Full Length Research Paper

Optimization of physical and biological parameters for transient expression of *uidA* gene in embryogenic callus of date palm (*Phoenix dactylifera* L.) via particle bombardment

Mousa Mousavi[1], Amir Mousavi[2]*, Ali Akbar Habashi[3] and Kazem Arzani[1]

[1]Department of Horticultural Sciences, Tarbiat Modares University (TMU), P.O. Box 14155-336 Tehran, Iran.
[2]National Institute of Genetic Engineering and Biotechnology, P.O. Box 14155-6343, Tehran, Iran.
[3]Agricultural Biotechnology Research Institute of Iran, P.O. Box 31535-1897, Karaj, Iran.

The objective of this study was to establish an efficient genetic transformation system in date palm (*Phoenix dactylifera* L.) using particle bombardment. Somatic tissues derived from offshoots' meristem cultures were bombarded with genetic constructs harboring the *uidA* gene under control of the CaMV 35S or Act1 promoter. The effects of different physical, biological and DNA parameters were evaluated by comparing the number of blue spots obtained from the histochemical GUS assay. Optimal transient expression of the *uidA* gene in embryogenic calli was observed using the following conditions: bombardment at 1100 psi, 9 cm target distance, a 1.6 µm gold particle size coated with 2.5 µg of DNA, 26 inHg vacuum pressure, 3 mm distance between the rupture disk and macrocarrier and osmotic pretreatment of 0.4 M mannitol followed by 60 min of air desiccation. Significantly, higher expression rates were observed when the construct carrying the Act1 promoter was employed. The highest number of blue spots obtained in this protocol was 1500 blue spots per 1 cm^2 of bombarded tissue. Achievement of these optimized conditions considered as the first report of its kind is expected to provide valuable information for the generation of transgenic date palm plants.

Key words: Particle bombardment, date palm, *Phoenix dactylifera* L., transient expression.

INTRODUCTION

Date palm (*Phoenix dactylifera* L.), a perennial dioecious monocotyledon of the Arecaceae family has a high socio-economic importance due to its food value and providing many other products such as shelter, fiber, clothing and furniture. Moreover, it shows high natural tolerance to extremely adverse environmental conditions including drought, salinity and high temperatures (Bakheet et al., 2008).

Date palm is one of the oldest fruit crops, mainly culti-vated in the Middle East and North Africa and consists of a large number of well-adapted ecotypes locally called cultivars (Rhouma et al., 2008). In 2007, the area of harvested date palm in the world was 1.1 million ha yielding 6.42 million tons, with Egypt and Iran being the major producers (FAO, 2007). Over the past years, date cultivars have been mainly selected by growers based on fruit quality that were clonally propagated by offshoots (Carpenter and Ream, 1976) and so far, no typical new cultivar has been released through conventional breeding programs. This is mainly due to limited and long gene-ration cycle of the date palm. On the other hand, some of the date cultivars have become susceptible to a series of disorders in particular areas. For example, in north Africa a vascular fusariosis named Bayoud disease caused by the filamentous fungus (*Fusarium oxysporum* f. sp. *albedinis*) destroyed palm plantations more than a century ago and majority of the date palm groves in this

---

*Corresponding author. E-mail: m-amir@nigeb.ac.ir. Tel: +98-21-44580368. Fax: +98-21-44580347.

region of the world are now infected (Quenzar et al., 2001). Red palm weevil is also considered as one of the most important pests of date palm in the world. It is native to southern Asia, but since early 1980s, it is rapidly spread throughout the Middle East, North Africa and Mediterranean areas (Ferry and Gomez, 2002).

Therefore, the development of efficient genetic transformation methods to obtain new cultivars with the desired traits in facing future challenges in production of date palms is an important task. The new technology of genetic manipulations, allows the transfer of selected gene(s) to a specific genotype in only a single generation (Sartoretto et al., 2002) that would not be possible by conventional breeding. Several different transformation methods have been described amongst which biolistic-mediated transformation, a species-independent method for direct DNA transfer is commonly used in monocots (Altpeter et al., 2005). Parveez et al. (1997, 1998) and Majid and Parveez (2007) optimized different physical and biological parameters for transient expression of GUS and GFP reporter genes in oil palm through particle bombardment. Similar experiments were also conducted on selectable markers and reporter gene expressions in banana by Sreeramanan et al. (2006, 2005), whilst Becker et al. (2000) successfully reported stable transformation of Cavendish banana (*Mousa* spp. AAA group) via particle bombardment.

Up till now, no efficient protocol has been described in the literature for genetic transformation of date palm. In this study, we investigated the possibilities of gene transfer and expression into embryogenic callus of a commercially important date palm cultivar using the particle delivery system. In order to achieve this objective, the effect of several physical parameters together with other biological and transgene elements that are expected to have a strong effect on DNA delivery and efficient expression of the *uidA* reporter gene were assessed.

MATERIALS AND METHODS

Plant materials and establishment of embryogenic calli for bombardment

Plant material obtained from the mature commercial date palm trees cultivar `Kabkab' grown at the date palm collection orchard, Date Palm and Tropical Fruit Research Institute, Ahwaz, Iran. Embryogenic calli were initiated from 1 - 2 years old offshoot meristems on an induction medium containing MS salts and vitamins (Murashige and Skoog, 1962) supplemented with 100 mg/l of 2,4-D, 3 mg/l of 2ip, 40 mg/l of adenine, 30 g/l of sucrose and 3 g/l of activated charcoal. All cultures were incubated in the dark at 26°C and transferred to a fresh medium every 4 weeks for 16 - 20 weeks. The resultant embryogenic calli were collected from induction cultures and maintained by subculturing on a medium with the same composition except for 2,4-D, which was reduced to 10 mg/l.

Embryogenic calli were precultured in MS medium containing 10 mg/l of 2,4-D, 3 mg/l of 2ip, 40 mg/l of adenine and 0.4 M mannitol (as an osmoticum treatment) for 24 h in the dark before bombard-

ment. They were arranged in the center of petri dishes (in a 2 cm diameter circle) and partial desiccation was carried out by removal of the plate covers in a laminar flow bench for 30 - 60 min prior to bombardment.

Plasmid DNA

The plasmids used for the transient expression experiments consisted of pBI221 (Clontech, USA), a 5.6 kb construct harboring the *uidA* gene coding for β-glucuronidase (GUS) under control of the constitutive cauliflower mosaic virus 35S (CaMV 35) promoter, pCAMBIA 3301 (Cambia, Australia), a 11.307 kb construct harboring the *uidA* and bar genes, both under the control of CaMV 35S promoter and pAct1-D (McElroy et al., 1990), a 7.5 kb construct, harboring the *uidA* gene with the 5´ region of the rice actin1 promoter.

Establishment of parameters for particle bombardment

Optimization of the physical parameters for particle bombardment was carried out under the following conditions, acceleration pressure (900, 1100 and 1350 psi), distance from rupture disk to the macrocarrier (3, 9 and 18 mm), distance from macrocarrier to target tissue (6, 9 and 12 cm), vacuum pressure (24, 26 and 28 inHg), particle type (gold and tungsten), particle size (gold in diameters of 0.6, 1.0 and 1.6 µm), coating agents (spermidine, $CaCl_2$ and both), number of bombardments (single and double) and time of partial desiccation prior to bombardment (0, 30 and 60 min). Other DNA and biological parameters included were plasmid type (pAct1-D, pBI221 and pCAMBIA 3301), DNA concentration (0.5, 2.5, 12.5 and 25 µg per bombardment), tissue type (embryogenic callus, somatic embryos, leaf and root), osmoticum type (mannitol, sorbitol, sucrose and glucose) and osmoticum concentration (0.0, 0.2, 0.4 and 0.6 M mannitol).

Plasmid DNA was precipitated into gold or tungsten particles and bombarded according to the protocols supplied for the Biolistic PDS-1000/He particle delivery system (BioRad, USA) with minor modifications. While vigorously vortexing 50 µl of particle solution (prepared in 50% glycerol), 10 µl of DNA, 50 µl of 2.5 M $CaCl_2$ and 25 µl of 0.1 M spermidine were added and the mixture was vortexed for 2 min. The microcarriers were allowed to settle for 4 min and then pelleted by spinning for 3 s in a microfuge. After removal of the liquid, the pellet was washed twice (without vortexing) with 140 µl of 70 and 100% ethanol, respectively. After adding 82 µl of 100% ethanol and resuspension of pellet by vortexing, 12 µl of the DNA-coated microcarrier suspension was loaded into the center of a macrocarrier, air dried and used for bombardment.

Transient GUS expression assay and data analysis

Bombarded calli remained on the same plates for 72 - 94 h before being assayed for GUS expression by transferring them to 2.5 ml of X-Gluc staining solution, followed by incubation for 24 h at 37°C, as described by Jefferson et al. (1987) with some modifications. The staining solution (100 ml) consisted of 500 µl of 1 M NaH_2PO_4, 10 mg of chloramphenicol, 100 µl of triton X-100, 2 ml of methanol and 88 mg of X-gluc (dissolved in DMSO). The pH of the final solution was adjusted to 8.0. Transient GUS activity was recorded as the number of blue spots (irrespective of size) in each treatment under a Stemi 2000-C binocular microscope (Zeiss, Germany) and then photographed.

Statistical analysis of data (with at least 3 replicates for each experiment) was conducted using one-way ANOVA by the PROC-GLM program of SAS. Analysis of variance was performed for each

treatment. Means were separated at the 5% probability level with the Duncan's multiple range test when a significant F ratio occurred (P < 0.05). Significant differences are indicated by different letters above the bars.

RESULTS AND DISCUSSION

Transient expression of the *uidA* gene (defined by number of blue spots) was used during this study as an indicator to monitor the effects of various conditions on the efficiency of particle bombardment-mediated transformation in embryogenic callus tissues of date palm. Results obtained from analysis of variance of each treatment are summarized in Table 1. Significant differences (P < 0.05) between treatments/variables were obtained for all experiments except bombardment number.

Effect of helium pressure

It was observed that an helium pressure of 1100 psi give significantly the highest (512 ± 39) transient GUS gene expression in embryogenic calli of date palm, as compared to 900 and 1350 psi which resulted in 38 ± 16 and 242 ± 39 blue spots, respectively (Figure 1a and Table 1). Similarly, a higher level of transient GUS expression has been reported in oil palm (Parveez et al., 1997), banana (Sreeramanan et al., 2005), maize (Petrillo et al., 2008), cowpea (Ikea et al., 2003) rice (Anoop et al., 2004; Jain et al., 1996) and cotton (Banerjee, 2001) using a 1100 psi rupture disk.

The ability of the microcarrier particles to successfully penetrate different cell types is highly dependent on the helium gas pressure (Kikkert, 1993). Bombardment with lower or higher pressures did not result in any significant enhancement in transient GUS expression. Lower expression at reduced pressures could be correlated to the poor penetration capability of the microcarriers as they approach the recipient tissues. On the other hand, at higher pressures, the increased penetration force of the particles might injure the cells (Janna et al., 2006).

Selection of appropriate distances from rupture disk to the macrocarrier and stopping screen to the target tissue

The effect of the gas shock wave on microcarrier velocities is determined in part by the gap between the rupture disk and macrocarrier. The smaller the distance, the more powerful the effect of the gas shock wave on macrocarrier acceleration. In this study, a 3 mm distance between the rupture disk and macrocarrier gave a significantly higher (477 ± 90) transient expression as compared to 9 mm with 205 ± 85 and 18 mm with 48 ± 25 blue spots (Figure 1b and Table 1). Previous investigation on oil palm has shown similar results (Parveez et al.,

1997). In practice, due to extra shock wave causing disruption of the target tissue and stopping screen, in case of bombardment with 3 mm distance between rupture disk and macrocarrier, we used 9 mm distance in all experiments.

The distance from the stopping screen to the target tissue can affect the velocity of microparticles and consequently transformation rates (Petrillo et al., 2008). In this study, a significantly higher number of blue spots (291 ± 99) were detected when calli were placed 9 cm away from the stopping screen, followed by 157 ± 12 and 66 ± 21 blue spots when distances of 6 and 12 cm were used, respectively (Figure 1c and Table 1). Similar observations were reported for banana (Sreeramanan et al., 2005) and rice calli (Ramesh and Gupta, 2005).

### Effect of CaCl$_2$ and spermidine on precipitation of DNA into the microcarrier

Calcium chloride and spermidine are 2 important components added to the solution mixture during precipitation of DNA into microcarriers. In this study, CaCl$_2$ and spermidine were added together or alone in the precipitation mixture. It was found that high transient GUS expression (128 ± 30) was observed when both CaCl$_2$ and spermidine were used compared to CaCl$_2$ (24 ± 5) and spermidine (10 ± 5) alone (Figure 1d and Table 1). Yee et al. (2008) and Janna et al. (2006) found a higher level of transient expression in *Oncidium* and *Dendrobium* when spermidine was used alone.

Determination of particle type and size

This experiment was performed in 2 steps; first, transient expression of the GUS gene was studied after delivering 2 types of particles including, 1 μm gold and 1.1 μm tungsten. In the second step, gold particles with diameters of 0.6, 1 and 1.6 μm were used. The results indicated that the number of blue spots per shot with gold particles were significantly higher (319 ± 48) than tungsten particles (99 ± 6) and 1.6 μm gold particles showed a significantly higher level of transient GUS gene expression (775 ± 64) when compared to 1 μm with 454 ± 68 and 0.6 μm with 4 ± 2 blue spots (Figures 1e and 1f, Table 1). Gold particles are often preferred since they have more round and uniform sizes than tungsten particles. They are biologically inert, non-toxic and do not degrade DNA bonds. Tungsten particles, on the other hand, are highly heterogeneous in size and shape, potentially toxic to some cell types and are subjected to surface oxidation. They can also acidify and degrade DNA bonds (Sanford et al., 1993). However, there are some reports on successful application of tungsten particles in plant cells transformation (Klein et al., 1988; Cabrera-Ponce et al., 1995; Jarl, 1999; Sartoretto et al., 2002). But most researchers have used gold particles

Table 1. Summary of the bombardment conditions used for each parameter experiment.

Bombardment conditions	Plasmid type and conc. (μg/shot)	Osmotic treatment	Partial desiccation (min)	Rupture disk (psi); target distance (cm)	Rupture disk-macrocarrier distance (mm)	Chamber vacuum (inHg)	Particle type and size (μm)	Bombard-ment number	Tissue type	Recommended
Osmoticum type	pAct1-D; 12.5	mannitol, sorbitol, glucose, sucrose (0.4 M)	-	1100; 6	9	26	gold; 1.6	1	callus	mannitol
Osmoticum concentration	pAct1-D; 12.5	mannitol (0, 0.2, 0.4, 0.6 M)	-	1100; 6	9	26	gold; 1.6	1	callus	0.4 M
Partial desiccation	pAct1-D; 12.5	mannitol (0.4 M)	0, 30, 60	1100; 6	9	26	gold; 1.6	1	callus	60 min
Helium pressure	pAct1-D; 12.5	mannitol (0.4 M)	60	900, 1100, 1350; 6	9	26	gold; 1.6	1	callus	1100 psi
Target tissue distance	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6, 9, 12	9	26	gold; 1.6	1	callus	9 cm
Vacuum pressure	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	9	24, 26, 28	gold; 1.6	1	callus	26 inHg
Rupture disk-macrocarrier distance	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	3, 9, 18	26	gold; 1.6	1	callus	9 mm
Coating agents	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	9	26	gold; 1.6	1	callus	spermidine + $CaCl_2$
Particle type	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	9	26	gold (1), tungsten (1.1)	1	callus	gold
Particle size	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	9	26	gold; 0.6, 1, 1.6	1	callus	1.6 μm
Bombardment number	pAct1-D; 12.5	mannitol (0.4 M)	60	1100; 6	9	26	gold; 1.6	1, 2	callus	1
Tissue type	pAct1-D; 12.5	mannitol (0.4 M)	60	1350; 6	9	26	gold; 1.6	1	callus, somatic embryo, leaf, root	callus
Plasmid type	pAct1-D, pCAMBIA3301, pBI221; 12.5	mannitol (0.4 M)	60	1100; 6	9	26	gold; 1.6	1	callus	pAct1-D
DNA concentration	pAct1-D; 0.5, 2.5, 12.5, 25	mannitol (0.4 M)	60	1100; 6	9	26	gold; 1.6	1	callus	2.5-12.5 μg
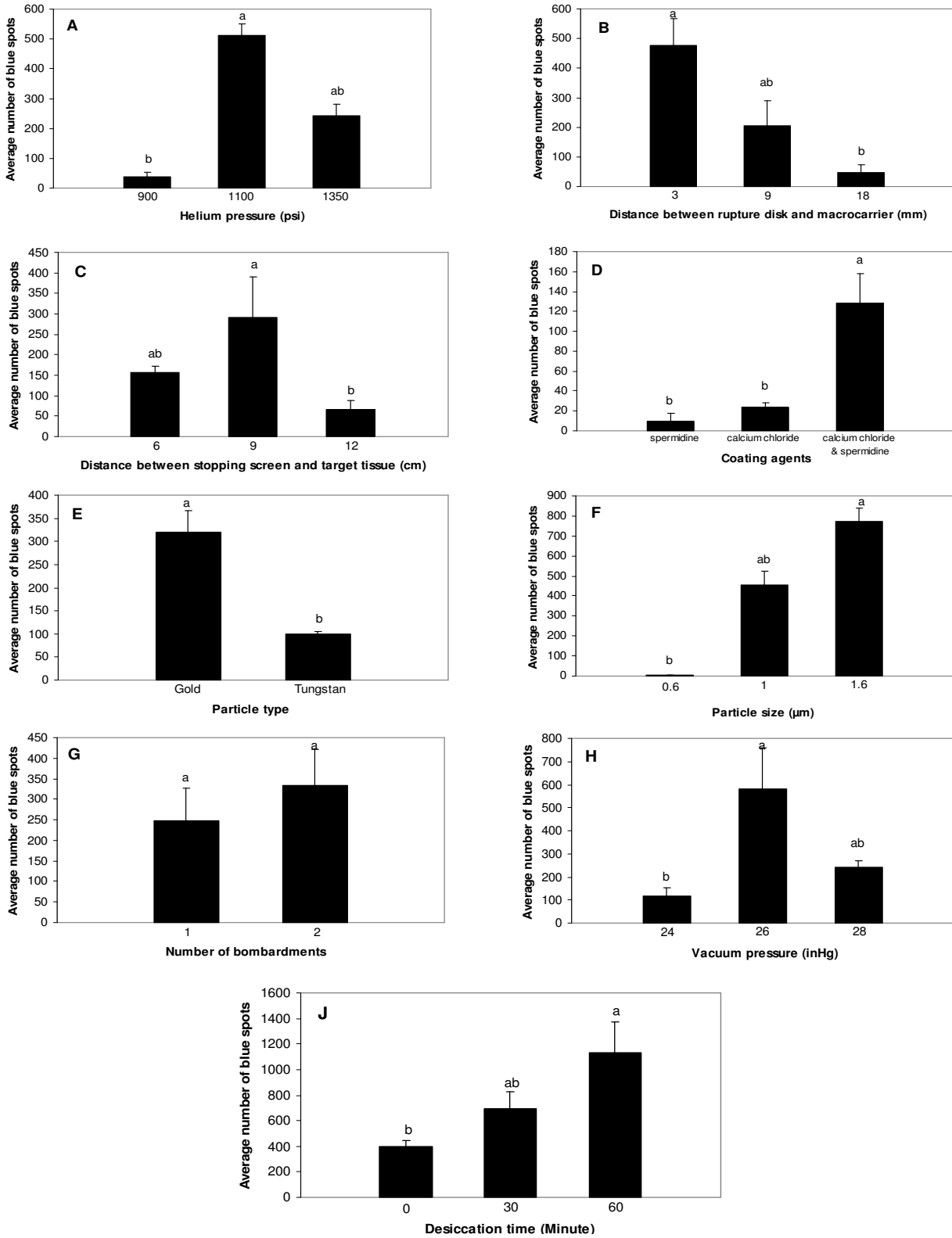
Figure 1. Effect of physical parameters on transient expression of the GUS gene in bombarded date palm embryogenic calli. A, helium pressure (psi); B, distance from rapture disk to macrocarrier; C, distance from stopping screen to target tissue; D, calcium chloride and spermidine as coating agents; E, particle type; F, particle size; G, bombardment number, H, vacuum pressure and J, partial air desiccation time. Data represent the mean ± SD determined from at least three replicates and were analyzed using one-way ANOVA (PROC-GLM program of SAS). Different letters indicate significant differences at the level of $P < 0.05$ within a parameter.

for gene delivery to plant tissues. Depending on the tissue type and bombardment parameters, different sizes have been used successfully. Li et al. (1994) and Takumi et al. (1994) have found that the 1.6 µm gold particle is better than 1.0 µm for transformation of white spruce embryogenic suspension and einkorn wheat, respectively. Okada et al. (2002) have also used 1.6 µm gold particles for transformation of sweet potato.

Effects of bombardment number and vacuum pressure

There was no significant difference observed between single (249 ± 96) and double (334 ± 145) bombardments (Figure 1g and Table 1), consistent with what have already been reported for oil palm (Parveez et al., 1997) and *Dendrobium* (Janna et al., 2006). Contrary to our results, double bombardment has been shown to increase the efficiency of transient expression of the GUS gene in banana (Sreeramanan et al., 2005), Brazilian maize inbred lines (Pettrillo et al., 2008) and cassava (Schopke et al., 1997). Double bombardment (by rotating the plate by 90°) especially at a short distance between macrocarrier and target tissue, results in better coverage of the target area and increases the efficiency of transformation. However, double bombardment can also cause higher tissue damage particularly with higher helium pressures.

The vacuum in the bombardment chamber reduces the frictional drag of mirocarriers as they are accelerated toward the target cells. Among 3 vacuum pressures tested in this study, 26 inHg significantly increased the efficiency of transient GUS expression (580 ± 179) followed by 2 other vacuum pressures of 28 and 24 inHg resulting in 243 ± 25 and 115 ± 36 blue spots, respectively (Figure 1h and Table 1). Sreeramanan et al. (2005) have reported that there were no significant differences among 26, 27, 28 and 29 inHg regarding the bombarded single bud of banana but if corm slices were bombarded, a significant difference was observed between 26 and 28 inHg. In the protocorm-like-body (PLB) of *Dendrobium*, a vaccum pressure of 27 inHg was also found to initiate more effective expression (Janna et al., 2006). Tian et al. (2006) have also successfully transformed tall fescue under a 26 inHg pressure.

Effect of partial air desiccation

In this experiment, embryogeic calli were bombarded after air desiccation for 0, 30 and 60 min in a laminar air-flow cabinet. During this treatment, partial desiccation of explants led to evaporation of excessive water surrounding the target cells. The results showed significant differences among the 3 durations of desiccation, with 60 min being more effective in increasing efficiency of tran-

sient GUS expression (1129 ± 245) followed by 30 min with 694 ± 130 and no desiccation with 395 ± 46 blue spots (Figure 1j and Table 1). These results were obtained on calli that previously remained for 24 h on media containing 0.4 M mannitol. In other reports, osmoticum treatment has mostly been used (as discussed above), but in this study both osmoticum treatment and partial desiccation of date palm recipient tissues were used successfully. Partial desiccation has also been reported to promote embryogenesis in *Pinus kesiya* (Malabadi et al., 2004) and regeneration from callus in rice (Sharma et al., 2004).
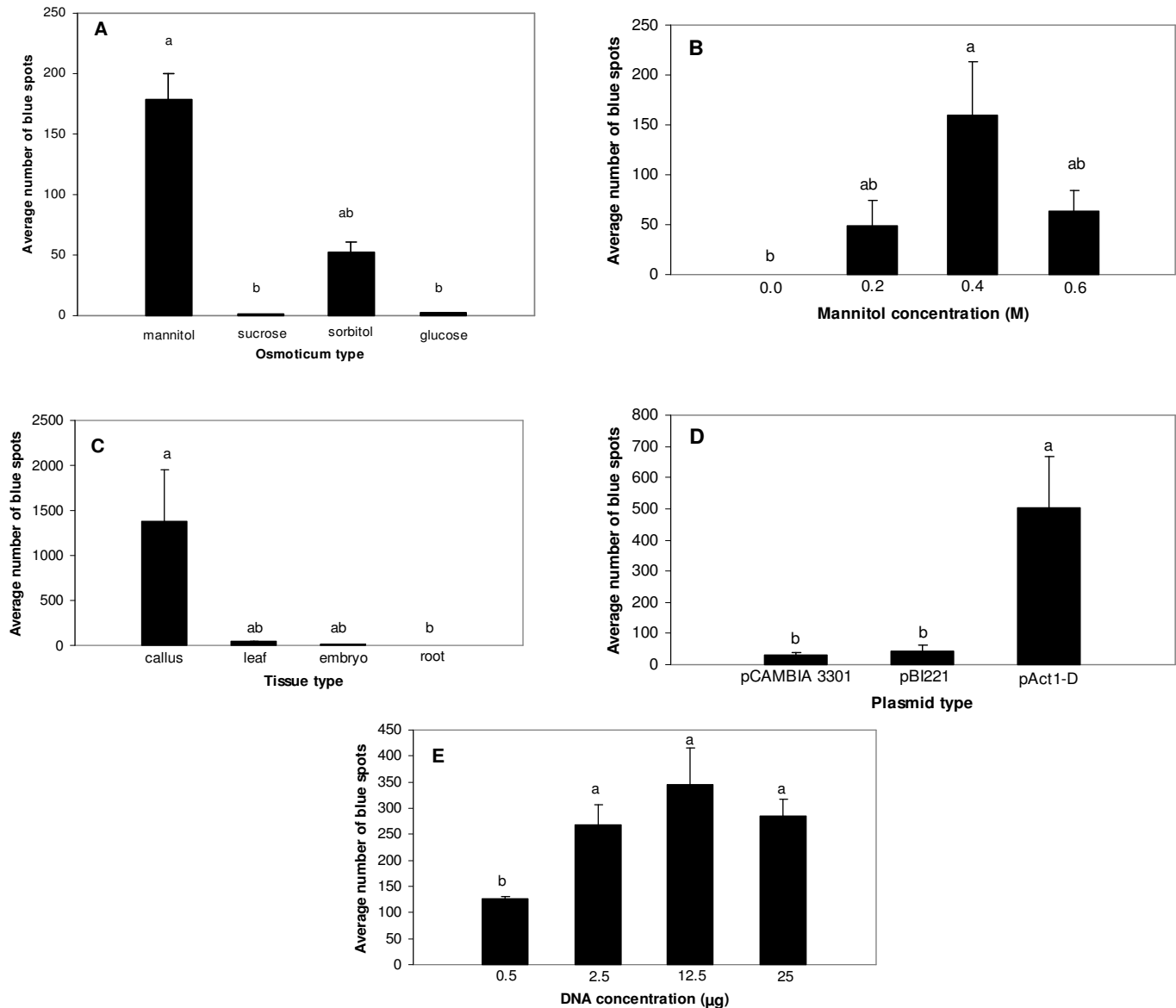
Effect of osmoticum type and concentration

24 h before bombardment, the embryogenic calli were transferred to MS medium supplemented with 10 mg/l of 2,4-D, 3 mg/l of 2ip, 40 mg/l of adenine and 4 types of osmoticum agents including mannitol, sorbitol, sucrose and glucose, each at 0.4 M. After bombardment, the calli remained on these media for 4 days, followed by GUS staining. The results showed a significant difference between different osmoticum agents. Mannitol with 179 ± 21 blue spots was found to be more effective than sorbitol (52 ± 9), glucose (2 ± 0.5) and sucrose (1 ± 0) with respect to transient expression of the GUS gene (Figure 2a and Table 1). Effect of mannitol (alone or together with sorbitol) on increasing efficiency of transient GUS expression has been reported by several investigators for different plants such as barely (Obert et al., 2008), pearl millet (Patell et al., 2008), wheat (Chawla et al., 1999), cassava (Schopk et al., 1997), coffee (Gatica-Arias et al., 2008) and *Alstromeria* (Kim, 2005).

The effect of different concentrations of mannitol (0, 0.2, 0.4, and 0.6 M) as osmoticum treatments was also investigated. The media used were the same as the osmoticum type procedure and the embryogenic calli were maintained 24 h on this media prior to bombardment. It was found that a 0.4 M concentration of mannitol with 160 ± 52 blue spots significantly increased the level of transient GUS expression when compared to 0.6 and 0.2 M with 63 ± 21 and 49 ± 25 blue spots, respectively. There were no blue spots observed in the absence of mannitol (control) as indicated in Figure 2b and Table 1. Similar results have been reported for oil palm (Parveez et al., 1998) and maize (Vain et al., 1993).

Determination of target tissue type

In order to determine the appropriate target tissue in date palm for transformation using the biolistic gene delivery system, 4 types of tissues including embryogenic calli, somatic embryos, leaves and roots (the latter 2 tissues were derived from tissue culture plantlets) were evaluated for GUS gene activity after particle bombardment.

Figure 2. Effect of biological parameters on transient expression of the GUS gene in bombarded date palm embryogenic calli. A, osmoticum type; B, osmoticum concentration; C, target tissue type; D, plasmid type and E, DNA quantity per shot. Data represent the mean ± SD determined from at least three replicates and were analyzed using one-way ANOVA (PROC-GLM program of SAS). Different letters indicate significant differences at the level of P <0.05 within a parameter.

Significant differences were observed among the tissues tested. As shown in Figure 3, the embryogenic calli showed the highest average number of blue spots per shot (1383 ± 565) compared to leaf (44 ± 3) and somatic embryos (9 ± 3), while no spots appeared after bombardment of the roots (Figure 2c and Table 1). Similar results have also been obtained by Kanchanapoom et al. (2008) in oil palm embryogenic calli where 100% survival of hygromycin resistant explants was observed after bombardment of embryogenic calli with pCAMBIA1302 at 850 and 1550 psi. In sugarcane, a high level of GUS activity was observed after bombardment of leaves with the Ubi-1 promoter (Gallo-Meagher and Irvine, 1993). In

orchids, the tissue type employed in the bombardment process can have a significant effect on transformation efficiency where half-moon PLBs were effective in maximizing transformation. In general, for *Cymbidium* hybrids, the outer layers of the PLB appear to be the most appropriate recipient tissue than any other organ (Chai and Yu, 2007).

Effect of promoter type

The actin promoter of rice is widely used in monocot transformation. It was, therefore interesting to check the
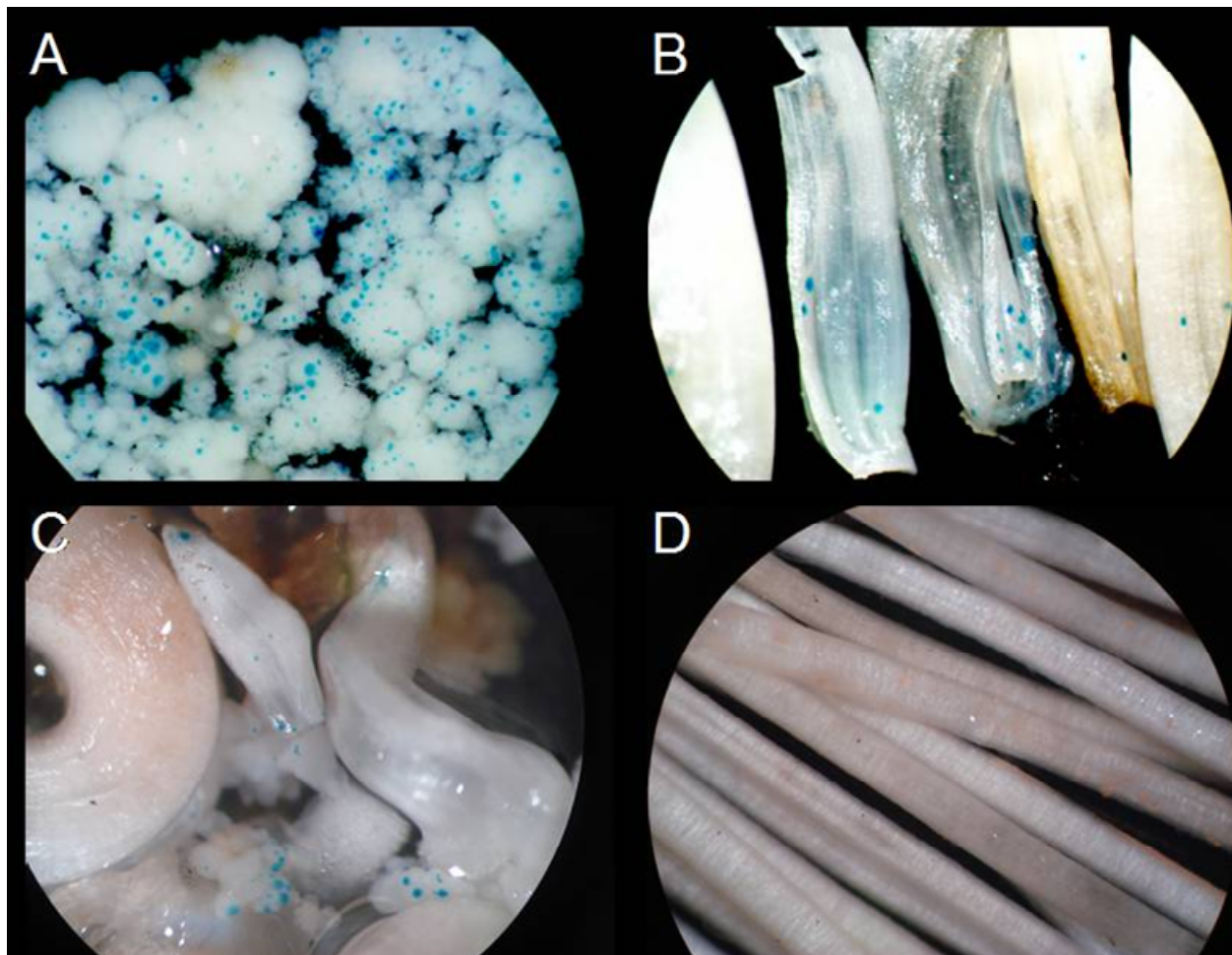
Figure 3. Transient GUS expression in different date palm tissues bombarded with the construct carrying the Act1 promoter. A, embryogenic calli; B, leaves; C, somatic embryos and D, roots.

effectiveness of this promoter in directing GUS expression in date palm tissues in comparison to common constitutive promoters. Three plasmids with 2 different promoters (pCAMBIA 3301 and pBI221 harboring CaMV 35S, and pAct1-D harboring rice Act1) carrying the GUS reporter gene were bombarded after precipitation into 1.6 µm gold particles. Among the 3 plasmids tested, pAct1-D showed a significant effect in increasing GUS expression efficiency by more than 10-fold (505 ± 162), whereas no significant differences were observed between the 2 other plasmids, pBI221 (44 ± 20) and pCAMBIA3301 (32 ± 5) (Figure 2d and Table 1). Gallo-Meagher and Irvine (1993) have reported a higher level of transient GUS activity by use of the Act1 promoter as compared to that of the CaMV 35S promoter in sugarcane. The Act1 promoter has also demonstrated the same effectiveness as the CaMV 35S promoter in some *Brassica* cultivars (Tuan and Garg, 2001). However, in another study, significant differences were observed between *Pinus nigra* cotyledons bombarded with the Act1 and CaMV 35S promoters (Lopez et al., 2000). Basu et al. (2003)

have demonstrated a low level of transient GUS activity by using the CaMV 35S promoter in the monocot *Agrostis palustris* when compared to ubiquitin promoters of corn and rice. Schenk et al. (1998) have found that after bombardment of maize and sorghum leaves with different promoters, the CaMV 35S promoter shows the lowest level of GUS and GFP gene expression in both plants as compared to those of the Act1 and ubiquitin promoters. The higher potential of the actin promoter in driving *uidA* gene expression as compared to CaMV 35S, suggests that Act1 can be employed to direct the expression of desirable agronomic genes in date palm transformation projects.

Effect of DNA quantity per bombardment

To obtain the appropriate amount of DNA for better coating into the microcarrier prior to bombardment, an experiment with different plasmid concentrations was performed. In this experiment pAct1-D at 4 concentra-

tions of 0.5, 2.5, 12.5 and 25 µg per shot was tested. After adding DNA, other reagents were added as described in materials and methods. From the results, a significant difference was observed between 0.5 µg of DNA and the other 3 concentrations. 0.5 µg per shot showed the lowest average number of blue spots (126 ± 4) per shot when compared to 2.5, 12.5 and 25 µg concentrations with 268 ± 39, 345 ± 70 and 285 ± 32 spots, respectively (Figure 2e and Table 1). Increasing the concentration of DNA from 2.5 µg up to 25 µg per shot increased the efficiency of transient GUS expression but with no significant difference among them. Our results are consistent with the findings of Sreeramanan et al. (2005) in banana, Parveez et al. (1998) in oil palm, Yee et al. (2008) in *Oncidium* and Khalafalla et al. (2005) in soybean.

ACKNOWLEDGMENTS

REFERENCES

Altpeter F, Baisakh N, Beachy R, Bock R, Capell T, Christou P, Daniell H, Datta K, Datta S, Dix PJ, Fauquet C, Huang N, Kohli A, Mooibroek H, Nicholson L, Nguyen TT, Nugent G, Raemakers K, Romano A, Somers DA, Stoger E, Taylor N, Visser R (2005). Particle bombardment and the genetic enhancement of crops: myths and realities. Mol. Breed. 15: 305-327.

Anoop N, Gupta AK (2004). Transgenic indica rice CV IR- 50 over-expressing vigna aconitifolia –Pyrroline -5-carboxylate synthase cDNA shows tolerance to high salt. J. Plant Biochem. Biotechnol. 12: 109-116.

Bakheet SA, Taha HS, Hanafy MS, Solliman ME (2008). Morphogenesis of sexual embryos of date palm cultured *In vitro* and early identification of sex type. J. Appl. Sci. Res. 4(4): 345-352.

Banerjee AK (2001). In vitro regeneration and genetic transformation studies in Indian cultivars of cotton. PhD thesis, Pune University, Maharashtra, India.

Basu C, Kausch AP, Luo H, Chandlee JM (2003). Promoter analysis in transient assays using a GUS reporter gene construct in creeping bent grass (Agrostis palustris). J. Plant Physiol. 160: 1233-1239.

Becker DK, Dugdale B, Smith MK, Harding RM, Dale JL (2000). Ge-netic transformation of cavandish banana (Musa spp. AAA group) cv 'Grand Nain' via micreoprojectile bombardment. Plant Cell Rep. 19: 229-234.

Cabrera-Ponce JL, Vegas-Garcia, Herrera-Estrella L (1995). Herbi-cide resistant transgenic papaya plants produced by an efficient particle bombardment transformation method. Plant Cell Rep. 15: 1-7.

Carpenter JB, Ream CL (1976). Date palm breeding, a review. Date Grower's Ins. Rep. 53: 23-33.

Chai D, Yu H (2007) Recent advances in transgenic orchid production. Orchid Sci. Biotechnol. 1(2): 34-39.

Chawla HS, Cass LA, Simmonds JA (1999). Expression of anthocyanin pigmentation in wheat transformed with anthocyanin regulatory genes. Curr. Sci. 76(10): 1365-1370.

Ferry M, Gomez S (2002). The red palm weevil in the. Mediterranean area, (formerly Principes). Palms. 46(4): 172-178.

Gallo-Meagher M, Irvine JE (1993). Effects of tissue type and promoter strength on transient GUS expression in sugarcane following particle bombardment. Plant Cell Rep. 12: 666-670.

Gatica-Arias AM, Arrieta-Esquivel G, Esquivel ME (2008). Plant regeneration via indirect somatic embryogenesis and optimization of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuai. Electron. J. Biotechnol. 11(1): 1-12.

Ikea J, Ingelbrecht I, Uwaifo A, Thottappilly G (2003). Stable gene transformation in cowpea (*Vigna unguiculata* L. walp.) using particle gun method. Afr. J. Biotechnol. 2(8): 211-218.

Jain RK, Jain S, Wang B, Wu R (1996). Optimization of biolistic method for transient gene expression and production of agronomically useful transgenic ice plants. Plant Cell Rep. 15: 936-968.

Janna OA, Maziah M, Ahmad Parveez GKA, Saleh K (2006). Factors affecting delivery and transient expression of β-glucuronidase gene in *Dendrobium* Sonia protocormlike- body. Afr. J. Biotechnol. 5(2): 88-94.

Jarl CI (1999). Plant regeneration and transient expression after particle bombardment of different barley (*Hordeum vulgar* L.) genotypes. Hered. 130: 83-87.

Jefferson RA, Kavanagh TA, Bevan MW (1987). GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO. J. 6(13): 3901-3907.

Kachanapoom K, Nekkaew A, Kachanapoom K, Phongdara A (2008). Determination of most efficient target tissue and helium pressure for biolistic transformation of oil palm (*Elaeis guineensis* Jacq.). Songkanakarin J. Sci Technol. 30(2): 153-157.

Khalafalla MM, Rahman SM, El-Shemy HA, Nakamoto Y, Wakasa K, Ishimoto M (2005). Optimization of particle bombardment conditions by monitoring of transient sGFP (S65T) Expression in Transformed soybean. Breed. Sci. 55: 257-263.

Kikert JR (1993). The biolestic PDS-1000/He device. Plant Cell Tissue Organ Cult. 33: 221-226.

Kim JB (2005). Development of efficient regeneration and transformation system in *Alstroemeria*. PhD thesis, Wageningen University, The Netherlands.

Klein TM, Harper EC, Svab Z, Sanford JC, Fromm ME, Maliga P (1988). Stable genetic transformation of intact Nicotiana cells by the particle bombardment process. Proc. Natl. Acad. Sci. 85:8502-8505.

Li Y, Tremblay FM, Seguin A (1994). Transient transformation of pollen and embryogenic tissues of white spruce (*Picea glauca* (Moench.) Voss) resulting from microprojectile bombardment. Plant Cell Rep. 13: 661-665.

Lopez M, Humara JM, Rodriguez R, Ordas RJ (2000). Transient *uidA* gene expresión in electroporated cotyledonary protoplasts of *Pinus nigra* ssp. Salzmannii and in bombarded cotyldones. Can. J. Res. 30: 448-455.

Majid NA, Parveez GKA (2007). Evaluation of green fluorescence protein (GFP) as a selectable marker for oil palm transformation via transient expression. Asia Pac. J. Biol. Biotechnol. 15(1): 1-8.

Malabadi RB, Choudhury H, Tandon P (2004). Initiation, maintenance and maturation of somatic embryos from thin apical dome sections in *Pinus kesiya* (Royle ex. Gord) promoted by partial desiccation and Gellan gum. Sci. Hort. 102(4): 449-459.

McElroy D, Zhng W, Cao J, Wu R (1990). Isolation of an efficient actin promoter for use in rice transformation. Plant Cell. 2: 163-171.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco cultures. Physiol. Plant 15: 473-497.

Obert B, Middlefell-Williams J, Millam S (2008). Genetic transfor-mation of bareley microspores using anther bombardment. Biotechnol. Lett. 30: 945-949.

Okada Y, Saito A, Nishiguchi M, Kimura T, Matsuda Y, Murata T (2002). Microprojectile bombardment-mediated transformation of sweet potato (*Ipomoea batatas* (L.) LAM). Sabro. J. Breed. Genet. 34(1): 1-8.

Parveez GKA, Chowdhury MKU, Saleh NM (1997). Physical para-meters affecting transient GUS gene expression in oil palm (*Elaeis guineensis* Jacq.) using the biolistic device. Ind. Crop Prod. 6: 41-50.

Parveez GKA, Chowdhury MKU, Saleh NM (1998). Biological parameters affecting transient GUS gene expression in oil palm

Elaeis guineensis Jacq.) embryogenic calli via microprojectile bom-(bardment. Ind. Crops. Prod. 8: 17-27.

Patell VM, Pradesh A, Krishnan RK, Chandran DK, Rayapuram N (2008). A process for generating genetically modified pearl millet through agrobacterium and biolistic transformation. Eur. Pat. EP. 1 876 242 A2.

Petrillo CP, Carneiro NP, Purcino AAC, Carvalho CHS, Alves JD, and Carneiro AA (2008). Optimization of particle bombardment parameters for genetic transformation of Brazilian maize inbred lines. Pesq. Agropec. Bars. Brasilia. 43(3): 371-378.

Quenzar B, Trifi M, Bouachrine B, Hartmann C, Marrakechi M, Benslimane AA, Rode A (2001). A mitochondrial molecular marker of resistance to Bayoud disease in date palm. Theor. Appl. Genet. 103: 366-370.

Ramesh M, Gupta K (2005). Transient expression of β-glucuronidase gene in indica and japonica rice (*Oryza sativa* L.) callus cultures after different stages of co-bombardment. Afr. J. Biotechnol. 4(7): 596-600.

Rhouma S, Dakhlaoui-Dkhil S, Salem AOM, Zehdi-Zouzi S, Rhouma A, Marakchi M, Trifi M (2008). Genetic Diversity and polygenetic relationships in date-palms (*Phoenix dactylifera* L.) as assessed by random amplified microsatellite polymorphism markers (RAMPOs). Sci. Hort. 117: 53-57.

Sanford J, Smith FD, Russell, JA (1993). Optimizing the biolistic process for different biological applications. Methods Enzymol. 217: 483-509.

Sartoretto LM, Cid LPB, Brasileiro ACM (2002). Biolistic transformation of *Eucalyptus grandis* ×*E. urophylla* callus. Funct. Plant Biol. 29: 917-924.

Schenk PM, Elliott AR, Manners JM (1998). Assessment of transient gene expression in plant tissues using the green fluorescent protein reference. Plant Mol. Biol. Rep. 16: 313-322.

Schopke C, Taylor NJ, Carcamo R, Beachy RN, Fauqet C (1997). Optimization of parameters for particle bombardment of embryo-genic suspension cultures of cassava (*Manihot esculenta* Crantz) using computer image analysis. Plant Cell Rep. 16: 526-530.

Sharma V, Yadav RC, Yadav NR (2004). High frequency plant regeneration from desiccated calli of *Indica rice* (*Oryza sativa* L.). Afr. J. Biotechnol. 3(5): 256-259.

Sreeramanan S, Maziah M, Abdullah MP, Rosli NM, Xavier R (2006). Potential selectable marker for genetic transformation in banana. Biotechnol. 5(2): 189-197.

Sreeramanan S, Maziah M, Abdullah MP, Sariah M, Xavier R, Nor'Aini MF (2005). Pysical and biological parameters affecting transient GUS and GFP expression in banana via particle bombardment. Asia Pacific J. Biol. Biotechnol. 13(1): 35-57.

Takumi S, Otani M and Shimada T (1994). Effect of six promoter-intron combinations on transient reporter gene expression in einkorn, emmer and common wheat cells by particle bombardment. Plant Sci. 103: 161-166.

Tian L, Huang C, Yu R, Liang R Li Z, Zhang L, Wang Y, Zhang X, Wu Z (2006). Overexpression AtNHX1 confers salt-tolerance of transgenic tall fescue. Afr. J. Biotechnol. 5(11): 1041-1044.

Tuan VD, Garg GK (2001). Gene transformation in *Brassica* sp. using particle bombardment technique. Omonrice. 9: 36-40.

Vain P, McMullen MD, Finer JJ (1993). Osmotic treatment enhances particle bombardment-mediated transient and stable transformation of maize. Plant Cell Rep. 12: 84-88.

Yee NC, Abdullah JO, Mahmood M, Basiron N (2008). Co-transfer of *gfp*, CHS and *hpt*II genes into *Oncidium* Sharry Baby PLB using the biolistic gun. Afr. J. Biotechnol. 7(15): 2605-2617.