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Optimization of Some Factors Affecting Genetic Transformation of Semi-Dry Egyptian Date Palm Cultivar (Sewi) Using Particle Bombardment

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ABSTRACT

Establishment of regeneration/transformation system for date palm is a key step towards improvement of date palm via genetic engineering. Herein, we reported the establishment of a particle bombardment transformation system for the Egyptian date palm cultivar (Sewi). A construct harboring cholesterol oxidase gene, which render plant resistance against insect attack was introduced into embryogenic date palm callus using PDS1000/He particle bombardment system. Three sorts of calli colonies out of 200 putative transformants microcalli colonies, which have been bombarded with DNA-coated particles, gave positive *GUS* expression. The successful integration of *GUS* gene in *GUS*-positive clones was verified by PCR. The reported system involves the establishment of embryogenic callus cultures from shoot tip explants, followed by shooting of the embryogenic callus by DNA coated particles under the optimized physical conditions. The developed system can be seen as a first step towards production of transgenic date palm autoresist notorious pests.

Key Words: *Phoenix dactylifera*, Somatic embryogenesis, Direct gene transfer

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INTRODUCTION

Date palm *(Phoenix dactylifera* L.*)* tree is considered one of the most important commercial crops in the Arab world *(FAO, 1984)*. This tree is a target for so many notorious pests such as red palm weevil *(Rhynchophorus ferrugineus)* and Bayoud disease caused by *Fusarium oxysporum (Salma and Saker, 2002)*. The surface application of pesticides to control weevil is ineffective as the larvae persist in the trunk of the tree. Similarly the use of chemical fungicide is ineffective in bayoud management, as the fungus is soil borne. Moreover, the application of conventional breeding to produce new date palm breeds resistant to pests is time consuming and tedious endeavor. It usually takes up to 30 years to make three backcrosses and to obtain the first offshoot *(Saker and Moursy, 2003)*. On the other hand, modern biotechnology creates unprecedented opportunities to improve agricultural productivity, decrease our dependence on potentially harmful chemical pesticides and enhance our ability to produce food in arid and dry regions of the world. Accordingly there is great demand for the development of transformation system for date palm, as a first step towards engineering new date palm breeds auto-resist notorious pests. This strategy proved to be effective and promising in other crops *(Boller et al., 1983; Lin et al., 1995; Barton et al., 1987; Kramer and Muthukrishnan, 1998; Cho et al., 1995; Schroeder et al., 1995; Morton et al., 2000 and Valencia et al., 2000)*.

Over the years, many plants, including several important crops, have been genetically modified using *Agrobacterium tumefaciens*-mediated transformation. Nevertheless the use of *Agrobacterium* for transformation is still limited in some plants, e.g., monocotyledons, due to the lack of available and efficient transformation and regeneration systems. On the other hand, direct gene transfer via bombardment method is successfully used in the production of the transgenic plants either monocots or dicots *(Christou, 1992)*. Survey of published data indicated that there is no one published report on the transformation of Egyptian date palm, except the abstract of *Saker and Ghareeb, (2007)*. They studied factors influencing transient expression of *Agrobacterium*-mediated transformation of *GUS* gene in embryogenic callus of date palm. They reported that date palm embryogenic callus can be infected with *Agrobacterium*. The putative transformants showed positive *GUS* expression. However, the promising progress of oil palm transformation *(Parveez et al., 1998)* encouraged us to establish regeneration/ transformation system for the Egyptian date palm using particle bombardment. To target this goal, a tissue culture system was developed and physical factors affecting the successful delivery of DNA were investigated.

MATERIALS AND METHODS

Tissue culture:

Date palm offshoots (cv. Sewi) were separated from donor mother tree during fruiting season and shoot tip explants were separated and sterilized as described by *Saker et al. (1998)*. Proliferation of embryogenic callus, shoot and root formation was carried out as described by *Saker et al. (2006a)*. Sewi is a semi-dry Egyptian date palm cultivar grown in oasis and new valley regions.

Plasmid:

A molecular construct (pBC4), harboring cholesterol oxidase gene (insect resistance gene), *GUS* (reporter marker gene) and kanamycin resistance gene under the control of 35 S promoter and NOS terminator *(Cho et al., 1995)* was used in the transformation experiments. The plasmid DNA was kindly provided by Prof. Ahmed El-Sharkawy, Genetics Dept., Faculty of Agriculture, Cairo University.

Bombardment:

Particle bombardment was carried out as described by *Klein et al. (1988)*, with minor modifications. Briefly, particles were prepared for bombardment as follows: 60 mg of particles were placed in microcentrifuge tube, vortexed vigorously in 1 ml of 100% ethanol for 5 min, soaked at room temperature for 15 min and centrifuged for 15 min at 10.000 rpm. The supernatant was decanted and the particles were washed 3 times in sterile distilled water, suspended in 2 ml of 50 % (v/v) sterilized glycerol and divided into 125 µl aliquots with 3.75 mg of particles per aliquot. Nine µg of plasmid DNA were added to microcentrifuge tube containing 3.75 mg of particles in 125 µl aliquots, vortexed and 125 µl of 2.5 M $CaCl_2$ were added and vortexed. Fifty µl of 0.1 M spermidine (freshly prepared and filter sterilized 0.1 M spermidine solution) were added and vortexed fro 4 min, kept on ice for 15 min, centrifuged for 20 sec at 1000 rpm and the supernatant was removed. Half ml of 75% ethanol was added, vortexed for 2 min, centrifuged for 20 sec at 1000 rpm and supernatant was removed and 0.5 ml of 100 % ethanol was added, resupended and 6 µl of the homogenous solution were used per shot. The negative controls were bombarded with uncoated particles in an identical fashion. The shelf carrying the target tissues was fixed at three different flight positions (7, 10 and 13 cm). The chamber vacuum was fixed at 25 Hg. Three different rupture-disc pressures (900, 1100, 1300 and 1500 psi) were tested.

Selection of putative transformants:

A preliminary experiment to determine the lethal concentration of kanamycin was carried out. Different concentrations of kanamycin, i. e. 25, 50, 75 and 100 mg/l were tested. One-Hundred mg/l kanamycin were found to be effective enough to prevent the growth of non-transformed embryogenic date palm callus cultures.

***GUS* assay:**

For rapid screening of putative transgenic calli, *GUS* expression was assayed using fluorescence detection method. Briefly, embryogenic micro calli colonies were squeezed in wells of ELISA plate in 100 µl GEP solution, then 100 µl MUG solution were add and incubated over night at 37°C, visualized under UV light (positive samples have a strong fluorescence). GEP solution (100 ml) consists of 50 mM Na_2HPO_4, 10 mM DTT or β-Mercaptoethanol, 1 mM Na 2 EDTA, 0.1% Na- Lauroylsarcosine and 0.1% Triton X-100. MUG solution (100 ml) consists of 20 mg MUG in 50 ml GEP Solution. Histcohmical detection of *GUS* activity was carried out typically as described by *Jefferson et al. (1987)*.

PCR analysis:

DNA isolation was done using Gentra DNA isolation kit (USA). DNA concentration was determined by electrophoresing 10 µl of the purified DNA along with serial dilutions of Lambda DNA in 0.8% agarose. PCR amplification of *GUS* gene was performed in a 20 µl reaction mixture containing 2 µl 10X amplification buffer, 200 µM dNTPs mix, 10 pmole each of upper and reverse primers, 40 ng template DNA, 1.0 unit Taq polymerase (Promega) and volume was completed to 20 µl using sterilized distilled water. The reaction mixture was assembled on ice and quickly transferred to preheated thermal cycler Biometra (Germany). The amplification was conducted for 35 cycles. The following temperature profile was followed: denaturation at 94°C for 1.5 min, annealing at 65°C for 1.5 min and extension at 72°C for 2 min and finally incubated at 72°C for 5 min. Amplification products were electrophoresed in 2% agarose using TAE buffer, for 2 hr at 70 volt, visualized under UV light after staining in 0.2 µg/ml ethidium bromide and photographed. Primers used for amplification of *GUS* gene were 5\ CCT GTA GAA ACC CCA ACC CG 3\ (forward) and 5\ TGG CTG TGA CGC ACA GTT CA 3\ (reverse). The expected size of the amplified band is 930-bp.

RESULTS AND DISSCUSION

Establishment of embryogenic callus cultures:

Contamination-free shoot tip explants were cultured on callus induction medium contained MS salts, B5 vitamins, 30g/l sucrose, 10 mg/l 2,4-D, 3 mg/l 2ip, 170 mg/l KH_2PO_4 and 3 g/l activated charcoal. Subculturing of explants to fresh medium was carried out every three weeks for 7 months. Shoot proliferation and rooting of proliferated shoots were carried out as described by *Saker et al., (2006a)*. Figure (1) shows the different steps of proliferation of embryogenic callus from shoot tip explants, shoot recovery and rooting.

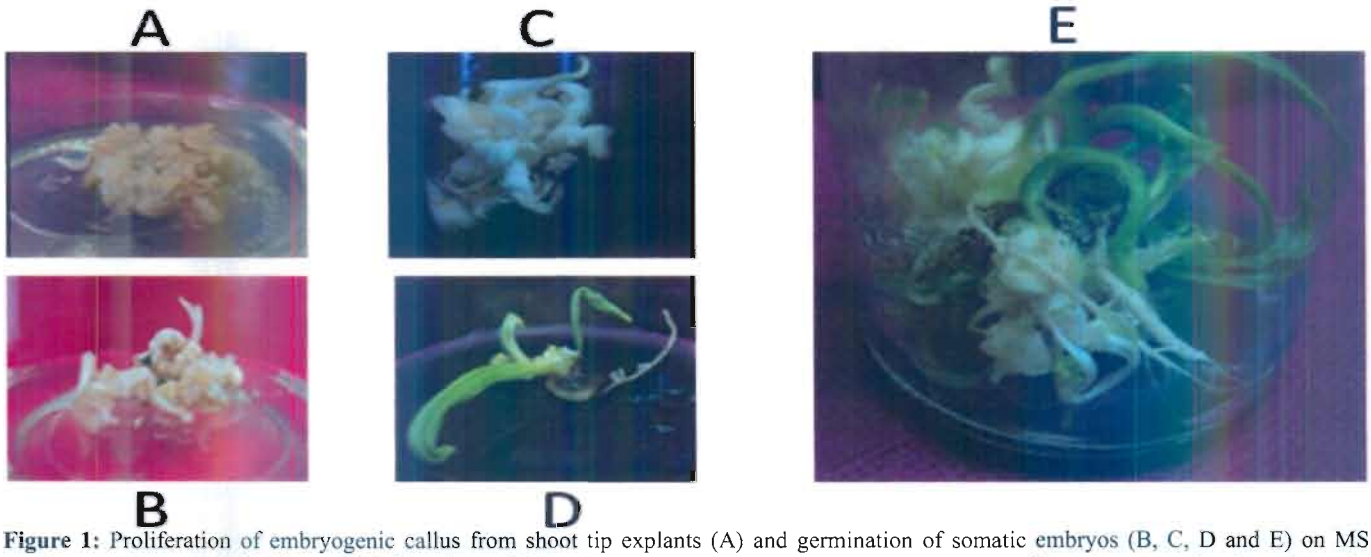

Figure 1: Proliferation of embryogenic callus from shoot tip explants (A) and germination of somatic embryos (B, C, D and E) on MS medium supplemented with 10 mg/l 2,4-D and 3 mg/l 2ip (proliferation of embryogenic callus) and basal MS medium (germination of somatic embryos).

Effect of microprojectile flight distance:

Preliminary investigations of physical factors influencing gene delivery using biolistic gun indicated that flight distance of microprojectiles and applied pressure were the most effective parameters (data not included her). Therefore, three flight distances (7, 10 and 13 cm) were tested with different helium pressures (900, 1100, 1300 and 1500). Flight distance is the distance between macrocarrier and stopping plate whereas the target tissue is settled (microprojectile flight distance). In this experiment, three different distances, i.e. 7, 10 and 13 cm were investigated. The best distance was identified based on the number of shooted explants showed positive transient *GUS* expression and percentage of explant survival on selective antibiotic containing medium. Data of table 1 indicated that the highest percentage of explants showed positive *GUS* expression (50) was recorded following shooting of explants with microprojectile at distance of 7 cm. Contrary, when the distance increased, i.e. 10 and 13 cm, a marked decrease in the percentage of explants showed positive *GUS* expression was recorded (Table 1). Data of this table also indicate that the highest percentage of explant survival on antibiotic containing medium was achieved following shooting with microoprojecticle at flight distance 10 cm. It In the light of explant survival on selective medium and the percentage of explants showed positive *GUS* expression, the best distance for successful shooting (DNA delivery) into date palm embryogenic callus is 10 cm. Therefore, a distance of about 10 cm was suggested to be near the optimal for date palm embryogenic callus transformation using biolistic device PDS-1000-He (Bio-Rad) because short flight distance (7 cm) reduced the transient *GUS* expression and caused tissue dislocation which may be associated with high tissue damage as explained by *Parveez et al. (1997)*. In this context, other reports have shown similar results *(King and Kasha, 1994)*.

Table 1: Data of physical parameters influencing DNA delivery in date palm callus cultures using Biolistic. A): Effect of different flight distances on explants survival and transient *GUS* expression.

The distance between the target and macrocarrier holder	Number of shooted explants	% of survival of shooted explants on selective medium	% of explants showed positive transient GUS expression
1/3 (7 cm)	100	4	50
1/4 (10 cm)	100	6	40
1/5 (13 cm)	100	5	30

Effect of helium pressure:

To identify the best pressure should be applied to achieve efficient DNA delivery into date palm callus culture, four helium pressures (psi) were tested. Transient *GUS* expression and explant survival on selective medium were used as indicators to identify the best pressure. Data of Table (2) indicated that bombarding of explants using 900 and 1500 psi is associated with notable decrease in the percentage of explant survival on selective media and positive *GUS* expression and the lowest values (2, 40 and 30, respectively) were recorded following bombarding callus tissues using 1500 psi. It was observed that 1100 psi gave the highest values of explant survival (4) and percentage of explants showing positive transient *GUS* expression (60). Finally it could be concluded that reducing the applied helium pressure resulted in notable decrease in *GUS* expression, which may be due to the reduction in the force and velocity of microprojectle to penetrate embryogenic callus tissue. Contrary, increasing the applied helium pressure up to 1500 psi dramatically reduce the *GUS* expression. This can be attributed to dislocation of target tissue and/or damage of cells as a result of very

high velocity and force of microprojectle. This result is in agreement with the most of the published data on embryogenic callus of oil palm *(Parveez et al., 1997)* and barley *(Ritala et al., 1993)*. In this context, many reports for most plant transformations using biolistic, indicated that the optimal pressure is around 1000 psi *(Sanford et al., 1993 and Saker et al., 2006b)*. In conclusion, optimal physical conditions affecting successful delivery of DNA into plant tissue were reported to be cell and tissue type dependent *(Iida et al., 1990)*. Also interaction among factors affect the successful delivery process, for instance microprojectile with small size will need lower force to penetrate the tissue while bigger size particles will need higher force and accordingly high helium pressure and short flight distance *(Li et al., 1994)*. Not only physical factor but also cell, tissue and plant type affect the successful delivery of DNA as mentioned by *Iida et al. (1990)*. Herein we reported for the first time physical factors, near optimal, which should be applied to achieve better DNA delivery in embryogenic date palm of the Egyptian cultivar Sewi.

Table 2: Data of physical parameters influencing DNA delivery in date palm callus cultures using Biolistic. B): Effect of different applied helium pressure on explants survival and transient gus expression.

Applied helium pressure (psi)	Number of Shooted explants	% of survival of shooted explants on selective medium	% of explants showed positive transient GUS expression
900	100	2	40
1100	100	4	60
1300	100	3	50
1500	100	2	40

Biochemical and molecular analysis of putative transgenic embryogenic callus:

For stable transformation, the aforementioned conditions for optimal delivery of DNA to embryogenic date palm callus cultures were used to deliver the cholesterol oxidase gene, which render recipient plants resistance against insects. A total of 200 microcalli colonies were bombarded with DNA-coated particles. Only 3 sorts of embryogenic calli were *GUS* positive (Figure 2). The same sorts gave *GUS* positive results in histochemical *GUS* assay after one month of shooting (Figure 2). Putative transgenic calli displayed positive *GUS* were subjected to PCR analysis using a pair of specific primers flanking DNA sequences of *GUS* gene. The obtained PCR amplification pattern presented in (Figure 2) confirmed the presence of the reporter gene *GUS* in *GUS*-positive calli and the same amplicons detected in positive control (about 900-bp) was also detected in transformants and was absent in non-transformed negative control.

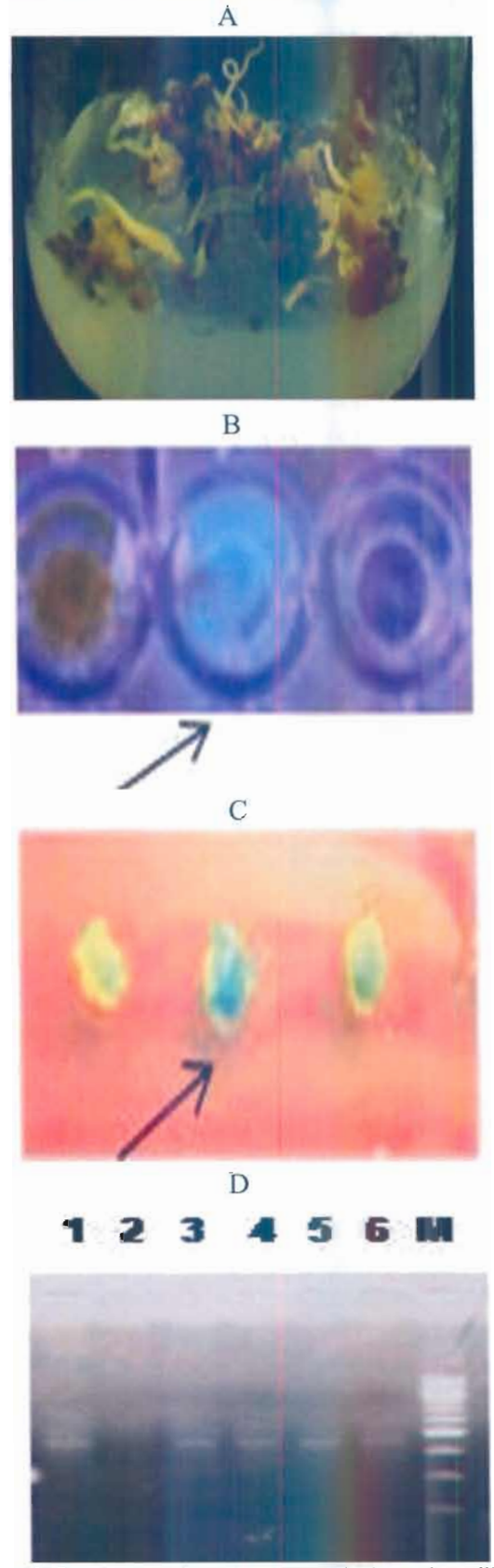

Figure 2: Putative transformed embryogenic date palm calli onto selective medium (A), fluorescence (B) and histochemical (C) *GUS* assay in putative transgenic date palm callus colonies and PCR amplification of *GUS* gene (D). Arrows point to positive *GUS* expression (bright blue) and PCR amplicon of interest. Lane 1 (positive control, i.e. plasmid used in bombardment), lane 2 (negative control, i.e. non-transformed), lanes 3-6 putative transformant and M is DNA marker.

Results of biochemical and molecular analysis of the present experiment confirmed the possible transformation of embryogenic date palm using gene gun-mediated transformation system. This preliminary report can be seen as a first step towards production of transgenic date palm resistant to notorious pests. Further studies are needed to increase the percentage of transformation and explant survival, especially it was noted that the survival of explants on, even, antibiotic free medium is notably low (preliminary data not shown her). This observation was also reported by *Saker et al. (2006b)* in rice. The negative effect of bombarding callus, on callus growth and survival on antibiotic-free medium was previously attributed to a cytotoxicity of unknown-nature. Recently this side effect has been studied in details by *Krysiak et al. (1999)*. He reported DNA double-strand breaks and inhibition of somatic embryogenesis by tungsten microprojectile in wheat.

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