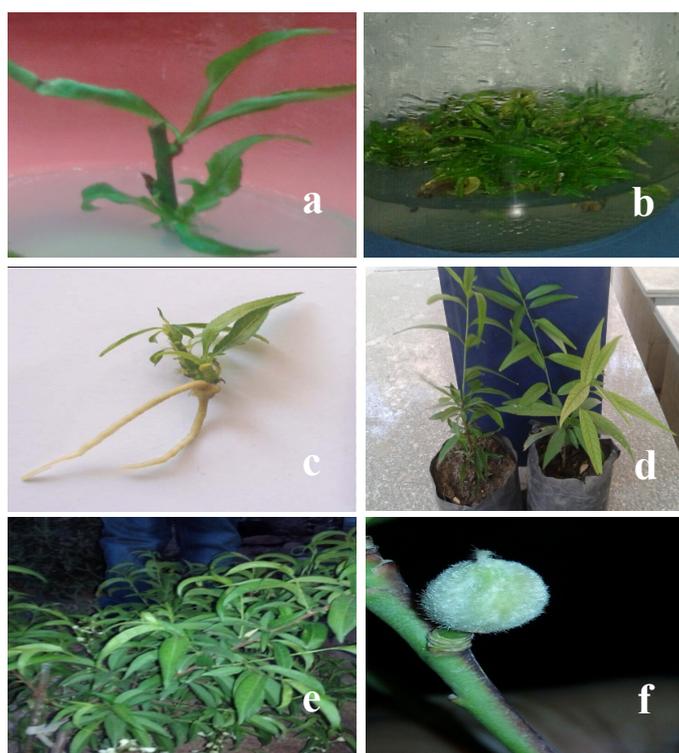
regulator for shoot multiplication in other plants, such as *Centaurea pauciflorus* (Cueneca et al., 1999), *Centaurea junoniana* (Hammat and Evans, 1985), *Solenostemma argle* (Abd Alhady, 2011), *Amygdalus communis* (Sharifinoghaddam et al., 2011) and Nemaguard, okinawa peach rootstocks (Edriss et al., 2014) and Volkamer lemon (Ahmed et al., 2017). For Nemaguard peach rootstock, BAP proved to be the most effective cytokinin for shoot multiplication but not for shoot elongation (Table 2). The increase in number of shoots of Nemaguard peach rootstock may be due to the physiological role of BAP, which is thus to break the apical dominance and stimulate growth of new shoots (Pruski et al., 2005). Shoot elongation decreased slightly with increasing BAP concentration. This result suggests an inverse relationship between the number of shoots and shoot elongation. The reduced shoot elongation was observed in other propagation protocols (Ault, 1994 and Cueneca et al., 1999).

### 3. Induction of Rooting and Acclimatization

About 3-4 cm long shoots with 3-4 leaves, harvested from *in vitro* proliferated shoots, were placed in half strength MS medium supplemented with different concentrations of IBA or NAA (0.0, 1.0, 2.0 and 3 mg/L). The shoots showed different responses to rooting after six weeks of culture (Table 3). The highest percentage of rooting (90%) was obtained on half strength MS medium supplemented with 3.0 mg/L IBA (Fig. 1c). On the other hand, in case of NAA, the highest rooting percentage of Nemaguard peach rootstock shoots (70%) was obtained at a concentration of 3.0 mg/L NAA. As shown in table (3), no rooting percentage was obtained on half strength MS medium without auxin. This result is in harmony with that obtained by Durkovic (2008). Also, Komalavalli and Rao (1997 and 2000) noticed that from the three auxins; IAA, IBA and NAA tested to induce rooting of *Gymnema sylvestris*, IBA (3 mg/L) was the most effective for root induction and survival in the field. The maximum number of root/shoot was also obtained when IBA were added to half strength MS medium at concentration of 3.0 mg/L. On the other hand, the maximum root length (8.5 and 7.2 cm) was obtained when IBA or NAA were supplemented to half strength MS medium at 3.0 mg/L.

**Table (3).** Effect of IBA and NAA on the *in vitro* rooting of Nemagard peach rootstock after six weeks of culture on ½ MS medium.

Treatments (mg/L)		Rooting percentage (%)	Average roots/shoot	Average root length (cm)
IBA	NAA			
0.0	0.0	0	0.0	0.0
1.0	0.0	70	2.5d	5.5e
2.0	0.0	80	3.7b	7.0c
3.0	0.0	90	5.8a	8.5a
0.0	1.0	50	1.5f	4.5f
0.0	2.0	60	2.0e	6.5d
0.0	3.0	70	3.5c	7.2b

**Fig. (1).** *In vitro* propagation of Nemagard Peach rootstock; **(a)** establishment of Nemagard Peach rootstock, **(b)** multiplication of shoots, **(c)** *in vitro* rooted plantlet after six weeks of culture, **(d)** plantlets acclimatized in greenhouse, **(e)** *in vitro* Nemagard Peach rootstock planted under saint Catherine conditions and **(f)** plants producing seeds.

Endogenous hormones might have a role in promoting plants to root. Until the hormonal balance reached its optimal level to push the roots to grow and develop in the presence of exogenous hormones, since increasing auxin concentration promotes root formation on shoot bases (George and Shermington, 1984). The use of half-strength MS medium for root induction of Nemaguard peach rootstock was supported by Beena et al. (2003), who reported that half-strength MS medium induced more roots compared to full-strength MS in *Ceropegia candelabrum*. In the root meristem, auxin is implicated in regulating the pattern of cell division and differentiation (Friml, 2003). According to Puente and Martin (1997); if the shoots are competent to root, rooting rate could be increased easily. It has been reported that shoot characteristics sent as size and shoot culture origin fall to attain a stabilized growth phase or apparent rejuvenation can also lead to a variable rooting response (Marks and Simpson, 2000). The beneficial effect of IBA on rooting has been observed in many plant species (Linh, 2001; Amri et al., 2010; Cheniany et al., 2010 and Ahmed et al., 2017). Lack of rooting morphogenesis may be due to lack of cell sensitivity to respond to morphogenesis (Hartmann et al., 1997). Similarly, auxins are involved in the process of adventitious root formation in many woody plants; IBA is commonly used to promote root initiation.

Plantlets with well-developed root system were transferred to plastic pots covered with translucent plastic bags to ensure high humidity around the plants. The use of this procedure during the acclimatization phase ensured that most plantlets transplanted to *ex vitro* conditions continued growing vigorously. After six weeks and when the plastic bags were removed, 90% of the plantlets survived in the greenhouse and showed no sign of water stress (Fig. 1d). Thereafter, the regenerated plants showed normal growth. The *in vitro* Nemoguard peach rootstocks were planted in Saint Catherine, South Sinai, Egypt where chilling requirements for producing the rootstock seeds are available for commercial production (Fig. 1e and f). Hence, it is encouraged to grow Nemoguard peach rootstock under Saint Catherine conditions for commercial seed production.

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## الإكثار المعملّي لأصل الخوخ (نيما جارد)

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وحدة زراعة الأنسجة، قسم الأصول الوراثية، مركز بحوث الصحراء، المطرية، القاهرة، مصر

أجريت هذه الدراسة للإكثار الدقيق لأصل الخوخ النيماجارد، حيث أستخدمت الأجزاء الساقية البرعمية كمنفصلات نباتية للتأسيس المعملّي. حققت المنفصلات النباتية المزروعة على بيئة مورايشيج وسكوج (MS) والمزودة بـ 0.5 ملليجرام/لتر بنزيل أدنين (BAP) + 0.2 ملليجرام/لتر أندول حمض الخليك (NAA) 100% نسبة حياة مع أعلى نسبة نمو بالنسبة للمنفضلات الحية مع أعلى متوسط طول للفرع. أوضحت الدراسة أيضًا أن أعلى متوسط عدد للأفرع الناتجة بالنسبة لكل منفصل نباتي تم الحصول عليه على بيئة مورايشيج وسكوج المزودة بـ 3 ملليجرام/لتر بنزيل أدنين + 0.5 ملليجرام/لتر أيزوبنتنيل أدنين (2iP)، في حين أن البيئة الخالية من أي منظمات نمو أعطت أعلى متوسط طول للفرع، مقارنة بالمعاملات الأخرى، كما أن متوسط طول الفرع انخفض بزيادة تركيز البنزيل أدنين.

تم الحصول على أعلى نسبة تجذير (90%) عندما زرعت الأفرع الناتجة من مرحلة التضاعف على بيئة مورايشيج وسكوج المزودة بـ 3 ملليجرام/لتر أندول حمض البيوتريك (IBA) والحصول على نبات كامل. وكان عدد الجذور لكل فرع (5.8 جذر/فرع). على الجانب الآخر تم الحصول على أقصى طول للجذر (7.2-8.5 سم) عندما أضيف 3 ملليجرام/لتر أندول حمض البيوتريك أو نقتالين حمض الخليك إلى نصف قوة بيئة مورايشيج وسكوج أعلى نسبة حياة للنبات تم الحصول عليها عندما نقلت النباتات الناتجة إلى ظروف الصوبة. تم في هذا البحث عملية الإكثار الدقيق بالأجزاء الساقية البرعمية لأصل الخوخ (نيماجارد) بنجاح دون ضرر للنبات الأم، وفي النهاية النباتات الناتجة معملّيًا تم زراعتها تحت ظروف سانت كاترين، جنوب سيناء، مصر. وقد تحقق للنباتات المزروعة إحتياجاتها من البرودة وأزهرت وأثمرت. وإزهار وإثمار النباتات المزروعة تحت ظروف سانت كاترين تشجع على الإنتاج التجاري لبذور النيماجارد وإنتاجها محليًا وعدم استيرادها من الخارج.