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Full Length Research Paper

Factors affecting delivery and transient expression of β-glucuronidase gene in *Dendrobium* Sonia protocorm-like-body

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The effect of the biolistic device parameters and other factors affecting delivery and expression of *uid*A gene in *Dendrobium* Sonia was investigated. Three week old protocorm like body (PLB) were bombarded with gold microparticles coated with *pAHC25* plasmid harbouring the *uid*A gene which encodes *β*-glucuronidase. The factors investigated were the helium pressure, target distance, macrocarrier flight distance to stopping screen, distance from stopping screen to target tissues, vacuum pressure, gold microparticles size, spermidine and calcium on DNA precipitation, and the number of bombardments. Two days after bombardment, the PLB were subjected to histochemical GUS assay, and transient GUS activity was recorded as blue spots using a Leica stereomicroscope. All the factors tested showed significant effects ($p<0.05$) on the delivery of DNA and expression of the *uid*A gene in *Dendrobium* PLB except for calcium. Surviving PLBs were able to grow and regenerate normally into plantlets.

Key Words: biolistic transformation, orchid, *Dendrobium*, protocorm like body, *β*-glucuronidase assay.

INTRODUCTION

Orchid growing in Malaysia is a multi-million ringgit industry, most notably in the cut–flower trade (Mohd. Khairol and Noor Auni, 1991). Approximately 24.3 million stalks of orchid cut-flowers were produced in 2000 with *Dendrobium* topping the list at 13.1 million stalks (FAMA, 2000). A major issue faced by the orchid industry in Malaysia is associated with the lack of varieties. In order to keep up with the ever-changing tastes of consumers, there is an urgent need to create new and better varieties of orchids. Genetic engineering allows the transfer of selected gene into the orchid plant to create varieties. The biolistic device is still preferred over *Agrobacterium* as the transformation vehicle because the former has the advantage that it does not require construction of a particular vector and thus is host-independent. Considering that the nature of the DNA delivery system (PDS 1000 / He apparatus) is highly variable (Taylor and Vasil, 1991), it is therefore necessary to assess the system itself for a more efficient delivery of plasmid DNA into target tissues. The first report on using the biolistic device to introduce plasmid DNA into *Dendrobium* orchid was made by Kuehnle and Sugii (1992). Since then, other groups have reported transformation of the same genus using the device (Chia et al., 1994; Men et al., 2003; Tee et al., 2003). Recently, optimization of the biolistic parameters for callus cultures of *Dendrobium* Sonia 17 has been reported using GFP and GUS as transformation reporters (Tee and Maziah, 2005). In this study, we assessed the biolistic device parameters (helium pressure, distance from the macrocarrier to stopping screen, distance from stopping screen to target

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Abbreviations: PLB, protocorm-like-body; MS medium, Murashige and Skoog medium.

tissue, and vacuum pressure) together with other factors such as gold microparticles size, spermidine and calcium on DNA precipitation, and the number of bombardments, affecting the delivery and expression of *uid*A gene in PLB cultures of orchid. We also tested the ability of the PLB to regenerate after the bombardment.

MATERIALS AND METHODS

Plasmid

The plasmid used *pAHC25* (9.7 kb) was obtained from Christensen et al. (1992). It carries the *uid*A and *bar* genes, driven by the maize polyubiquitin promoter plus the first intron-exon. Preparation of gold microcarrier and precipitation of plasmid DNA onto the gold microparticles were carried out according to supplier's instructions (Bio-Rad) for the Biolistic PDS/1000 Helium System.

Biolistic transformation

The Biolistic™ PDS/1000 Helium System (BioRad, USA) was used in this study. The biolistic device parameters analyzed were as follows: rupture disk pressure (helium pressure of 450, 650, 900, 1100, 1350, 1550 psi); macrocarrier to stopping screen distance (6, 11, 16 mm), stopping plate to target tissue distance (60, 90, 120 mm); vacuum pressure (26, 27, 28 inches of mercury (inHg)). Other parameters analyzed include gold microparticles size (0.6, 1.0 and 1.6 µm), presence of spermidine and calcium in DNA precipitation step, and number of bombardment (1X, 2X, 3X) per target tissue plate.

Tissues preparation for transformation

Young shoots of *Dendrobium* Sonia were excised from three-year-old matured plants. Following excision, the shoots were placed under running tap water for 30 min, and then surface sterilized in 1.58% (v/v) sodium hypochloride for 15 min. The shoots were then transferred to fresh sodium hypochlorous solution for another 15 min of sterilization. The shoots were then rinsed off three times with sterile distilled water for about 5 min each time and subsequently placed in a sterile Petri dish. Apical meristems were isolated to induce PLB on hormonal-free half-strength MS basal salt medium (Murashige and Skoog, 1962). After two weeks, primary PLB appeared. As the PLB grew, secondary PLB formed on the primary PLB. The induced primary and secondary PLB were then multiplied in liquid basal ½ MS medium, supplemented with 2% (w/v) sucrose, on a rotary shaker at 120 rpm, 25 ± 0.5°C and 16 h illumination provided by fluorescent tubes with a photon flux density of 150 µmol/m^2/s^1. After three weeks of culturing, individual PLB of approximately 3-6 mm (length), measured along the longest diameter, were plated on solid basal ½ MS medium (Murashige and Skoog, 1962), pH 5.8, supplemented with 2% (w/v) sucrose and 2.5 g/L phytagel. A total of 20 individual PLB were centrally placed on each 90 mm diameter Petri dish. For each parameter tested, three independent bombardments (replicates) were carried out. The controls include non-bombarded tissues and tissues bombarded with gold microcarriers only (no DNA). All bombarded and non-bombarded tissues were subjected to transient GUS histochemical assay two days after bombardment.

GUS histochemical assay

GUS assay was performed according to Jefferson (1987). Transient GUS activity was recorded as blue spots (irrespective of size) using a Leica stereomicroscope and photographed either using a Nikon UFX-DX system.

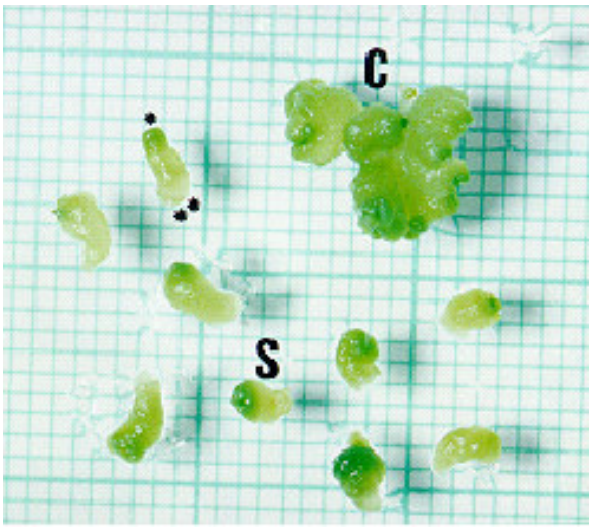

Figure 1. Induced protocorm-like-body (PLB). In clump form (marked "C") and separated into individual tissue (marked "S"). Each PLB gives rise to one plantlet. * indicates the shoot region, ** for the root region.

RESULTS AND DISCUSSION

The initiation of protocorm like body (PLB) tissue from the shoot meristem was observed after two weeks on phytohormone-free medium containing half-strength MS basal salts. Secondary PLB grew on top of the primary PLB to form a clump (Figure 1). The clump of PLB was cultured on fresh liquid medium for proliferation. For bombardment, each individual PLB was separated using scalpel and plated on fresh medium in a Petri dish. Transient *uid*A gene expression was used throughout this work as an efficiency indicator for assessing the biolistic device parameters affecting delivery of plasmid DNA into the target tissues, PLB. Each blue dot which was taken as an expression unit of the *uid*A gene (defined by Klein et al., 1988) was recorded to indicate GUS activity within a single cell or group of cells.

Effect of helium pressure

A helium shock wave is used to propel the plastic macrocarrier disk carrying DNA coated microparticles towards the target tissues. Ability of the microparticles to penetrate the different cell layers or tissue types is greatly dependent on the propelling force of the helium gas (Kirkkert, 1993). Results showed that changes in helium pressures were found to affect transient GUS expression significantly ($p<0.05$; Figure 2). The highest transient expression was observed at 1100 psi, consistent with

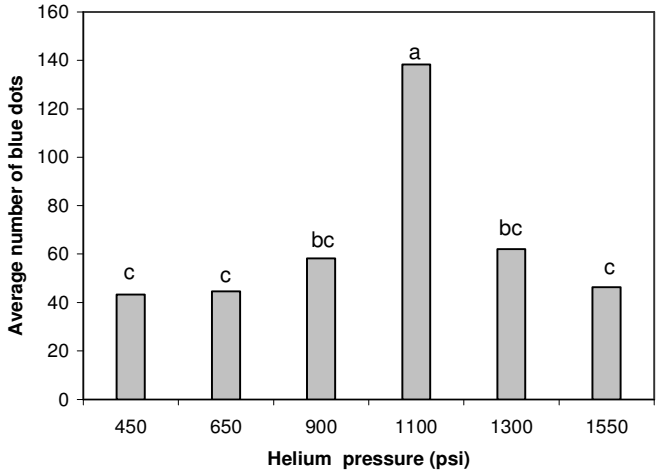

Figure 2. Effect of helium pressureon tra nsient GUS expression in PLB. Each value is the mean of three replicates. Any two means having a common alphabet (a, b or c) are not significantly different at p = 0.05 using Duncan's multiple range test across the different pressures.

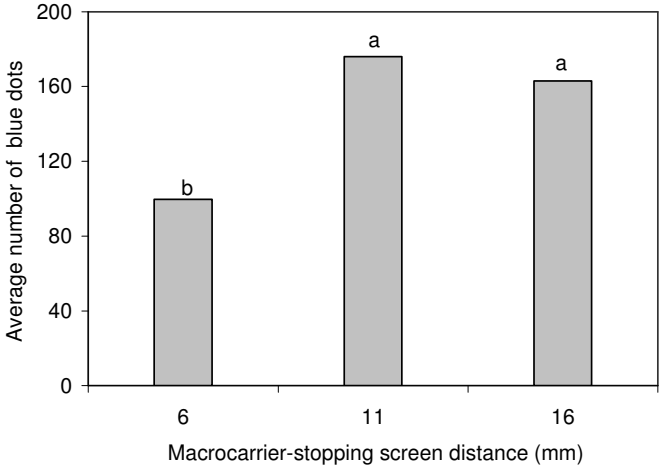

Figure 3. The effect of distance (mm) from the macrocarrier to the stopping screen on transient GUS expression in PLB. Data presented is the mean of three replicates. Any two means having a common alphabet (a or b) are not significantly different at p = 0.05 using Duncan's multiple range test.

observations made in rice (Zhang et al., 1996; Ramesh and Gupta, 2005), cassava (Schopke et al., 1997) and oil palm (Parveez et al., 1997).

Bombarding using lower or higher pressures did not result in any significant increased in transient GUS expression. Lower expression at lower pressure could be attributed to the poor penetration capability of the microparticles as they moved towards the tissues. While at higher pressures, the high penetrating force of the microparticles might be injurious to the tissues.

Effect of macrocarrier flight distance to stopping screen

Varying the macrocarrier flight distance (6, 11, or 16 mm) to the stopping screen was compared. Figure 3 showed that higher GUS expression was seen with longer flight distances but no significant differences were recorded with the 11 and 16 mm distances. Microcarrier velocities were reported to be affected by the distance that the macrocarrier travels, that is, the greater the distance, the higher is the velocity achieved (Kikkert, 1993). A high velocity is required for the release of the microcarriers from the macrocarrier, upon its impact on the stopping screen, in order for the micro particles to continue forward until they penetrated the target tissues. The low level of GUS expression accorded by the shorter distance (6 mm) implied that most of the micro particles were dispersed out with insufficient propelling force to penetrate the target tissues. While reduced GUS expression at higher 16 mm distance is probably due to instability of the macrocarrier resulting in variability and uneven distribution of microcarriers.

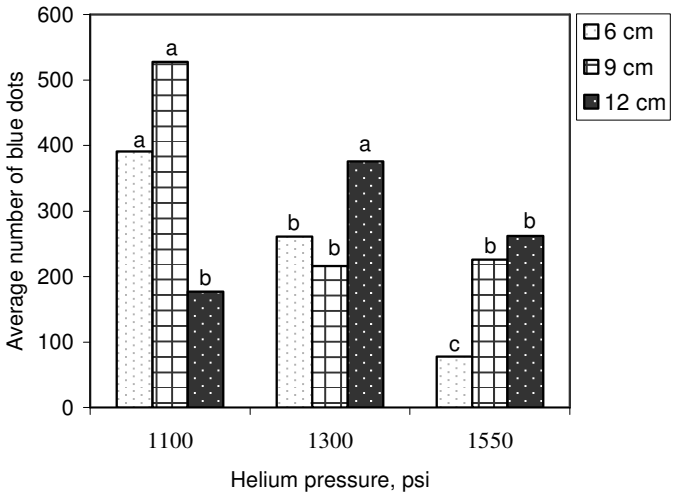

Figure 4. Effect of distance from the stopping screen to the target tissues on transient GUS expression in PLB. Each value is the mean of three replicates. Means with the same alphabet (a, b or c) are not significant at p = 0.05 using Duncan's multiple range test.

Effect of the distance from stopping screen to target tissues

Figure 4 compares the effect of flight distances of the microparticles on transient GUS expression at three different helium pressures. Changes in both helium pressure and distance were found to affect the level of transient GUS expression. For 1100 psi, the highest expression was observed at 9 cm although no significant

differences were observed with the 6 cm distance. Schopke et al. (1997) reported similar result of using a 1100 psi with 9 cm combination that gave higher expression in their cassava cultures. For the same psi at 6 cm, a lower expression level was observed, which could be due to tissue damage as tissue dislocation was observed at this closed-up range. While at 12 cm, the expression level was significantly reduced. This could be due to decreased velocity of the microparticles with the long flight distance giving reduced penetration force and thereby fewer cells receiving the oncoming DNA. Increasing flight distance resulted in reduced transient expression was also reported by Oard et al. (1990) and Parveez et al. (1997). For 1300 psi, the highest expression was observed at 12 cm while slightly lower expression levels were observed at 6 and 9 cm. For 1550 psi, closed-up range of 6 cm resulted in drastic reduction of GUS expression possibly due to massive tissue damage as exemplified by a high frequency of tissue dislocation during bombardment.

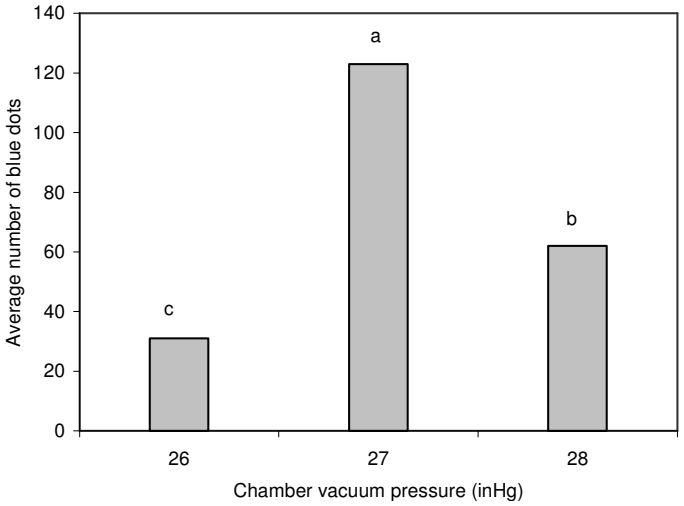

Figure 5. Effect of different vacuum pressures (inHg) on transient GUS expression in singled PLBs. Data is the mean of three replicates. Means with the same alphabet (a, b or c) are not significantly different at p = 0.05 using Duncan's multiple range test.

Effect of vacuum pressure

The vacuum in the bombardment chamber plays an important role in determining the drag forces that act on the micro particles during flight, thereby affecting the rate at which the micro particles lose velocity with distance. In short, the higher the vacuum, the better the microcarriers can maintain velocity (Kikkert, 1993). However, there is a drawback of maintaining a high vacuum pressure in the chamber because too high a vacuum (< 200 millibars) will cause the tissue to lose moisture rapidly and thereby reduced cell viability (McCabe and Christou, 1993). The best vacuum pressure that gave the highest significant ($p<0.05$) transient GUS expression in the PLB was accorded by 27 inHg (Figure 5). The lowest transient expression was observed at 26 inHg. This is not surprising because as the vacuum pressure decreases, the velocity of the micro particles is slowed down; thereby the chances of getting a hit or a good penetration into the target tissues are greatly reduced.

At 28 inHg, there was a significant ($p<0.05$) reduction in transient GUS expression. This is probably because the higher vacuum pressure is detrimental to the targeted cells due to higher velocity and a narrow distribution of micro particles over the targeted areas causing greater physical damage to the target cells.

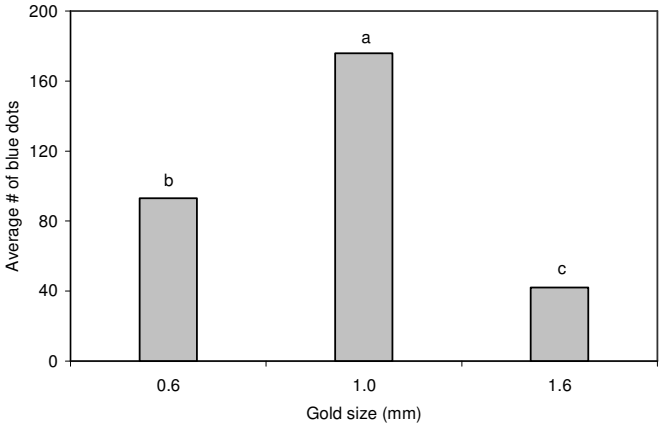

Figure 6. Effect of different gold microcarriers size on transient GUS expression in PLB. Means with the same alphabet are not significantly different at p = 0.05.

Gold microcarrier size

Different gold micro particles sizes (0.6, 1.0 and 1.6 µm) were compared for their efficiencies in delivering DNA into the target tissues. The intermediate size (1.0 µm) gave significantly ($p<0.5$) the highest expression level compared to other sizes used (Figure 6). This result is consistent with the observations obtained by Parveez et al. (1997), Schopke et al. (1997), Xiao and Ha (1997), and Kamo and Blowers (1999) in oil palm, cassava and creeping bent grass and *gladiolus* plants, respectively. Yang et al. (1999) showed that larger micro particles size decreases transient GUS expression in their *Cymbidium* orchid PLB. Folling and Olsen (2002) also reported higher damaging effect with larger micro particles size in their wheat transformation.

$CaCl_2$ and spermidine in DNA-microcarrier precipitation

Calcium and spermidine were components added in the solution mixture during DNA-gold micro particles

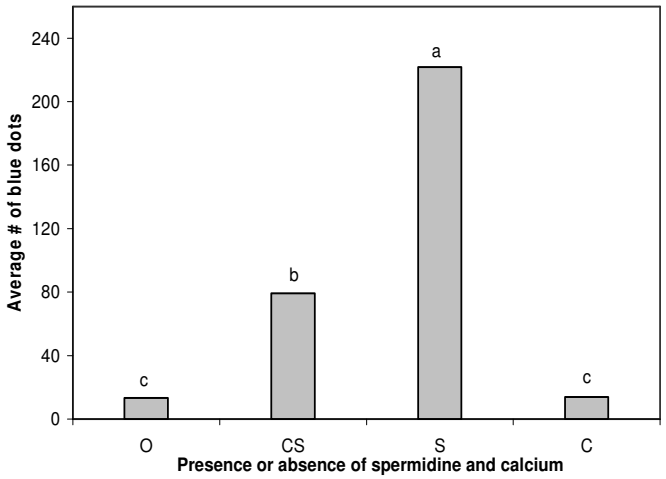

Figure 7. Effect of $CaCl_2$ and spermidine in the DNA-microcarrier cocktail mixture on transient GUS expression in PLBs**.** O = no spermidine or calcium; CS = both calcium and spermidine were added; S = Spermidine only; C = calcium only. Means with the same alphabets are not significant at p = 0.05.

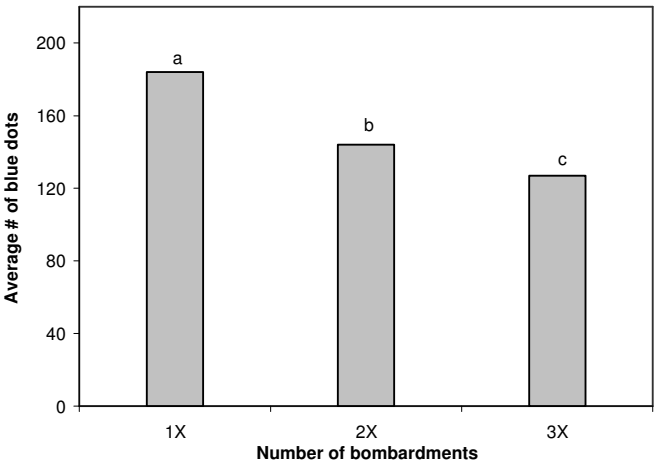

Figure 8. Effect of the number of bombardments on transient GUS expression in PLB. Means with the same alphabet are not significant at p = 0.05.

preparation in order to aid in the binding of DNA molecules to the gold micro particles. In this study, the highest transient GUS activity was obtained when spermidine was used in the preparation step (Figure 7). Exclusion of spermidine resulted in a 16-fold dropped in transient GUS expression. Studies by Moore et al. (1994) showed that spermidine had no effect on transient expression in their soybean transformation work. The addition of calcium showed unusually low GUS expression in this study, with or without spermidine. It is unknown why calcium did not help in the binding process of the DNA molecules onto gold microcarriers.

Table 1. Summary of optimised parameters for transformation of *Dendrobium* Sonia PLB.

Parameters	Optimised Settings
Helium pressure	1100 psi
Distance from macrocarrier to stopping screen	11 mm
Distance from stopping screen to target tissues	9 cm
Vacuum pressure	27 inHg
Gold microcarrier	1.0 µm
Presence of $CaCl_2$ and spermidine in DNA-microcarrier precipitation	With spermidine only
Gold microcarrier size	1.0 µm
Number of bombardments	1X

Number of bombardments

Increasing the number of bombardments significant ($p<0.5$) decreased transient GUS expression in the PLB (Figure 8). This is not surprising because rapid penetration of gold microparticles into the target tissues by the biolistic gun method tends to create mechanical injuries to the tissue (Hunold et al., 1994). So, by increasing the number of shots would undoubtedly increased the injuries capacity of the targets thereby decreasing the number of surviving cells capable of expressing the gene. Advantage of multiple bombardments in increasing GUS expression was reported by Reggiardo et al. (1991) in maize and by Clemente et al. (1992) in peanut. This shows that rather than decreasing the number of surviving cells, the second bombardment would seem to compensate for the first slight misfire of the gun (King and Kasha, 1994). Parveez et al. (1997), however, observed no significant differences between single and double bombardments in their oil palm although double bombardment gave higher transient GUS value. Takahashi et al. (1998) reported using two shots per target sample for their Eustoma grandiflorum while Marchant et al. (1998) used one shot per target sample in roses.

Optimised bombardment conditions

Bombardment using the optimised condition (summarised in Table 1) did not affect the regeneration capacity of the bombarded PLB as shown in Figure 9. The presence of the microcarriers (gold) could be seen around the GUS-expressing regions (Figure 9B). The percentage of transformed and proliferated PLB (clump) expressing

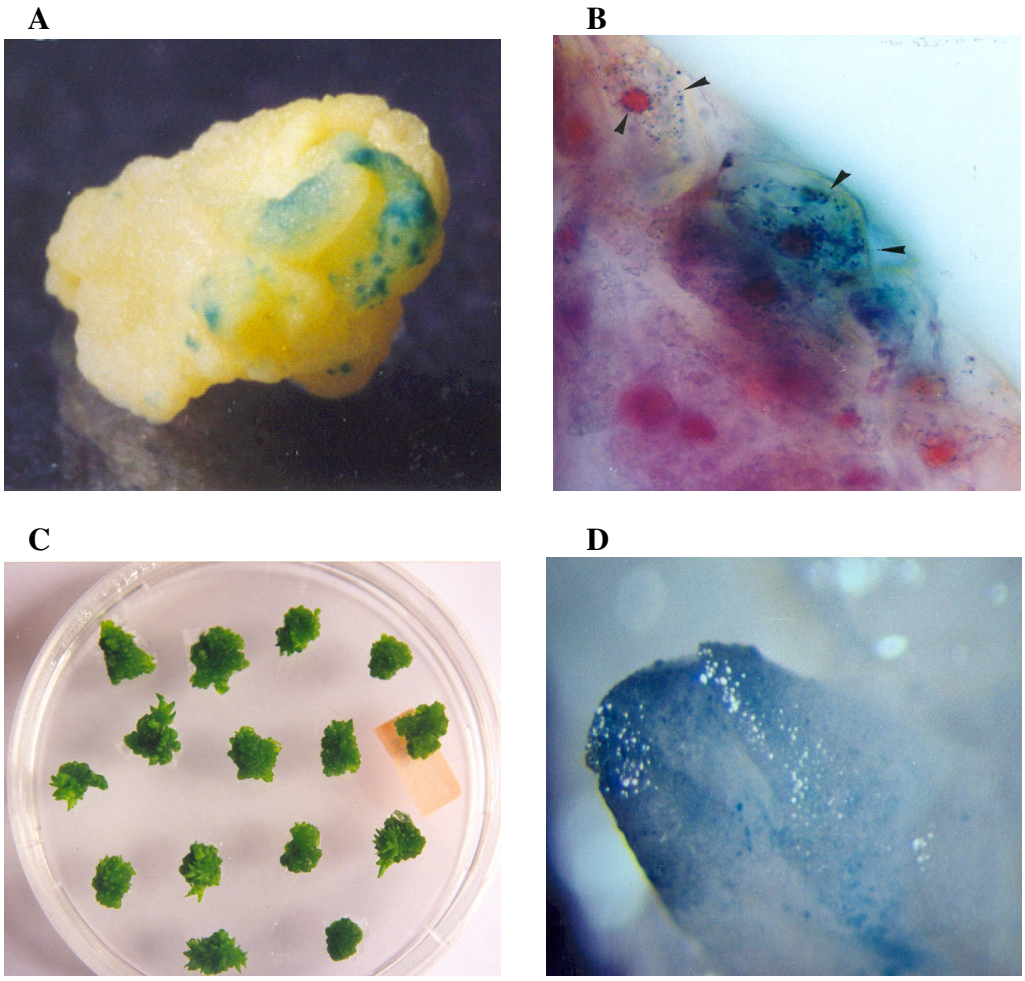

Figure 9. GUS expression in bombarded PLB. **(A)** GUS-positive (blue colour) PLB decolourised with ethanol; **(B)** squashed PLB stained with safranin; note the red-stained nuclei and intracellular distribution of gold particles (arrowheads) in GUS-expressing region (blue); magnification: 400X; **(C)** proliferated and regenerated PLB; **(D)** young shoot decolourised with ethanol.

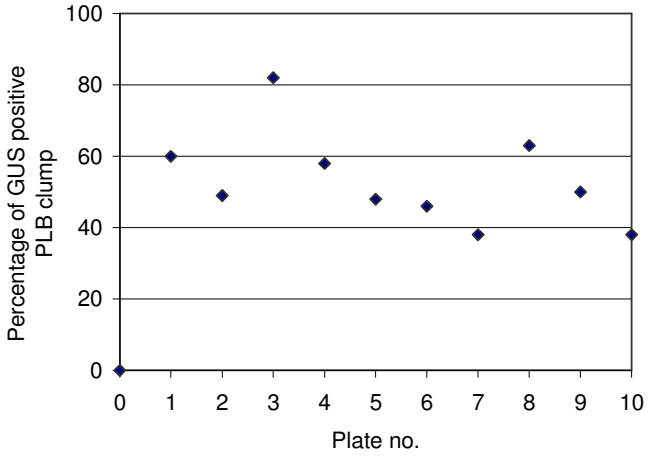

Figure 10. Percentage of transformed and proliferated PLB clumps expressing GUS activity one month post-bombardment. A total of 200 clumps from ten plates (independent bombardments) were assayed. Plate 0 = untransformed control as a check that there were no endogenous (background) GUS activity under the standard assay conditions (pH 7.0). The percentage of expression was calculated as the number of clump expressing GUS from a total number of 20 samples per plate regardless of the number of PLB in a clump.

GUS in ten independent bombarding events ranges from 40 to 80% (Figure 10).

CONCLUSION

We found that the parameters of the delivery device and other factors involved in the preparation of DNA for bombardment, except for calcium, significantly affect plasmid DNA delivery into PLB of *Dendrobium* Sonia.

The bombarded tissue, PLB, was able to regenerate. Hence, by choosing the suitable parameters we are able to transform this orchid hybrid with potential genes yielding certain characteristic traits that are of commercial value.

ACKNOWLEDGEMENT

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