Establishment of Regeneration system for Taif peach (*Prunus persica* L. Batsch) cultivar (Balady cultivar) in Taif, KSA.

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Abstract: Peach (*Prunus persica* L. Batsch) is one of the most important stone fruits in different parts of the world. Peach trees are essentially infected by many different pests and diseases. One of basic requirement for successful transformation system is reliable high-frequency regeneration. Shoot organogenesis could be induced from stored mature embryos of Taif peach (*Prunus persica* L.) cultivar *in vitro*. In this study, the influences of different cytokinin (TDZ) levels and dark incubation periods on shoot regeneration were determined. Taif peach cultivar regenerated in the presence of TDZ and darkness for the first 13 days of the culture. Dark incubation at the early stage of culture was critical for obtaining higher regeneration efficiencies from stored Taif peach embryos. Media containing 3.6 µM TDZ with 2.5µM IBA was more effective than other TDZ levels in inducing shoot regeneration. The highest regeneration frequency was obtained with concentrations of 3.6 µM TDZ (62.5%) in combination with dark incubation. Root percentage of the regenerated shoots was 38.2% by addition of 10.74 µM NAA in the medium. Regeneration of adventitious shoots from stored mature embryos of Taif peach cultivar is obtained. Therefore, it could be suggested that Taif peach cultivar has a potential for genetic transformation through this regeneration protocol.

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1. Introduction

Peach trees are actually plagued by many different pests and diseases whereas about 20% or more from peach fruits were lost through packaging and transportation processes, due to its soft skin and juicy flesh. Thus, peach need more attention to improve their fruit quality i.e. (delay softening, retard overall ripening and extended shelf life) as well as to increase trees productivity.

Breeding fruit tree *via* conventional procedures is difficult and expensive process for several reasons including heterozygosity, polyploidy, long breeding cycles, and long field trial procedures. Genetic transformation and gene modern manipulation are alternative tools to improve fruit trees.

Improvement of peach through traditional breeding methods is a long– term effort, because of its long generation time, absence of divers' germplasm, as illustrated by peach cultivars, also limited progress in peach improvement. Therefore, new approaches are needed to expand the germplasm base for peach plant gene transfer to offer the peach breeder the opportunity for transfer specific genes into peach germplasm. The first step to improve fruit plant through genetic modification is to study their capability for regeneration and transformation systems, one requirement for successful transformation system which is reliable high-frequency regeneration. Genetic transformation protocol has been reported for peach (*P. persica*) (Wu *et al.*, 2006). Development of an effective genetic transformation system for peach depends largely on the availability of an efficient and reliable regeneration system and a development of reliable regeneration systems from mature tissues is a prerequisite for the application of gene transfer techniques to the improvement of woody species (Litz and Gray, 1992).

Several important aspects of regeneration of European plum from hypocotyl explants were studied. Multiple shoots were induced and full plants were recovered for a large number of plum varieties. This indicates that European plum species is, in general, very responsive to *in vitro* regeneration from hypocotyls. Shoot organogenesis could be induced from both mature and immature seed explants but regeneration efficiency was higher when immature seeds were used. Rooting efficiency for varieties with low rooting tendency could be greatly increased by addition of naphthaleneacetic acid in the medium (Lining *et al.,* 2007).

Plant regeneration from a tissue culture system is often the most critical step in the success of various biotechnological techniques of any plant

improvement program. The induction of somatic embryogenesis directly on explants tissues or in callus cultures has been reported in many fruit trees species including the peach (Hammerschlag, 1986). In peach (*Prunus persica* L.), shoot regeneration from embryonic tissues has been reported (Meng and Zhou, 1981; Pooler and Scorza, 1995) but no regeneration of plants from mature explants has been obtained so far (Gentile *et al*., 2002).

There are a number of reports of regeneration of adventitious shoots from various explants of peach; where, regeneration of adventitious shoots has been achieved from immature and mature cotyledons of peach (*Prunus persica* L.) (Pooler and Scorza, 1995 Yan and Zhou, 2002 and Wu *et al*., 2005;) and, regeneration using mature embryo slices has been reported for El- Sheikh Zewaied peach cultivar plants (Nagaty *et al*., 2007).

Using stored seed, although not clonally propagated cultivars are yet to be developed for many species and at least allow for transformation and regeneration year – round (Mante *et al*., 1991). Although transformation of seed-derived material is of limited use in improving vegetative propagated peach scion cultivars, it could have an impact on the development of new seed-propagated lines used in some countries (Perez *et al*., 1993).

Among growth regulators used in peach tissue culture media are the cytokinins, kinetin and BA, and the auxins IBA, IAA and NAA (Hammerschlag, 1986). BA has been generally used for *in vitro* shoot proliferation of peach (*Prunus persica* L. Batsch) (Almehdi and Parfitt, 1986 and Hammerschlag *et al*., 1987).

Canli and Tian, 2008 and 2009, maintained that TDZ was more effective in inducing shoot regeneration from mature stored cotyledons for Japanese plum and sweet cherry than BA and dark incubation significantly increased the regeneration frequencies.

Nemaguard peach rootstock achieved 95% rooting after 6 weeks by subculturing shoots on MS medium supplemented with 50 mg/L ascorbic acid and 0.1 mg/L NAA (Miller *et al*., 1982). Cultured shoots of Nemaguard rootstock on MS medium supplemented with IAA, IBA or NAA, NAA resulted in best rooting (Hammershlag *et al*.,1987). NAA proved to be superior to IBA with Okinawa and Nemaguard rootstocks and increasing auxin concentrations increased root number especially with NAA (Guindy, 1990). With two peach genotypes, Antonelli and Chiariotti (1988) showed that, chilled explants rooted more easily, while using activated charcoal strongly inhibited rooting, even in the presence of auxins.

Induced shoots of European plums *in vitro* usually exhibited low rooting efficiency (Tian *et al*., unpublished results), which is the major obstacle for full plant recovery, given the low transformation efficiency of European plum (Gonzalen-Padilla *et al*., 2003).

The aim of this study is develop *in vitro* peach regeneration method for Taif peach cv. through using mature explants (mature embryos). This study was carried out to assess the potential for induction of adventitious shoot regeneration from stored mature embryos of Taif peach cv. which cultivated by seed in Taif Governorate, KSA. The present regeneration developed protocol represents a potential system for genetic transformation of Taif peach cultivar. Fruit characteristics of Taif peach cultivar were studied before (Nagaty and El-Assal, 2011).

2. Materials and Methods:

2. 1. Shoot regeneration using mature peach embryo

Plant material:

Seeds obtained from ripe fruits of local Taif peach trees, which are mainly grown at Bani Saad region, Taif Governorate, KSA. Peach trees are originated from seeds; the full bloom date is usually at the third week of March and ripens at the fourth week of June. The embryo explants isolated from mature stored seeds according to Mante *et al*. (1991) method. Explants were cultured on shoot regeneration medium (SRM) as described by Lining *et al*. (2007).

Explant preparation:

Stones collected from ripe fruits washed with tap water, cleaned with a sodium hypochlorite solution (0.05%), and rinsed under running water for 5 min. Stones were dried at room temperature (20–25℃) for 3–4 days and then stored at 4℃ in plastic mesh bags. The stones were cracked open carefully with a nutcracker and the seeds were collected, disinfected with a 0.5% sodium hypochlorite solution containing 0.005% Tween 20 for 15 min and rinsed three times with sterile water. As recommended by Fred and Davis (1983), seeds were soaked in sterile water containing 3000 ppm GA_3 at room temperature overnight for breaking seed dormancy and softening tissue texture for easier dissection. Seed coat was removed with the aid of a scalpel. The embryonic axis was excised from cotyledons. The embryonic axis was cut into two slices across the axis (each was about 0.5–1 mm thickness; Fig. 1B) under a dissection microscope and slices were cultured on Shoot Regeneration Medium (SRM).

Shoot regeneration medium:

Shoot Regeneration Medium(SRM) consisted of MS salts (Murashige and Skoog, 1962), 555 µM inositol, 1.2 µM thiamine HCl, 4.1 µM nicotinic acid, 2.4 µM pyridoxine HCl, 2.5 µM indolebutyric acid (IBA), 25 g /1 sucrose and 2g / l gelrite (Lining *et al.*, 2007). The pH adjusted to 5.7-5.8 by 1M KOH and 1M HCl prior to gelrite addition before autoclaving for 30 min (in 1 L containers).

Thidiazuron (TDZ) at 0.0,1.8,3.6 and 7.2 µM concentrations were added to SRM medium before pouring approximately 35 ml into 100 mm X 25 mm Petri dishes. Eight embryonic axis segments were placed in each Petri dish (which was replicated 3 times). Explants were incubated on SRM at 26°C for 12-14 days in dark. Then, transferred to incubate at 26±1˚C under a 16/8-h (light/dark) photoperiod with a Photosynthetic Photon Flux of 50 µmol m^{-2} s^{-1}. Shoot induction was calculated after 1 month in culture. When shoots reached 0.5-1 cm in length, they transferred to shoot elongation medium [consist of basal SRM with 29 µM gibberellic acid (GA_3), 1ml (1.2 µM thiamine HCl)] and Cultures were grown in the culture room at 26±1˚C under 16/8-h (light/dark) photoperiod with light provided by white fluorescent tubes according to Nagaty *et al.*(2007). When shoots were 3cm tall, they were rooted by transferring it to rooting medium.

2.2. Rooting stage

In vitro peach shoots induced from explants were excised and transferred to a different rooting medium consisting of half strength MS salts supplemented with 25g/l sucrose, 555 µM inositol, 1.2 µM thiamine HCl, 2mg/l calcium pantothenate and 2g/l gelrite. Both IBA at 4.90 and 9.80 µM and NAA at 5.37 and 10.74 µM were used to induce roots from *in vitro* shoots. Medium pH was adjusted to 5.7-5.8, and autoclaved at 121°C (15 psi) for 20 min then poured into 100 mm ✕ 25mm Petri dishes. Cultures were incubated in total dark at 5ºC for 2 weeks followed by incubation under 16/8-h (light/dark) at 26±1˚C .This modification to enhance the rooting ability of *in vitro* peach shoots according to Nagaty *et al.*(2007).

2.3. Data analysis

The obtained data were evaluated by the analysis of variance method (Snedecor and Cochran, 1972). Duncan´s multiple range was used for the comparison among the means (Duncan, 1955). Computation was performed using MSTATC computer program package (Russell, 1986).

3. Results and Discussion:

In contrast to results obtained with immature peach seed (Mante *et al.*, 1989), preliminary experiments with mature seed indicated that two weeks of darkness was necessary for shoot initiation and regeneration.

3.1. Shoot regeneration

In vitro embryonic axis were detached from stored mature seeds (Mante *et al.*, 1991) and divided to slices, these segments cultured under aseptic conditions on shoot regeneration medium (SRM) to initiate callus induction and shoot formation (Fig. 1B). Embryonic axis segments expanded in size (swollen) and turned to white color (Fig. 1C). Embryonic axis segments produced small calli and adventitious white shoots started to become visible around 19-21 days after culturing (Fig. 1E) in SRM media containing TDZ. The shoots turned green when they were transferred to light (Fig. 1G-H). The adventitious shoot buds often emerged in clusters and developed on the surfaces of embryonic axis segments.

Regenerating mature embryos were characterized by a swelling of the embryonic axis segments at the proximal end after 1 to 2 weeks in culture, followed in some cases by callus production and shoot development from the proximal end. Embryos slices with little callus development generally produced only one to two large shoots per one embryonic axis slice.

The effect of TDZ concentrations on shoot regeneration from embryonic axis explants was found to be significant at $p \leq 0.05$ by ANOVA (Table 1). Most peach embryonic axis explants produced adventitious shoots when the explants were incubated in the dark on the TDZ containing medium. In general, the highest regeneration frequencies were obtained from dark incubated treatments containing 1.8 or 3.6 µM TDZ (Table 1). The highest regeneration frequency (62.5%) was obtained from the dark incubated explants of Taif peach cultivar at 3.6 µM TDZ (Table 1). Those findings were agreed with Canli and Tian, 2008 and 2009 publications.

Results from the current study indicate that the dark incubation significantly increased the shoot regeneration efficiency of Taif peach cultivar. Data emphasized that the dark condition is very critical factor for shoot regeneration at least for Taif peach cultivar. Embryonic axis segments *in vitro* subjected to dark condition at 26 °C for 2 weeks.

These results are in agreement with the previous reports on regeneration of shoots in *Prunus* species where the effectiveness of dark incubation in promoting callus formation and/or shoot regeneration from cotyledon explants was reported in *P. dulcis*

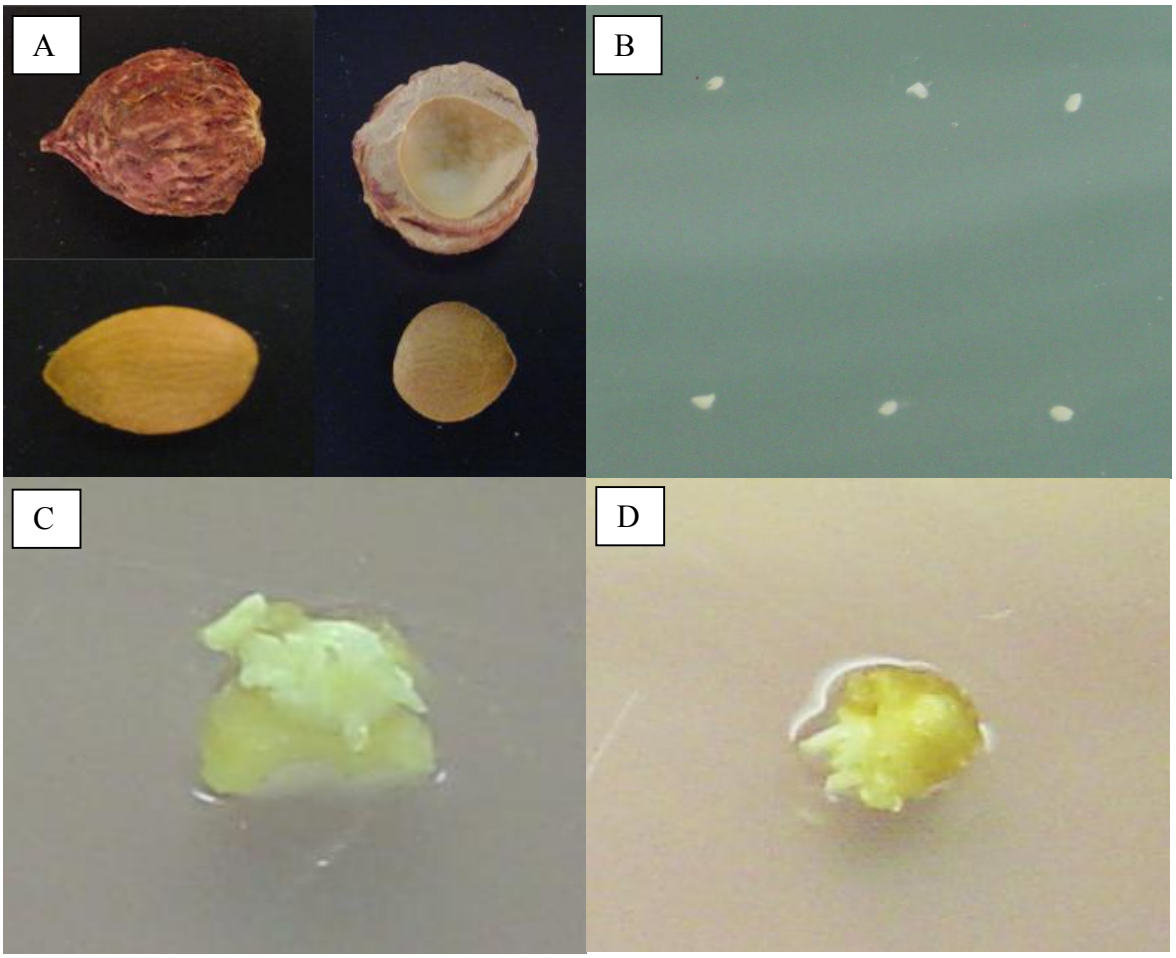
(Ainsley *et al*., 2001) and *P. armeniaca* (Nedelcheva and Tsoneva, 1998).

In the same time, it has been shown that dark incubation is critical for regeneration of shoots in some *Prunus* species; (Miguel *et al*., 1996). Dark incubation could be promoting organogenesis by increasing the levels of endogenous plant growth regulators such as indoleacetic acid (IAA). This hormone, known to influence cell division and organogenesis,which interacts with exogenously given plant growth regulators and promote regeneration (Pooler and Scorza, 1995; Miguel *et al*., 1996; Gentila *et al*., 2002). Espinosa *et al*. (2006) also reported that higher percentages of regeneration were obtained when leaf explants of *P. serotina* maintained in the dark for 3 weeks and then transferred to light for 2 weeks.

Here we showed that stored mature Taif peach embryos could be induced to regenerate shoots with high frequencies if they are incubated in the dark for the first 13-14 days of culture on the TDZ containing medium and TDZ was more effective for inducing adventitious shoot formation from mature stored embryos of Taif peach cultivar. Similarly, TDZ was found to be more favorable than BA in regeneration of adventitious shoots in many other species (De Bond *et al*., 1996; Sarwar and Skirvin, 1997; Hammatt and Grant, 1998). The effectiveness of TDZ in promoting shoot formation was also reported in *prunus* (Bhagwat and Lane, 2004; Matt and Jehle, 2005; Blando *et al*., 2007).

In spite of TDZ function as a cytokinin, it is precisely not known. On the other hand, the improved regeneration using TDZ rather than BA is well documented in many woody species (Huetteman and Preece, 1993).

Data for the number of shoots per regenerating explants was not presented because it was difficult to perform an accurate count of clusters for shoots and adventitious buds turning into new small shoots.



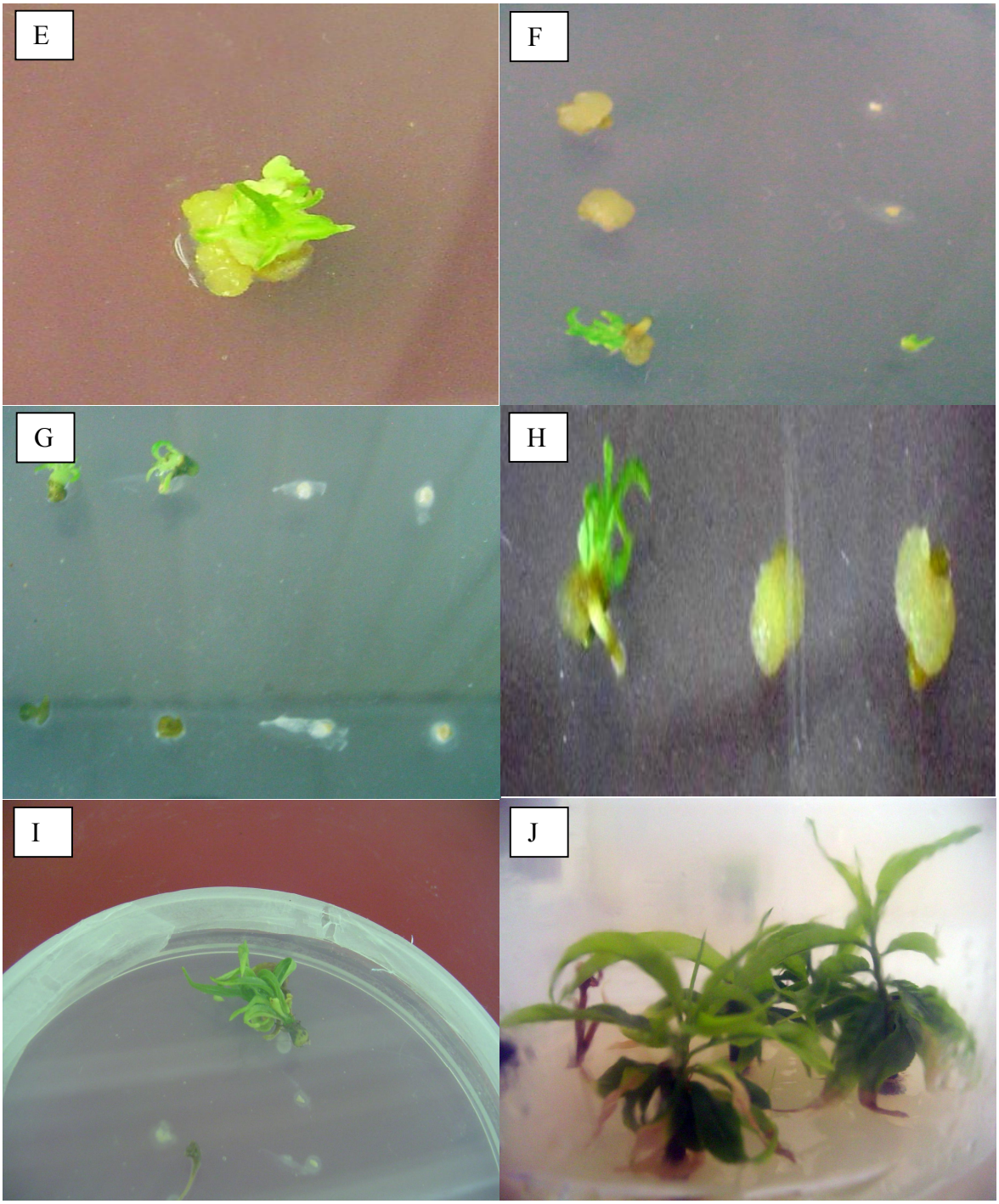


Fig.1. Regeneration steps of adventitious shoots from mature embryos of Taif peach (Prunus persica) cultivar (A) Taif peach cv stone was cracked and excided mature seed; (B) mature embryo segments of cv. Taif peach cultured on shoot regeneration media at day 1; (C-D) small calli and white shoots were proliferated from embryonic axis segments; (E-F) emergences of adventitious shoot primordia and distinguished shoots from 20 day-old mature embryonic axis explants of cv. Taif peach; (G-H) Close up of the shoot primordial and newly emerging shoots at day 20 and (I-J) Adventitious shoots of cv. Taif after 30 days from the culture initiation.

Table 1. Effect of TDZ levels on shoot regeneration frequency of Taif peach cultivar from mature embryos cultured on MS medium under dark incubated condition.

Treatments	% Regeneration
Full MS+IBA(2.5µM)+ TDZ (0)	16.6[c*]
Full MS +IBA(2.5µM)+ TDZ (1.8 µM)	33.3[b]
Full MS +IBA(2.5µM)+ TDZ (3.6 µM)	62.5[a]
Full MS +IBA(2.5µM)+ TDZ (7.2 µM)	37.5[b]

*Mean separation within column by Duncan's multiple range test at $p \leq 0.05$. Each treatment consisted of 3 plates with eight embryonic axis segments per plate.

3.2 Rooting stage

Individual shoots or shoot clusters induced were transferred to medium without TDZ for rooting**.** Table (2) and Fig.(2), indicate that the medium constituent of half strength MS salts supplemented with TDZ free and pH adjusted to 5.7-5.8 was the best medium for root formation especially with NAA at 5.37 and 10.74 µM (Fig.2 B-C), these concentrations were significantly differences than IBA at 4.90 and 9.80 µM. The present results in agreement with (Miller *et al*., 1982; Hammerschlag *et al*., 1987 and Lining *et al*., 2007) whereas, the auxin (NAA) proved to be superior than IBA with Kinawa and Nemaguard peach root stocks and increasing the concentration of auxin was positively affected the average number of roots/explant. In addition, the difference in rooting capacity may due to sort and level of endogenous rooting substances and rooting cofactors (Feliciano and de Assis, 1983).

When cultures were kept in the dark on half strength MS basal salts containing 4.90 and 9.80 µM IBA, the rooting frequency of Taif peach cultivar were 0 and 22.3%, respectively (Table 2). Root percentage were 26.4% and 38.2% for *in vitro* shoots cultured on ½ MS basal salts containing 5.37 and 10.74 µM NAA, respectively. Root numbers were greater by ½ MS basal salts containing 10.74 µM NAA (2.8±0.8) than ½ MS basal salts containing 5.37 µM NAA (1.5±0.6) (Table 2 and Fig. 2). This results in agreement with (Ainsley *et al*., 2001 and Gonzalen-Padilla *et al*., 2003).

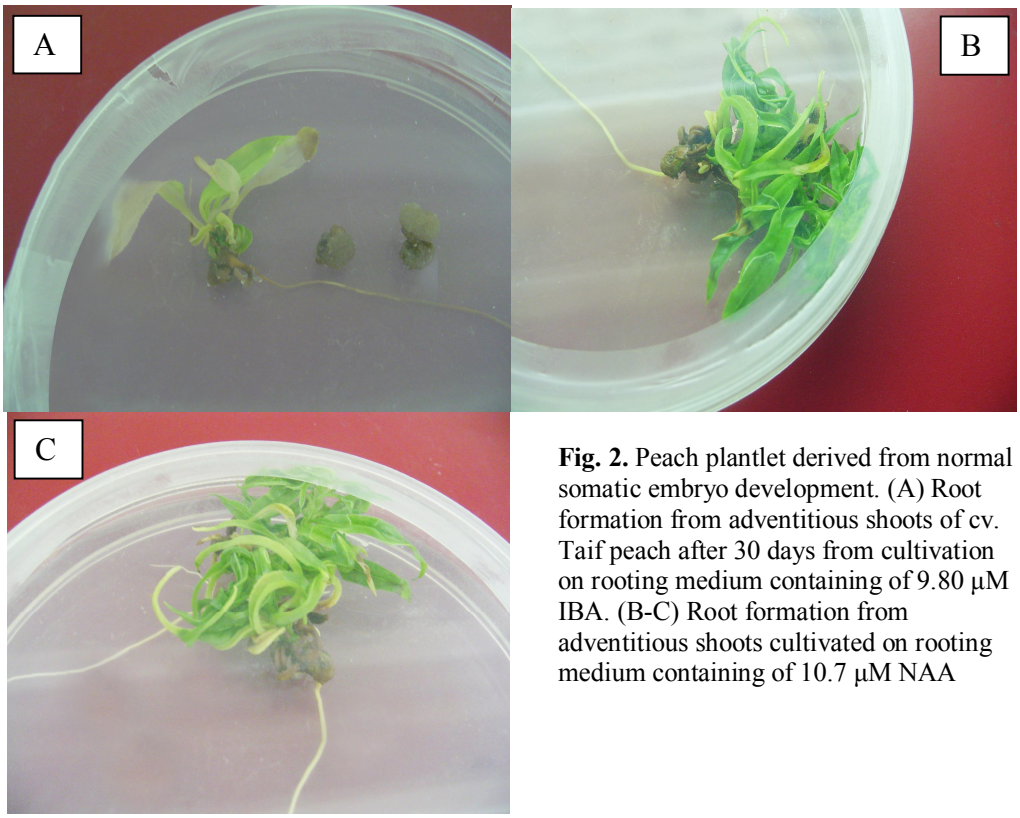


Fig. 2. Peach plantlet derived from normal somatic embryo development. (A) Root formation from adventitious shoots of cv. Taif peach after 30 days from cultivation on rooting medium containing of 9.80 µM IBA. (B-C) Root formation from adventitious shoots cultivated on rooting medium containing of 10.7 µM NAA

Table 2. *In vitro* rooting of peach shoots obtained from mature embryonic axis of cv. Taif peach on ½MS using different rooting growth regulators.

Treatments µM		Root %	Root number [a]
IBA	0.20	0	-
	0.40	22.3	1.2 ± 0.5
NAA	0.20	26.4	1.5 ± 0.6
	0.40	38.2	2.8 ± 0.8

[a] Average root number (mean ± S.E.).

3.3 Data analysis

Experiments were arranged in a completely randomized design and observations were recorded 4 weeks after culture initiation. The analysis of variance (ANOVA) was performed on the data with mean separation by Duncan´s multiple range test. Data were analyzed using MSTATC computer program package (Russell, 1986).

4. Conclusion and Recommendation

Seed material is useful for several strategies in genetic engineering of *Prunus* germplasm such as resistance assessments to insects, diseases and herbicides, gene silencing, gene expression studies, and marker gene assessments.

This report demonstrates that adventitious shoots could be induced from mature stored embryos of cv. Taif peach (*Prunus persica* L.). The present protocol might be a potential system for the improvement of the transformation efficiency in Taif peach cultivar, where regeneration of adventitious shoots with relatively high regeneration and efficiency rate were achieved from stored mature embryos of cv. Taif peach. This leads to be a potential candidate for genetic transformation for cv. Taif peach *via Agrobacterium* infection or particle gun bombardment.

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