

***In Vitro* Regeneration and Genetic Transformation of Peach (*Prunus Persica* L.) Plants**

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Abstract: Peach (*Prunus persica* L. Batsch) is one of the most important stone fruits in different parts of the world. Local peach trees located in North Sinai Governorate, Egypt are essentially infected by many different pests and diseases. Regeneration of adult plant material is one of the main limitations for successful peach (*Prunus persica* L.) transformation. Plant regeneration protocol was developed for direct organogenesis, using peach young leaf segments as explants, and accomplished by studying the influence of different concentrations and types of cytokinin and auxin hormones. The best regeneration for peach (64.8 %) was observed on WP medium with 3 mg/l TDZ and 1.0 mg/l NAA in darkness for two weeks, followed by transfer to the light for three weeks. The highest mean length of shoots (1.35 cm) was obtained on medium containing 3 mg/l TDZ + 0.5 mg/l NAA. Adventitious shoots were rooted (92%) and rooted plantlets survived after acclimatization to the greenhouse (91%) on liquid MS medium supplemented with 3% sucrose, 100 mg/L myoinositol, 2 mg/l IBA, 0.5 mg/l NAA and 162 mg/l phloroglucinol. An *Agrobacterium tumefaciens*-mediated transformation system was developed for peach. The binary vector pISV2678 with the *bar* and the *gus-intron* genes was transferred into young leaf segments of peach. The obtained putative transgenic plantlets were able to grow under medium containing 3 mg/l bialaphos. Transformation efficiency was 26.7% by using *Agrobacterium*-mediated transformation system. The presence of *gus-intron* (2070 bp) and *bar* (540 bp) genes was confirmed *via* polymerase chain reaction while their expressions were detected by histochemical GUS assay and leaf painting with 2 mg/L Basta herbicide. Southern blotting technique was carried out to confirm the integration of *bar* gene in the transformed tissues.

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1. Introduction:

The peach [*Prunus persica* L. (Batsch)] is a member of the family *Rosaceae*, all commercial cultivars belong to [*P. persica* (L.) Batsch]. Peach crop is considered to be one of the most important fruit crops for farmers in North Sinai. Peach acreage reached about 59257 feddans in North Sinai; these areas produce about 14000 ton / annually from fresh fruits. Peach trees are actually plagued by many different pests and diseases before and after harvest, and about 20% of peach fruits were damaged through packaging and transportation processes, due to its soft skin and juicy flesh. Therefore, it needs further breeding and genetics research to improve peach quality (delay softening, retard overall ripening and extended shelf life), productivity and value of peach crop (Nagaty *et al.*, 2007).

Genetic transformation provides the means for adding single horticultural traits in existing cultivars without modify their commercial characteristics. This capability is particularly valuable for perennial plants and fruit tree species, in which conventional breeding is hampered by their long generation time and juvenile periods, complex reproductive biology, high levels of heterozygosity, limited genetic sources and linkage drag of undesirable traits from wild relatives. In

addition, gene transfer technologies for fruit tree species take the inherent advantage of vegetative propagation used for their reproduction, which allowed for the application of a high scale production of the desired transgenic line starting from one successful transformed line. Genetic improvement of fruit trees is essential for increasing fruit production. For most of these species, the desired new varieties contemplate the presence of agronomic and horticultural traits related to propagation, yield, appearance, quality, disease and pest control, abiotic stress and shelf-life (Humberto, 2011).

Adventitious regeneration is a key in the application of genetic engineering techniques to accelerate traditional plant breeding. The perennial *Prunus* species is one of the most recalcitrant in producing adventitious shoots, although reports on the successful regeneration and transformation of different species have appeared in recent years (Song and Sink, 2005). Adventitious shoot regeneration using leaf explants has been established for several species of *Prunus*, such *P. domestica* (Bassi and Cossio, 1991; Nowak *et al.*, 2004), *P. persica* (Declerck and Korban, 1996; Gentile *et al.*, 2002), *P. armeniaca* (Petri *et al.*, 2008; Perez-Tornero *et al.*, 2000), *P. avium* (Bhagwat and Lane, 2004) and *P. serotina* (Espinoza *et al.*, 2006;

Liu and Pijut, 2008:). Transformation and regeneration using leaf tissue have also been reported for *Prunus*. A recent review (**Petri and Burgos, 2005**) indicates only few instances of successful transformations of clonal material from *prunus* resulting in transgenic plants. More recently, the stable transformation of *P. armeniaca* (**Petri et al., 2008**), *P. dulcis* (**Ramesh et al., 2006**) and *P. cerasus* (**Song and Sink, 2006**) has been reported. There are only a limited number of reports of in vitro adventitious shoot regeneration from leaf explants of *P. persica* and *P. davidiana*. One paper about regeneration from peach leaf tissue has been published to date (**Gentile et al., 2002**), which was the first to report adventitious shoot regeneration from mature leaf explants of *P. persica*. The aim of the present study is to develop a simple and reproducible method for *in vitro* propagation and regeneration of local peach (*Prunus persica* L.) from leaf explants. This study also aimed to developing a

reproducible protocol for transformation through *Agrobacterium*-mediated transformation system.

2. Material and Methods

The experiments of the present study were conducted in the Biotechnology and Tissue Culture Labs. at Marout Research station, Alexandria, Egypt, during 2009-2013.

Plasmids.

The plasmid pISV2678 (Unpublished data) harbors the *gus-intron* under the control of 35S promoter and *nos* terminator as well as the *bar* fused to the AMV Leader gene under the control of *nos* promoter, pAg7 terminator (**Becker et al., 1992**) was used to optimize the transformation system in peach via *Agro-bacterium* mediated transformation (**Horch et al., 1985**). The plasmid was kindly provided by Dr. P. Ratet, Institut des Sciences Vegetales (ISV), Centre National de la Recherche Scientifique (CNRS), Gif-Sur-Yvette, France (**Fig.1**).

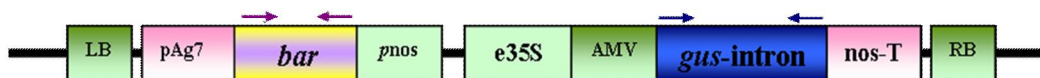


Figure 1. A diagram showing the physical map of the binary vector pISV2678 containing the *gus-intron* reporter and the *bar* selectable marker genes.

Micropropagation of local peach (*P. persica* L.).

Micropropagation of local peach (*P. persica* L.) was established *in vitro* from nodal sections of mature trees. Nodal sections (2–3 cm in length) were excised from 5 to 7 years-old mature trees of *P. persica* located in North Sinai Governorate, Egypt. Nodal sections were surface disinfested in 70% (v/v) ethanol for 30s, then in 15% (v/v) commercial bleach (5.25% sodium hypochlorite) for 20 min, followed by four rinses in sterile, deionized water. Nodal sections were placed vertically in glass jars containing 40 ml of MS medium with 3% (v/v) sucrose, 2 mg/l BA, 0.2 mg/l indole-3-butyric acid (IBA), and 2.5 g/l phytigel. The pH of the medium was adjusted to 5.7 with KOH before the addition of phytigel and was autoclaved at 1.1 kg cm² and 121°C for 20 min. Cultures were incubated at 26°C under a 16-h photoperiod provided by cool-white fluorescent lamps at an intensity of 32–40 μmol m⁻²s⁻¹. Explants were transferred to fresh medium every 3 weeks for induction of shoots.

Adventitious shoot regeneration.

Leaf segments (0.5–1.0 cm²) that included the petiole from 3-weeks –old *in vitro* grown shoots were wounded by several cuts transversely along the midrib, and cultured abaxial side up on medium. Leaf explants were placed on WP medium (**Lloyd and McCown, 1981**) supplemented with 1–5 mg/l TDZ in combination with 0.5–2 mg/l IBA or 0.5–1 mg/l NAA. The explants were placed in the darkness for two weeks before

exposure to light with a 16-h photoperiod (32–40 μmol m⁻²s⁻¹) at 25°C for three weeks. Regeneration percentage and number of adventitious shoots per leaf explant were recorded six weeks after the beginning of the experiment. Young shoots were transferred to fresh medium containing 1 mg/l BA and 0.5 mg/l 2iP. Shoots were maintained on WP medium and transferred to fresh media every four weeks.

Shoot multiplication.

For multiplication of shoots, the young shoot explants were cultured on solid WP medium supplemented with different concentrations of BAP (1.0 and 2.0 mg/l), 2iP (0.5 and 0.75 mg/l), kinetin (0.5 and 1.0 mg/l), 30 g/l sucrose and 2.5 g/l phytigel. All cultures were incubated in a controlled growth room at 26°C ± 2 under 8/16 h (dark/light) fluorescent lights. Shoots were maintained for root induction experiments.

Root formation and acclimatization of regenerated plants.

The elongated shoots were excised individually and transferred onto full strength MS salts with 3% sucrose, 100 mg/L myo-inositol and 162 mg/L phloroglucinol (liquid medium), containing different concentrations of IBA (1.0–3.0 mg/l) alone or in combinations with 0.5 mg/l NAA in the dark for 7 days. After induction period, shoots were transferred to a hormone-free medium under a 16 h photoperiod for one week; afterwards, shoots were transferred to a

rooting medium at $26^{\circ}\text{C} \pm 2$ under 8/16 h (dark/light) fluorescent lights for two weeks. After four weeks of culture on rooting medium, the effect of the different concentrations of auxins (IBA and NAA) on rooting percentage, number of roots and mean rooted shoots were evaluated in comparison to a hormone free medium. The plantlets were transferred to pots containing a mixture media of peat moss and sand (1:1v:v), covered with plastic bags to increase the humidity and grown under a photoperiod of 16/8 h light/dark in controlled greenhouse conditions. Plants were hardened by removing the plastic bags after 7–10 d.

Transformation system of peach (*P. persica* L.).

Herbicide bialaphos sensitivity test.

The herbicide resistance of non transformed peach tissues was determined, by planting

Leaf section explants on MS salts basal medium with different concentrations, i.e., 0, 1, 2, 3, 4 and 5 mg/l of Bialaphos (glufosinate ammonium), five leaf section explants for each concentration. The medium also contained 3 mg/l TDZ + 1.0 mg/l NAA to stimulate cell proliferation. Bialaphos was sterilized by filtration through disposable filters (0.22 μm) and incorporated into pre-cooled (45–50 $^{\circ}\text{C}$) autoclaved medium.

Agrobacterium mediate transformation.

Suspension of *A. tumefaciens* LBA4404-pISV2678 was prepared as follow: two ml Luria-Bertani (LB) broth that contained 100 mg/L kanamycin were inoculated with *Agrobacterium* and grown 16 h at 28 $^{\circ}\text{C}$ with vigorous shaking. An aliquot of 100 μl from the culture was used to inoculate 10 ml of a LB broth that contain 100 mg/L kanamycin and incubated at 28 $^{\circ}\text{C}$ with vigorous shaking until the absorbance at 550 nm was between 0.4 - 0.8 (equivalent to early log phase for most of the *Agrobacterium* strains). The cells were then pelleted and resuspended in an MS basal liquid medium. The leaf explants were inoculated by dunking in an *Agrobacterium* suspension for 10 - 15 min. After inoculation in this suspension, the explants were blotted dry on sterile filter paper, replated onto the suitable shoot formation medium and cocultivated for 3 days under optimized growth conditions. After cocultivation, the explants were transferred to a shoot induction media that contain 3 mg/l bialaphos and 300 mg/L carbinicillin (both antibiotics were added as filter sterilized solutions after autoclaving the media) to select transformed shoots and prevent bacterial growth.

After an additional six weeks of incubation in culture, the number of elongated shoots was counted. The elongated shoots were excised individually from the explants and subcultured on multiplication medium that containing 3 mg/l bialaphos. The experiment had

three replicates 30, 60 and 120 explants with a total number of 210 explants.

Histochemical GUS and leaf painting assays:

Histochemical GUS assay was carried out on transformed explants and developed shoots. The tested tissues were immersed in 1 mg/ml 5-bromo-4-chloro-3-indolyl glucuroinide (X-gluc) for 24 h and incubated at 37 $^{\circ}\text{C}$ over night. The upper surfaces of five fully expanded leaflets of plants grown in the greenhouse were thoroughly wetted by painting with BASTA herbicide at concentration of 2 g/L. Tolerance to bialaphos was scored two days after leaves painting for evaluate the expression of *bar* gene in transformed peach plants.

PCR assay:

The genomic DNA of peach was extracted from young leaves of putative transgenic plants by the method of **Delaporta *et al.* (1983)** and used in PCR analysis. Two pairs of specific primers were used to detect the putative transformed plants. The *bar* specific primers (namely P1, 5'AAA AGC TTC CAC CAT GAG CCC AGA ACG ACG3' and P2, 5'AAG GAT CCT CAG ATC TCG GTG ACG G') were designed to amplify 540 bp of the *bar* coding sequence, while the *gus* gene specific primers (namely, P3, 5'CCA GAT CTA ACA ATG CGC GGT GGT CAG TCC C3' and P4, 5'CCA GAT CTA TTC ATT GTT TGC CTC CCT GCT GC3') for amplifying the full *gus*-intron gene (2,070 bp). The PCR reactions were carried out in a total volume of 25 μl , containing 1 μl DNA, 20 pmol of each primer, 200 μM each dNTP, 0.5 unit *Taq* DNA polymerase and 3 μl 10x PCR buffer. The PCR temperature profile was as follows: initial denaturation of DNA at 94 $^{\circ}\text{C}$ for 5 min, 35 cycles comprised of 1 min denaturation at 94 $^{\circ}\text{C}$, 1 min annealing at 55 $^{\circ}\text{C}$ for *gus* gene or 60 $^{\circ}\text{C}$ for *bar* gene, 1 min elongation step at 72 $^{\circ}\text{C}$ followed by a final extension step at 72 $^{\circ}\text{C}$ for 7 min. Amplification products were analyzed by electrophoresis on 1 % agarose gels and detected by staining with ethidium bromide.

Southern blot analysis.

In this method, 25 μg of the genomic DNA from putative transgenic plants confirmed to be PCR-positive were digested with *Bam*HI then electrophoresed in 1% agarose gel and transferred to a positively charged Nylon membrane as described by **Sambrook *et al.* (1989)**. The PCR product of the *bar* gene (540 bp) was labeled with the DIG DNA Nonradioactive Labeling and Detection Kit (Roche) and used as a probe for hybridization experiments. Pre-hybridization, hybridization and immunological detection were carried out as recommended by the manufacturer. The membrane was incubated in 10 ml color solution (NBT/BCIP) under dark condition. After the appearance of the desired band, the reaction was

stopped by washing with 20 ml stop reaction buffer, supplied with the kit, for 5 min.

Statistical 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 Discussions

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* L.) (Wu *et al.*, 2006). Development of an effective genetic transformation system for peach depends largely on the availability of efficient and reliable regeneration systems. Development of reliable

regeneration systems from mature tissues is a prerequisite for the application of gene transfer techniques to improve woody species (Litz and Gray, 1992).

Adventitious shoot regeneration of peach (*P. persica* L.).

Several factors influence the efficiency of adventitious shoot regeneration, such as culture medium, genotype, growth regulators (cytokinin and auxin), size, age, position of the explants, and photoperiod, or light intensity. Regeneration in *Prunus* occurred more frequently if leaves were cultured on woody plant medium (WPM) (Lloyd and McCown, 1981) rather than Driver and Kuniyuki (1984) walnut (DKW) medium (Hammatt and Grant, 1998). Young, expanding leaves respond better than mature leaves (Perez-Tornero *et al.*, 2000). Shoot regeneration was higher in cultures incubated with a 16-h photoperiod as compared to those incubated in continuous darkness (Bhagwat and Lane, 2004).

Table 1: Optimization of growth regulators to regenerate adventitious shoot from peach (*P. persica* L.) young leaf explants after four weeks.

Growth regulators (mg/l)	% of shoot regeneration	Mean No. shoots	Mean length of shoots (cm)
2 mg/l TDZ + 0.5 mg/l IBA	8.48	1.62 ^d	0.58 ^e
3 mg/l TDZ + 1.0 mg/l IBA	32.4	1.53 ^d	0.85 ^d
3 mg/l TDZ + 1.5 mg/l IBA	61.8	2.33 ^c	1.02 ^c
4 mg/l TDZ + 1.0 mg/l IBA	52.4	2.02 ^c	0.92 ^d
5 mg/l TDZ + 1.0 mg/l IBA	48.6	1.85 ^d	0.75 ^d
3 mg/l TDZ + 0.5 mg/l NAA	36.4	3.38 ^b	1.35 ^a
3 mg/l TDZ + 1.0 mg/l NAA	64.8	4.12 ^a	1.28 ^b
4 mg/l TDZ + 1.0 mg/l NAA	51.5	3.25 ^b	1.10 ^c
5 mg/l TDZ + 1.0 mg/l NAA	50.3	2.82 ^c	1.00 ^c

To obtain direct shoot organogenesis, young leaf segments of peach were used as explants by cultured on WP medium with different concentrations of TDZ in combination with IBA or NAA (Table 1). The highest mean number of shoots (4.12) for local peach occurred on 3 mg/l TDZ and 1.0 mg/l NAA with 64.8% regeneration efficiency (Fig. 2A). The highest mean length of shoots (1.35 cm) on 3 mg/l TDZ + 0.5 mg/l NAA. The mean numbers of direct shoot organogenesis varied between 1.53 to 3.38 with other concentrations of TDZ in combination with IBA or NAA (Fig. 2B). The frequency of shoot organogenesis and the number of shoots per explants were significantly less when IBA was used with TDZ, whereas, shoot regeneration was observed when NAA was used with TDZ. The best protocol for adventitious shoot regeneration of peach was to culture the young leaf explants on WP medium supplemented with 3 mg/l TDZ and 1.0 mg/l NAA in darkness for 2 weeks, followed by transfer to the light for three weeks. TDZ was the most important factor for adventitious shoot

regeneration, as no adventitious shoots developed on explants exposed to media without TDZ. Successful regeneration is a key step for *Agrobacterium*-mediated transformation of this species. Hammatt and Grant (1998) and Espinosa *et al.* (2006) they reported that *P. serotina* can be regenerated from juvenile source leaf explants by using TDZ and NAA with a regeneration efficiency of 62% and 38.3% depending on the study. Suresh and Aniket (2013) found that somatic embryos were induced from leaf and stem explants cultured on Murashige and Skoog medium supplemented with 0.5-3.0 mg/l thidiazuron and 20% coconut water. Development of a system for the production of genetically transformed fruit trees greatly depends on the establishment of a reliable and effective regeneration system. Adventitious shoot formation from leaf explants has been reported for a limited number of mostly commercial genotypes, including wild pear *P. communis* var. *pyraster* L. (Caboni *et al.*, 1999) and *P. syriaca* Boiss. (Shibli *et al.*, 2000). In all cases leaves from *in vitro* grown

plants have been used as explants and have been exposed to a sequence of dark (2 to 3 weeks) and light exposure. This technique involves a very limited callus phase at the site of wounding. Bud regeneration occurred in 5 to 6 weeks.

Shoot multiplication.

To achieve shoot multiplication, the young shoots was transferred to a medium containing only

cytokinin and lacking auxin to allow multiplication. The effect of the presence of the three cytokinins; BAP, 2iP and kinetin in WP medium was examined during the phase of propagation. The best cytokinin combinations for forming multiplied shoot was obtained on WP medium with BA and 2iP (Table 2).

Table (2): Effect of different combinations of plant growth regulators on shoot multiplication of peach (*P. persica* L.).

WP medium supplemented with (mg/l)			No. of shoots obtained from a single shoot	Length of shoots (cm)
BA	2iP	kinetin		
1.0	–	–	3.4 ^d	1.5 ^{cd}
2.0	–	–	5.2 ^b	1.2 ^d
1.0	0.5	–	4.3 ^c	2.4 ^b
2.0	0.5	–	6.4 ^a	2.5 ^b
1.0	0.75	–	4.4 ^c	3.2 ^a
2.0	0.75	–	5.8 ^b	2.8 ^{ab}
1.0	–	0.5	3.5 ^d	1.8 ^c
2.0	–	1.0	5.4 ^b	1.4 ^{cd}
1.0	0.5	0.5	4.6 ^c	2.6 ^b
2.0	0.5	0.5	6.8 ^a	2.7 ^{ab}

The number of shoots obtained from a single shoot growing on WP medium containing 2 mg/l BA, 0.5 mg/l 2iP and 0.5 mg/l kinetin was significantly, it recorded 6.8 (**Fig. 2D**) also, WP medium containing 2 mg/l BA and 0.5 mg/l 2iP was significantly, it recorded 6.4 (**Fig. 2C**) comparing to the other

treatments were less significant. However, using 1 mg/l BA + 0.75 mg/l 2ip gave the highest increase in axillary shoot length (3.2 cm) compared to the increase in length of the other treatments, it ranged 1.2 - 2.8 cm for peach.

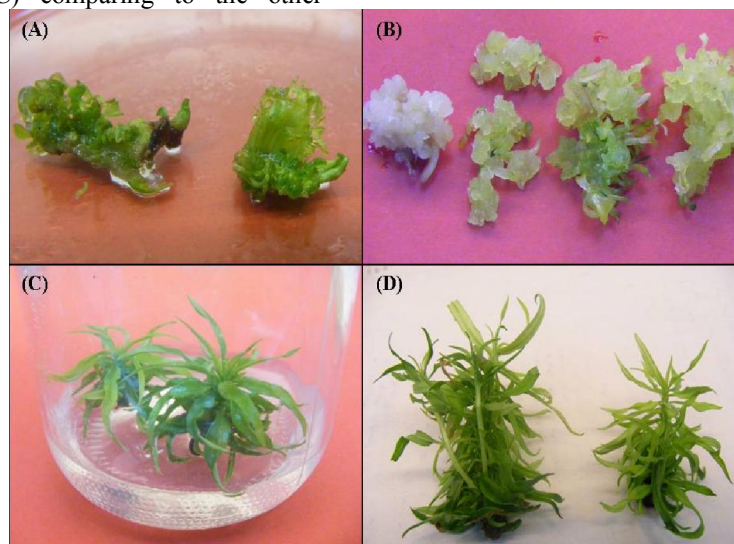


Figure (2): Adventitious shoot regeneration from *in vitro* leaves of peach. (A) Adventitious shoot on WP medium containing 3 mg/l TDZ and 1.0 mg/l NAA, (B) Adventitious shoot on WP medium containing 3 mg/l TDZ + 1.5 mg/l IBA. (C) Shoot multiplication on WP medium supplemented with 2 mg/l BA and 0.5 mg/l 2iP, (D) Shoot multiplication on WP medium supplemented with 2 mg/l BA, 0.5 mg/l 2iP and 0.5 kinetin.

Rooting of propagated shoots and transfer of plants into soil.

Root induction was enhanced on full strength MS salts with 3% sucrose, 100 mg/l myoinositol and

162 mg/L phloroglucinol (liquid medium). After examining auxins such as NAA and IBA we concluded that IBA combination with NAA were more suitable for optimal induction of roots and high percentage of

survival and acclimatization while, IBA alone had a low percentage of survival and acclimatization after transfer to the soil (Table 3). The liquid MS medium supplemented with 2 mg/l IBA and 0.5 mg/l NAA showed higher percentage of rooting in peach (92%) and the higher percentage of soil establishment (91%)

in the greenhouse after one month compared to the other treatments (Fig. 3). Similar results were reported with NAA in apple, and lack of vascular connections was implicated as one of the reasons for low plant survival (Yepes and Aldwinckle, 1994).

Table (3): *In vitro* rooting of peach shoots on MS supplemented with different growth regulators after 4 weeks, establishment of plants in the greenhouse and plants surviving for one month.

MS medium supplemented with (mg/l)		No. of			
IBA	NAA	Shoots transferred to medium	Plants rooted (%)	Plants transferred to greenhouse	Plants surviving (%)
1.0	–	60	24 (40%)	24	11 (46%)
1.5	–	60	28 (47%)	28	16 (57%)
2.0	–	45	26 (58%)	26	14 (54%)
3.0	–	50	25 (50%)	25	15 (60%)
1.0	0.5	60	25 (42%)	25	14 (56%)
1.5	0.5	40	19 (48%)	19	14 (74%)
2.0	0.5	50	46 (92%)	46	42 (91%)
3.0	0.5	50	35 (70%)	35	27 (77%)

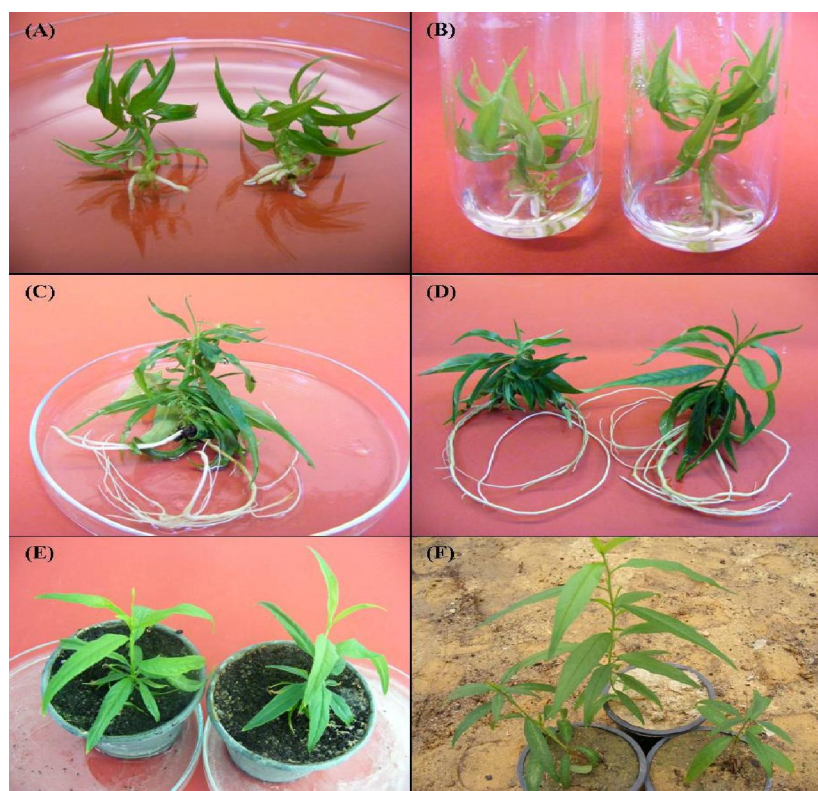


Figure (3). Root formation of peach (*P. persica* L.) on liquid MS medium supplemented with 2 mg/l IBA and 0.5 mg/l NAA after two weeks (A and B) and after four weeks (C and D). Acclimatized of peach plants under greenhouse after five weeks (E) and after two months (F).

***Agrobacterium*-mediated transformation of peach (*P. persica* L.).**

Efficient infection of *Agrobacterium* to plant cells and the subsequent transfer of T-DNA from *Agrobacterium* into plant cells are the first and also essential steps in the stable transformation process. The reporter gene expression can be used to evaluate

Agrobacterium infection and gene transfer into plants cells. Studied of the reporter gene expression can be very useful for evaluation of various factors for development and optimization of genetic transformation technologies (Chen *et al.*, 1998; Petri *et al.*, 2004).

Herbicide Bialaphos Sensitivity.

Prior to transformation it is necessary to study the sensitivity of growth and differentiation of peach (*P. persica* L.) tissues to bialaphos (glufosinate ammonium), which was used in the establishment of transformation through selection of transformed shoots. To determine the optimum concentration of bialaphos for the selection of transformed peach shoots, a kill curve experiment was carried out using non-transformed leaf sections of peach (*P. persica* L.). Leaf explants were cultured under the same conditions which were used for regeneration. Six selective media were prepared by adding filter sterilized 1mg/ml (active ingredient) stock solution of

glufosinate ammonium to autoclaved regeneration medium, in order to reach final concentration of 0.0, 1.0, 2.0, 2.5, 3.0, 3.5 and 4.0 mg/l (**Fig. 4**). Results showed that increasing in the bialaphos concentrations were accompanied by decrease in the percentage of the survival explants. The lethal dose of bialaphos was estimated at 3 mg/l. Where, 0.0, 1.0, 2.0 and 2.5 mg/l of bialaphos were recorded 94.3, 82.3, 65.3 and 24 %, respectively, and no survival was observed when 3.0, 3.5 and 4 mg/l bialaphos were used. The concentration of 3 mg/l bialaphos was then chosen as a selection marker for transformed tissues in peach (*P. persica* L.).

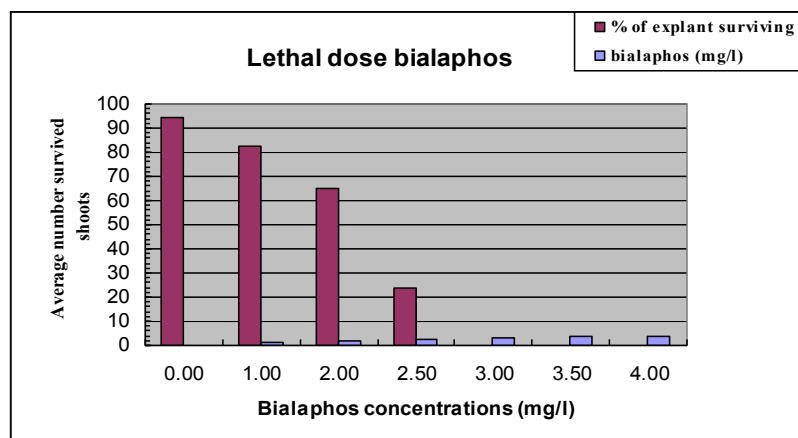


Figure (4): Effect of bialaphos concentrations on peach (*P. persica* L.) leaf segments

Evaluation of transformed plants with GUS assay.

The leaf explants of peach (*P. persica* L.) were infected with *Agrobacterium* strain LBA4404 containing the binary plasmid pISV2678. This construct carry the GUS-intron design and GUS expression is only activated in plant cells and the GUS expression cannot be due to the presence of *Agrobacterium* cells. Histochemical assay was conducted to evaluate GUS expression in explants infected by *Agrobacterium* strain LBA4404. GUS activity was detected in transformed explants and regenerated young shoots. Bialaphos-resistant shoots produced via *Agrobacterium*-mediated transformation strategy were randomly selected and subjected to a histochemical assay and visually

compared with non-transformed plant materials (**Fig. 5**). A percentage of about 85 % of the tested plant materials developed blue color whereas, there was no GUS expression detected in untransformed plants. PCR-positive plants were further analyzed by Southern blot analysis to confirm the presence of the transgenic peach plants and to study the integration patterns using *bar* gene as a probe, indicating that T-DNA was integrated into the plant genome. Genomic DNA was digested with *Bam*HI and data revealed that only one copy number was obtained but was absent with the negative control of the integration of *bar* gene into the genomic DNA of the transformants (**Fig. 9**).

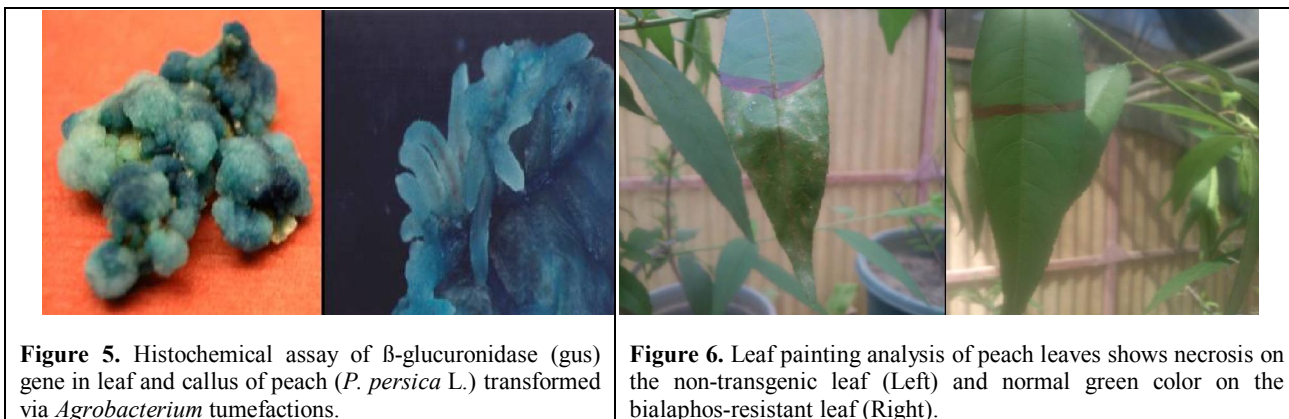


Figure 5. Histochemical assay of β -glucuronidase (*gus*) gene in leaf and callus of peach (*P. persica* L.) transformed via *Agrobacterium tumefaciens*.

Figure 6. Leaf painting analysis of peach leaves shows necrosis on the non-transgenic leaf (Left) and normal green color on the bialaphos-resistant leaf (Right).

Leaf painting assay.

The bialaphos-resistant plantlets were painted with Basta herbicide. As plasmid pISV2678 contain the *bar* gene, putative transgenic plants were treated with the recommended dose of basta herbicide (2 g/l basta) to prove the expression of the *bar* gene. The transgenic plant leaves were resistant to the herbicide (stay green), while non transgenic as well as control plant leaves became sensitive to the herbicide as they turned yellow then dark brown (necrosis) within two days (Fig. 6). The 0.2% of Basta applied is within the ranges reported for other crops and different crop plants have different responses to the herbicide (Gordon-Kamm *et al.*, 1990; Christou *et al.*, 1991).

Molecular analysis of transgenic plants.

Successful introduction of the transgenes (*bar* and *gus* genes) was confirmed by PCR using specific

primers for each of the transgenes. A fragment of 2070 bp (Fig. 7) was amplified from the DNA of the transgenic tissues by using the *gus-intron* specific primers, while a fragment of 540 bp (Fig. 8) was obtained with the *bar* specific primers. These bands were not amplified with non-transformed plants. It was observed that PCR-positive plants (26.7%) for *gus* gene were also positive for the *bar* gene, indicating that the two genes were successfully transferred to the explants by using the *Agrobacterium*-mediated transformation method. It was recorded that only 48 out of 180 of putatively transformed plants with pISV2678 were confirmed to be transgenic. The transformation efficiency was calculated to be 26.7% transformation percentage.

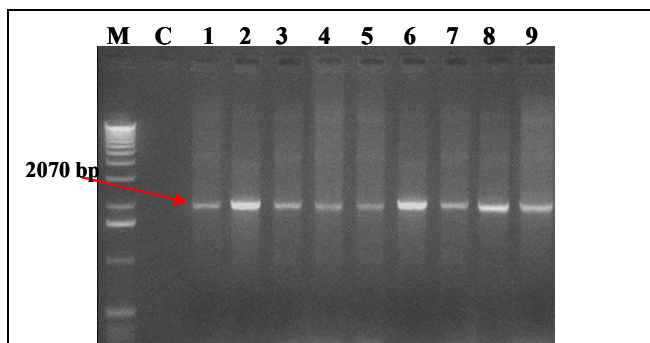


Figure (7). PCR screening for the presence of *gus-intron* (lane 1-9) gene in putative transgenic peach (*P. persica* L.) plants using *Agrobacterium* transformation. (c): non-transgenic plant. M: 1 kb DNA ladder.

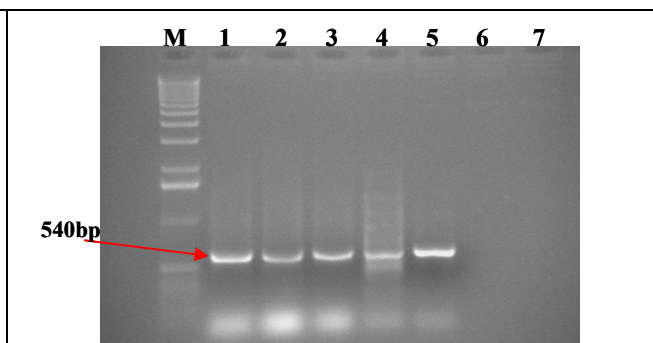


Figure (8). PCR analysis of putative transgenic peach plants (Lanes 1-7) using *bar*-specific primers to amplify 540 bp of the gene. Amplicons of the *bar* gene were presented in two out of the seven tested plants. M: 1 kb DNA ladder.

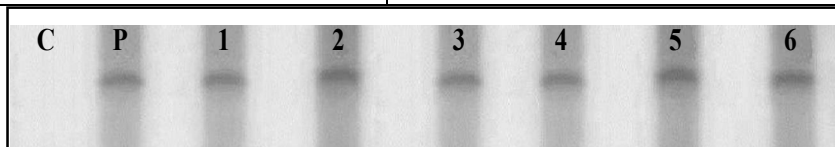


Figure 9. Southern blot analysis for the genomic DNA of PCR-positive transformed peach (*P. persica* L.) after digested with *Bam*HI using *bar* gene as a probe (Lanes 1-6), PCR-negative plants (Lane C) and positive PCR product of the *bar* gene (lane P).

Conclusion

The method developed here provides an efficient system of adventitious shoot regeneration from leaf explants of peach (*P. persica* L.) that will be useful for transformation. The results presented in this paper demonstrate an efficient methodology for high frequency genetic transformation of peach plants by co-cultivation of leaf explants with *Agrobacterium tumefaciens* engineered with *gus-intron* and *bar* gene inserts. All peach plants that were *gus* positive and resistant to Basta (0.2%) were also positive in Southern analysis using the *bar* probe.

Abbreviations: (MS) Murashige and Skoog's (1962) medium, (WPM) Woody Plant Medium, (IBA) Indole-3-Butyric Acid, (NAA) α -Naphthalene-Acetic-Acid, (*bar* gene) Phosphotricin-acetyl-transferase (gene), (*gus* gene) β -Glucuronidase (gene), (TDZ) Thiadiazuron (N-phenyl-N-1,2,3,-thiadiazol-5-ylurea), (X-gluc) (5-bromo-4-chloro-3-indolyl-B-D-glucuronide), (2iP) N⁶- Δ^2 -isopentenyl adenine, (PCR) Polymerase chain reaction.

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