UNIVERSIDADE FEDERAL DE LAVRAS

OPTIMIZATION OF SOYBEAN EMBRYOGENIC SUSPENSION CV. JACK WITH 606ASA2 PROMOTER USING PARTICLE BOMBARDMENT

MARCELO MURAD MAGALHÃES

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Thesis submitted to Universidade Federal de Lavras as a partial fulfillment of the requirements for the degree of Doctor in Food Science, area of concentration in Postharvest Physiology of Fruits and Vegetables.

Adviser

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LAVRAS
MINAS GERAIS - BRAZIL
1998

Ficha Catalográfica preparada pela Seção de Classificação e Catalogação da Biblioteca Central da UFLA

Magalhães, Marcelo Murad

Optimization of soybean embryogenic suspension cv. Jack with 606ASA2 promoter using particle bombardment / Marcelo Murad Magalhães. -- Lavras : UFLA, 1998.

56 p. : il.

Adviser: Admilson Bosco Chitarra.

Thesis (Doctor) - UFLA.

References.

1. Soybean. 2. Particle bombardment. 3. Transient expression. 4. 606ASA2 promoter. I. Universidade Federal de Lavras. II. Title.

CDD-633.34
-581.33

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APPROVED on February 6, 1998

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ACKNOWLEDGMENTS

I wish to express my gratitude and deep appreciation to Dr. Adimilson Bosco Chitarra and Dr. Jack M. Widholm for their valuable guidance throughout the course of this program at Federal University of Lavras and University of Illinois at Urbana-Champaign, and for their patience and assistance in preparing this thesis. Their guidance provided me invaluable professional experience.

My sincere thanks are expressed to my committee members, Dr. José Donizeti Alves, Itamar Ferreira de Souza and Amauri Alves Alvarenga, for their interest and contribution in finalizing my thesis. I am very gratefull to Dr. Pon Samuel Jayakumar for his knowledge and friendship.

I would like now to acknowledge my colleagues Rosemary, Luiz Carlos, Josivan, Eduardo, Silvanda, Jaime, Kelly, Sérgio, João, Gicelda in Brazil and Sonitichai, Sermissi, Hee Sook, Jeff, Lockman, Vera, Steve in Urbana. I am gratefull to Mirian Maluf, Oswaldo Francisco Pinto, João, Sérgio and Alexandre with whom I had brazilian culture in foreign country.

I would like to thank CNPq for providing funds during four years and half and University of Illinois during one year of my doctoral work.

Finally, I am eternally gratefull to my family.

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ABSTRACT

OPTIMIZATION OF SOYBEAN EMBRYOGENIC SUSPENSION CV. JACK WITH 606ASA2PROMOTER USING PARTICLE BOMBARDMENT

The anthranilate synthase gene from a tobacco suspension culture has a 606bp fragment in its promoter region that was fused with the GUS gene to use in transient expression assays in order to compare the performance of the Helium PDS/1000 Device and Particle Inflow Gun (PIG) in soybean embryogenic suspension cultures of the cv. Jack. The histochemical and fluorimetric enzymatic assay for GUS activity was carried out in embryos 2 days after bombardment. In most treatments the results from the histochemical and enzymatic assays were similar. The best conditions for the Helium PDS-1000 were 1100 psi, 13 cm flying distance, 1 mg of gold and 1 μg of DNA per shot. For the PIG 80 psi, 1 mg of gold and 13 cm target distance were the optimal conditions. The brief sonication of DNA suspension just before shooting increased the transient expression level. The sodium acetate DNA precipitation methodology was as effective as the $CaCl_2$/spermidine method and was quicker. When both guns were compared with their optimal conditions, the Helium PDS-1000 showed higher performance around 25% than the PIG using gold particles. Although the PIG was less effective than the Helium PDS/1000, it showed reproducibility and is a less expensive option.

RESUMO

MAGALHÃES, Marcelo Murad. **Otimização de suspensão embriogênica de soja cv. Jack com promotor ASA2 utilizando bombardeamento de partículas**. Lavras: UFLA, 1998. 53p. (Tese: Doutorado em Ciência dos Alimentos)*

O gene da anthranilase synthase isolado de cultura de suspensão embriogênioca de fumo, tem um fragmento de 606 bp na sua região do promotor, que foi ligado ao gene GUS para ser utlizado em ensaio de expressão transiente, e comparar a performance do aparelho de Hélio PDS/1000 e Pistola de Influxo de Partícula (PIG) em suspensão embriogênica de soja, cultivar Jack. Os ensaios histoquímicos e fluorimétricos da atividade GUS foram conduzidos 2 dias após bombardeamento. Na maioria dos tratamentos os resultados dos ensaios histoquímico e enzimático apresentaram a mesma tendência. As melhores condições para o aparelho de Hélio PDS-1000 foram 1100 psi, 13 cm de distância do alvo, 1 mg de ouro e 1 μg de DNA por tiro. Para o PIG, 80 psi, 1 mg de de ouro e 13 cm de distância do alvo foram os melhores parâmetros. Uma breve sonicação da suspensão de DNA, justamente antes do bombardeamento aumentou o nível de expressão transiente. A metodologia de precipitação de DNA utilizando-se acetato de sódio foi mais efetiva e rápida que a $CaCl_2$/

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esperimidina. Quando as duas pistolas foram comparadas utilizando-se as suas condições otimizadas, o revólver de Hélio PDS-1000 mostrou uma performance 25% superior ao PIG, utilizando partículas de ouro. Apesar do PIG ser menos efetivo do que o de Hélio PDS/1000, mostrou boa reproducibilidade e poderia ser uma opção mais barata.

1 INTRODUCTION

Soybean (*Glycine max* (L.) Merr.) is a source of oil and protein, and it is mainly grown on 35 million hectares in the United States and Brazil. In the United States only a few plant introductions have given rise to the major cultivars, and this narrow germplasm base might limit soybean breeding programs. Modification of soybean using genetic engineering techniques would facilitate the rapid development of new varieties with traits such as herbicide resistance, disease resistance, or seed quality improvement in a manner unobtainable by traditional plant breeding methods (Hinchee et al., 1989; Widholm, 1976).

Some transgenic soybean plants have been obtained using particle bombardment with the cryIA(b) gene (Parrott et al., 1994), with a synthetic *Bacillus thuringiensis* insecticidal crystal protein (Stewart et al., 1996) and chitinase (Chanprame, 1997). Some researchers attempting to study the properties and limits of co-transformation (Hadi et al., 1996), tested a seed specific promoter (Iida et al., 1995; Cho et al., 1997) using soybean embryogenic suspensions as target. Most of these publications used the Biolistic PDS-1000 Helium Gun. With the advance of this technology other models have been built (Gray and Finer, 1993). Among them, the Particle Inflow Gun (Vain et al., 1993), that in relation to the PDS-1000/He device does not need a microcarrier to carry the particles, thus reducing the consumables, and clean up and cycle time. However, there has not been much research with this model that has focused on preparations and conditioning methods using soybean embryogenic suspensions (Finer and McMullen, 1991). Thus information comparing both devices with

soybean embryogenic suspension cultures is practically nonexistent.

Most of the soybean transformation research has been carried out with the cauliflower mosaic virus 35S promoter (Benfey and Chua, 1990), and only a few publications have used different promoters such as the lectin (Cho et al., 1995) or conglycinin promoters (Iida et al., 1995). Actually the promoter options for soybean research are very limited. Recently the anthranilate synthase gene isolated from tobacco suspension cultured lines (Song et al., unpublished results) was found to contain a 606 bp fragment from the promoter region that seems to drive high expression in soybean embryogenic suspensions. This new promoter could be an option for soybean transformation research.

The objective of this study is to compare the performance of the Particle Inflow Gun and the PDS-1000/He device in transient expression with soybean embryogenic suspensions of cv. Jack using the 606 ASA2 GUS construct.

2 MATERIAL AND METHODS

2.1 Embryogenic suspensions

Pods from the soybean cultivar Jack were obtained from field-grown plants, sterilized by immersion in 1% NaOCl (20% commercial bleach, vol/vol.). After three washings with sterilized water, the zygotic embryos and embryonic axis were removed from immature seeds, and the cotyledons were placed on MSD40 containing MS salts (Murashige and Skoog, 1962) supplemented with B5 vitamins (Gamborg et al., 1968), 6% sucrose, 40 mg/L 2,4-D, pH adjusted to 7.0 and the medium solidified with 8.0 g/L agar. The cultures were kept at 28°C, under continuous light (80 $\mu E\ m^{-2}s^{-1}$ of light intensity). Every three weeks the

cotyledons were subcultured. Approximately 60 d later the globular stage somatic embryos were transferred to 125 ml - Erlenmeyer flasks containing Finer and Nagasawa (1988) medium, kept under continuous light and shaking speed at 125 rpm. Selective subcultures were made weekly until the embryos were green in color and globular in shape in order to be used for particle bombardment. The 606 bp promoter fragment from the anthranilate synthase gene was isolated from tobacco suspension cell lines (Song et al., 1997). The DNA was isolated from *E. coli* DH-5α using a Qiagen kit. This fragment was inserted in front of the GUS gene (Jefferson et al., 1987) with a NOS terminator in pUC19 vector. This new construct was transformed into *E. coli* DH-5α and the plasmid DNA isolated by Qiagen kit. The DNA final concentration was adjusted to 1 μg/μl to be used for bombardment.

2.2 Particle bombardment

One day before bombardment around 1 g of embryos were placed in a Petri dish containing filter paper without liquid. The DNA was precipitated according to calcium/spermidine methodology (Klein et al., 1988) and sodium acetate (Morikawa et al., 1987). The tungsten and gold particle size were 1.0 and 1.2 μm, respectively. From this mixture for both elements, 10 μl was pipetted onto the macrocarrier for the PDS-1000/He device (Du Pont) and 15 μl onto the Millipore screen in the Particle Inflow Gun (PIG). After bombardment the samples were kept at 28° for 2 d before GUS assay. Optimized conditions for the Helium PDS-1000/He device were determined by modifying the pressure, DNA, gold and tungsten amount, distance from the target tissue, sonication and sodium acetate effect, while for the Particle Inflow Gun the pressure and distance were

altered. Finally the best for each gun was chosen and their performance was compared in a separate experiment.

2.3 GUS assays

Fourty-eight hours after bombardment the embryos were transferred to GUS buffer with the substrate (Jefferson et al., 1987) overnight at 37°C, followed by four washings with 95% ethanol. The tissue was placed under a screen with 1 mm^2 mesh, and the blue foci counted using a Nikon microscope.

For the fluorimetric assay around 0.5 g of embryos were homogeneized in 600 µl of GUS extraction buffer, the mixture was centrifuged and the supernatant was used for protein concentration measurement according to Bradford (1976). One hundred µl of the extract was added to 500 µl of prewarmed (37°C) MUG assay buffer (Jefferson, 1987). Each reaction was stopped at 0, 30 and 60 min by the addition of 0.2 M Na_2CO_3. The amount of 4 MU in the samples was measured with excitation at 320-390 nm and emission at 415-490 nm in a Hitachi F-2000 fluorescent spectophotometer. The specific activity of the GUS enzyme was calculated as pmol 4-MU $min^{-1}mg^{-1}$ total protein.

Samples in an experiment was completely randomized with four replications. For histochemical assay ten readings were made for each treatment and four replicates were used for both GUS assays. The data were subjected to ANOVA and means compared by t test at 5% level of probability.

3 REVIEW OF LITERATURE

3.1 Particle Bombardment

The particle bombardment or biolistics process uses high velocity microprojectiles to deliver nucleic acid into intact cells and tissues (Sanford et al., 1987; Sanford et al., 1990). This technology has been used to transform animal cell lines (Zelenin et al., 1989), chloroplasts of tobacco and various plant tissues including cell suspensions, calli, immature embryos, mature embryos parts, meristems, leaf pieces, micropores and pollen (Sanford et al., 1993).

This process offers some advantages over *Agrobacterium* mediated transformation like non-host specificity and plasmid construction is simplified, since the DNA sequences essential for T-DNA replication and transfer in *Agrobacterium* are not required. In addition it is possible to obtain false positive results due to the growth of *Agrobacterium* in the host tissue. It is possible to introduce multiple plasmids (co-transformation), and the large plasmid containing multiple transforming DNA is not necessary using particle bombardment. The biolistics protocols for transformation are simplified, since the complex bacteria plant interrelationships that vary with each system, are eliminated (Gray and Finer, 1993).

The potential of this technology has stimulated the development of prototype devices by several laboratories. One of these models is PDS-1000/He device in which the DNA is delivered to cells in association with microscopic metal particles called microcarriers, that are propelled at high velocities toward target tissues. The microcarriers are accelerated on a plastic disk, called a macrocarrier, which is driven by a shock wave of helium gas. In this model, the

optimization parameters like pressure, flying distance, DNA precipitation methodology, nature of microcarriers and the use of meshes could be important to increase the transformation rates (Sanford et al., 1993). Some biological parameters are also important for successful biolistic transformation such as the appropriate gene construct with a strong promoter to express in a desired target tissue. The second parameter, the target cells must be in a receptive stage for transformation, and finally there must be high rates of particle penetration and cell survival and growth after bombardment (Sanford et al., 1993).

Another available gun is the Particle Inflow Gun (PIG) that was first described by Takecuchi et al. (1992). Basically, the PIG accelerates particles in a stream of low pressure helium. It does not need a macrocarrier to carry the particles, and the force necessary to accelerate them was reduced (Vain et al., 1993). For this model the use of prechamber, baffle, pressure and distance from target are related with tissue displacement, particle penetration and cell survival. Results from preliminary research suggests that a prechamber is more associated with stable transformation due to a reduction of damage in a target tissue (Vain et al., 1993).

The PIG has been compared with the Helium Device with barley. Using the helium gun highest transformation rates were reached with cultivar Golden Promise (1.4%), whereas the transformation rates for other genotypes were fairly low (0.3% for Sera and Corniche, and 0% for all other cultivars). By application of the PIG, stable transformed plants were recovered from genotypes Corniche and Salome at frequencies (1.2% and 1.5%, respectively) comparable to the transformation frequency of Golden Promise. The transformation frequency of Femina (0.6%) achieved with the PIG was higher than the frequencies of Dera and Corniche transformed with the Helium PDS (Koprete et al., 1996). This

result could be due to lower gas blast and acoustic shock caused by the inflow gun (Vain et al., 1993). The tissues remained viable and formed embryogenic structures in frequencies comparable to nontransformed controls (Koprete et al., 1996). In recent work with the PIG, the B-glucuronidase (GUS) expression driven by the CaMV 35 S promoter has been induced in apple cotyledonary explants from immature seeds (Yang et al., 1997). This model has also been used with maize and stable transformed plants were recovered (Vain et al., 1993). The helium device has been used in a larger number of crops like *Pinus radiata* (Walter et al., 1997), oil palm (Choowdhury et al., 1997), tomato (Van Eck et al., 1995), rice (Jain et al., 1996, Sivamani et al., 1996), barley (Stiff et al., 1996) and wheat (Altpeter et al., 1996). In relation to the PIG the model PDS-1000/He is safer, cleaner and allows better control over bombardment power, distributes the microcarrier more uniformly over the target cells, and it seems to be more consistent from bombardment to bombardment according to Kikkert (1993). However, the absence of macrocarrier, stopping screen and rupture disk in the Particle Inflow Gun reduces consumables, the clean up and cycle time are shorter and it is less expensive to obtain. The use a baffle jointly with a prechamber is recommended in order to reduce the tissue injury in the center of the plate. In some cases the results obtained with the helium gun are similar to those found with the PIG (Vain et al., 1993; Koprek et al., 1996).

In the case of soybean transformation, the first work was done with a particle acceleration device by Christou et al. (1988). Immature embryos were cultured and three weeks after the bombardment were selected with 50 mg/l kanamycin. However plants could not be regenerated from these transformed cultures. Meristems from immature soybean seed embryonic axes have also been bombarded with gold particles containing CaMV35S, **npt** II and **nos**, or

CaMV35S, uidA, nos gene constructs (McCabe et al., 1988). The tissues were then cultured on a high cytokinin (13.3μm BA) medium to induce multiple shoot formation in the dark for 1 to 2 weeks. Three to eight shoots were recovered from each axis. Around 2% of the shoots contained some transformed tissue with the npt II and uid A genes, indicating integration of both genes at a single site.

The embryogenic suspension system has been used in particle bombardment studies mainly with the Biolistics Particle Delivery System from DuPont. The first report used the tungsten particles coated with DNA carrying hph and/or uidA genes driven by the CaMV35S promoter (Finer and McMullen, 1991). Each bombardment produced about three stable transformed clones. These results give a stable transformation versus transient expression frequency of about 0.4%. The regenerated plants expressed GUS activity and contained the transformed genes. A few years later, "Fayette" embryogenic suspension cultures produced GUS positive globular somatic embryos and plants (Sato et al., 1993). An average of four independent transgenic lines were generated per bombarded flask of an embryogenic suspension. Histological analysis showed that in the somatic embryos the proliferation occurred from the first cell layer (Sato et al., 1993). Recently scientists from the same laboratory investigated some of the properties and limits of co-transformation, when 12 different plasmids were introduced into soybean cv. Jack embryogenic suspensions via particle bombardment (Hadi et al., 1996). The DNAs used for co-transformation included 10 plasmids containing RFLP markers for maize and 2 plasmids separately encoding hygromycin resistance. The resistant clones were isolated 8 weeks after bombardment. Southern hybridization analysis of 26 hygromycin resistant embryogenic clones verified the presence of the introduced plasmid DNAs. All co-transforming plasmids were present in most of transgenic

soybean clones and there was no preferential uptake and integration of any of the plasmids (Hadi et al., 1996).

Some successful attempts have been made to introduce the Bt gene into soybean embryogenic suspensions via particle bombardment (Parrott et al., 1994; Stewart et al., 1996). In this latter publication, embryogenic suspension cultures of the cv. Jack was transformed using particle bombardment with a synthetic *Bacillus thuringiensis* insecticidal crystal protein gene (Bt cry1Ac) driven by the 35S promoter and linked to the hph gene. Approximately 10 g of tissue was bombarded, and three transgenic lines were selected on hygromycin containing medium and these were converted to plants. The Cry 1Ac protein accumulated up to 46 ng mg^{-1} extractable protein. In a detached leaf bioassay, plants with an intact copy of the Bt gene, and to a lesser extent those with a rearranged copy, were protected from damage from corn earworm *(Helicoverpa zea)*, soybean looper *(Pseudoplusia includens)*, tobacco budworm *(Heliothis virescens)*, and velvetbean caterpillar *(Anticarsia gemmatalis)*. Corn earworm produced less than 3% defoliation on transgenic plants, compared with 20% on lepidopteran-resistant breeding line Gat 1R81-296, and more than 40% on susceptible cultivars.

In contrast with earlier studies in which primary transformants had decreased growth rates, abnormal leaf morphology, or reduced fertility (Finer and McMullen, 1991; Parrott et al., 1994), and the progeny obtained, if any, lost fertility in successive generations, the transgenic lines produced in this study did not show any morphological abnormalities and did maintain fertility. There are two methodological differences between this research and the earlier papers: culture age and selection regime (Finer and McMullen, 1991; Parrott et al., 1994). In this research, young cell lines (3 to 4 months) were used, in contrast

with cell lines that were 1 year old or older. Another modification was to initiate postbombardment selection on solidified MSD20 medium instead of FN liquid medium, and the selection begun the day after instead of 1 or 2 weeks after bombardment. The concept of this strategy compared with liquid medium is to promote slower growth of transgenic cells and slower death of nontransgenic cells. In addition to these facts, the embryo clumps are staticly placed on solidified medium, that could decrease the probability of toxic leachates from the dying clumps from interfering with the growth of living clumps (Stewart et al., 1996).

Recently the embryogenic suspensions have been used to test constructs and seed specific genes. In one of these publications, cultures of the soybean cv Jack were transformed with the B-glucuronidase and hygromycin phosphotransferase genes under the control of the cauliflower mosaic virus (CaMV) 35S promoter on different plasmids (Cho et al., 1997). GUS activity was detected in 45% of the transgenic clones showing that cotransformation occurred at high frequency. Stable integration of both the GUS reporter gene and HPT selectable marker, was confirmed by PCR amplification of both genes using double primer sets together in the same reaction. The Southern blot hybridization also showed the presence of foreign genes in the genomic DNA. A problem with low plantlet regeneration frequency and sterility of the regenerated plants was found in this study. The cultures used in this research were 1.5 years old (Cho et al., 1997). It seems that soybean embryogenic suspension cultures should not be older than 1 year at the time of bombardment (Hadi et al., 1996; Stewart et al., 1996).

The other study using soybean cv. Jack embryogenic suspension aimed to test a seed specific promoter by transient and stable gene expression (Cho et al.,

1995). The GUS gene driven by the lectin control regions was expressed with and without the lectin signal sequence. The GUS gene showed the developmental and tissue specific expression of the lectin promoter in developing somatic embryos derived from the transformed soybean embryogenic cultures. The use of these lectin cassettes in the embryogenic system will enable future studies in time specific gene regulation and will help to create new varieties with improved seed protein levels.

The embryogenic suspension is not an ideal method yet, but it is not very genotype specific and can be used to study constructs and seed specific genes. Although this method is low frequency, it requires labor, expensive equipment, and time and skill for initiation. Some transgenic plants with useful genes have been obtained with this methodology, but improvement in plant regeneration needs to be done.

3.2 Soybean Regeneration

The soybean [*Glycine max* (L.) Merr.] can be regenerated by somatic embryogenesis (Barwale et al., 1986; Wright et al., 1986). In the former publication the authors observed that MS (Murashige and Skoog, 1962) medium with NAA concentrations ranging from 21.4 to 60μM stimulated normal embryogenesis and somatic embryos underwent normal development. The cultivar Williams showed the best results with 54% embryogenic response obtained with 43μM NAA. When embryos of different sizes were plated, those from 4 to 7 mm in length, gave the highest response with the genotypes tested, and the highest embryogenic frequency was obtained with cv. Williams, around 65%. Other genotypes ranged from 30 to 65% (Barwale et al., 1986).

The results from histological studies at different times after plating in MS (Murashige and Skoog, 1962) with 43μM NAA and 0.2μM thiamine (Barwale et al., 1986), showed that meristematic regions could be seen on the surface of cotyledons as early as 5d after culture, and the further development of this embryo was very similar to that of zygotic embryos. It was observed that the mature embryo did not possess a well defined apical meristem, as would be present in zygotic embryos. Furthermore, other additives like glycine, nicotinic acid, pyridoxine.HCl and myo-inositol were tested, but only nicotinic acid had a favorable effect; increasing the embryogenesis by 18% (Barwale et al., 1986). For the best condition for germination, the embryos at the torpedo stage were placed on MSR medium and upon further growth were transferred to tubes with MS medium without any growth regulators. The green plants were transferred to Hoagland's solution no.1 and then transplanted into a soil mixture. All green plants set seeds and the R_1 seeds developed normally (Barwale et al., 1986).

In other basic and important research on the development of soybean somatic embryos, the cultivars J103 and McCall were used to test the effect of five auxin-sucrose treatments (Hartweck et al., 1988). For J103 explants in 25 mg/l 2,4-D + 3% (D25) or 10 mg/l NAA+ 1.5% sucrose media (N10), the greatest number of embryos were produced from the central region of abaxially oriented explants on D25, but only 5% of these embryos were normal in shape. The greatest number of embryos were produced from the marginal region on adaxially oriented explants on D25. For McCall explants on D25 or N10 media a number of normal embryos were produced from margins of abaxially oriented explants. This marked orientation effect on the frequency of normal somatic embryos initiated by high 2, 4-D, presumably reflects not just an effect of auxin on embryo development but an effect of initiation site, marginal initiation being

associated with a higher frequency of normal morphology than initiation from the central adaxial tissue. These interactions between tissue orientation with auxin treatment have been observed on other species like coffee (Pierson et al., 1983), pecan (Merkle et al., 1986) and sweet potato (Liu and Cantliffe, 1984). These effects probably involve differences in auxin transport or conjugation or both, as well as differences in cell type or age.

Based in histological analysis there were three patterns of cell division stimulated by auxin that form the somatic embryo. In direct embryogenesis, after NAA treatment or low 2,4-D treatment, certain single cells in the epidermis and subepidermis divide transversely and unequally to form two cells. This kind of cell is most frequently seen after NAA treatment. In the second case, the embryogenic tissue gave rise to embryos after rounds of cell divisions. On N10 medium, embryogenic tissue originated from the epidermal and subepidermal layers along the periphery of the explant. In the third case, the non-embryogenic meristematic zone is formed by divisions of the subepidermal and interior tissues. Generally they are associated with vascular tissue and tend to form files of homogeneous small cells with large nuclei and cytoplasm somewhat more darkly staining than that of adjacent nonmeristematic cells. In the explants, meristems became organized to form roots, often with internal tracheary differentiation. These structures were more common on explants from immature seeds about 5 mm in length, or on N10 adaxial explants, especially those that did not form somatic embryos (Hartweck et al., 1988).

These same researchers, also tested the effects of hormones and culture manipulation in soybean somatic embryogenesis (Lazzeri et al., 1987). When NAA and 2,4-D were compared at similar concentrations (25 and 23 μM respectively), 2,4-D produced larger numbers of somatic embryos; however the

embryogenesis efficiency was improved in media containing from 100 to 150 μM NAA. Some researchers suggest that soybean cotyledon tissue has a high diffusive resistance (Gifford and Thorne, 1985) so high external concentrations of auxin may be required for inductive concentrations to accumulate within the explant. Other evidence from this publication, indicates that somatic embryo induction from cotyledonary tissue requires only exogenous auxin since treatments with exogenous cytokinin (BAP) or ABA did not improve embryogenesis efficiency. These data suggest that exogenous ABA inhibits auxin-induced embryogenesis, although an effect on somatic embryo development was observed as cotyledons of some 2,4-D induced embryos became leaf-like in the presence of 0.38μM ABA. Previous work showed that ABA could inhibit development depending upon the embryo stage (Ackerson, 1984).

The type of auxin used can cause morphological differences. Somatic embryos treated with NAA showed bipolarity, with distinct radicle and hypocotyl regions, well defined cotyledons and a shoot apex visible from an early stage of development. It was noted that the most common abnormalities seen in NAA-induced embryos are loss of one or more cotyledons. The embryos induced by 2,4-D are in general horn-shaped, with fused cotyledons. The shoot apex is frequently underdeveloped in otherwise "mature" embryos (Lazzeri et al., 1987). Generally embryos with normal morphology germinate readily, while abnormal embryos are recalcitrant and often require long periods of incubation or culture manipulations for germination (Ackerson, 1984).

Another interesting suggestion from Lazzeri et al. (1987) is to improve the efficiency of somatic embryogenesis by pre-incubating the explants on 2,4-D medium before transfer to NAA medium. The 5-day exposure to 2,4-D gave the

highest efficiency value. Longer exposure to 2,4-D progressively reduced the frequency of normal embryos.

Another important aspect of soybean regeneration was the effect of genotype. Komatsuda and Ko (1990) screened 29 soybean genotypes. After removing the seed coats and embryonic axis, the pairs of cotyledons were placed on 10 ml of embryogenesis medium containing MS salts, B5 organics, 3% sucrose, 10 mg/l NAA and 0.8% gelrite at pH 7.0 and the numbers of somatic embryos were counted 6 weeks later. From 295 genotypes tested, 22 genotypes produced more than 2.0 embryos per pair of cotyledons. The highest embryo producer was the Brownier DL/64/177 which produced 8 somatic embryos per pair of cotyledons. Among the somatic embryos 28% germinated and 20% grew into plantlets (Komatsuda and Ko, 1990).

A similar study was done by Bailey et al. (1993), who examined soybeans genotypic effects on induction and maintenance of proliferative embryogenic cultures, and on yield, germination and conversion of mature embryos. Somatic embryos were induced from eight genotypes by explanting 100 immature cotyledons per genotype on MSD40 induction medium composed by MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 6% sucrose, 40 mg/l 2,4-D and pH 7.0. However this step was not a limiting factor for plant regeneration because induction frequency in the least responding genotype was sufficient to initiate and maintain proliferative embryogenic cultures. From this step, six genotypes were selected to initiate embryogenic cultures in liquid medium, 10A40N, under shaking. It is important to observe the qualitative aspects in suspensions like a green color and a nodular, homogeneous and compact morphology of the embryos. At this level the genotype ranking, from the highest to lowest quality embryo was: PI417138 > Lee = Peking = Davis >

Hutcheson > Century. The final fresh weight ranged from 68 to 176 mg, representing a 5- to 10-fold increase over the initial inoculum of 15±3 mg. Partially desiccated embryos were placed on MSO medium for 25d. The responses were grouped in four categories: a) no roots or shoots, b) roots only, c) shoots only, or d) roots and shoots. Successful germination was considered to be those structures with roots and shoots and shoots only. Germination frequency varied with genotype and ranged from 66% for Century to 78% for PI417138. The conversion frequency was different among genotypes and ranged from 8% for Century to 34% for PI417138. Based on this research the genotype PI417138 was clearly the most regenerable, judging by superior embryo yield, germination and conversion. The genotype Century, that has more agronomical importance, needs optimization of the limiting steps such as embryo yield, by improving selection for homogenous cultures (Bailey et al., 1993).

In order to associate some biochemical characteristics with cell division in carrot embryogenesis, Tsukahara and Komamine (1997) transferred embryogenic clusters to the induction medium. After 3 to 4d, the actively dividing cells were separated from cells that were not synthesizing DNA, by maceration of cell clusters and centrifugation in a percoll density gradient. The ^{35}S methionine labeled proteins extracted from the two types of cell were analyzed by SDS PAGE. Three polypeptides of 69, 98 and 100 kD were found only in cells that were actively synthesizing DNA. The authors suggested that these polypeptides could be candidates for markers of the DNA synthesis that is specific to embryogenesis. This kind of information is not available yet for soybean embryogenesis.

In spite of some research that has been done with soybean via somatic embryogenesis (Ranch et al., 1985; Barwale et al., 1986), the number of

transgenic plants obtained with this methodology (Finer and McMullen, 1991; Sato et al., 1993; Parrott et al., 1994; Stewart et al., 1996) is not very high and this method is far from routine.

One aspect of this system that has not been evaluated extensively in transformation studies is the induction of somatic embryos from immature cotyledons, which seems to be a very inefficient step. Recently Santarem et al. (1997) used somatic embryos induced from immature cotyledons of the cultivars Jack, Thorne, Resnick, and Chapman on medium composed of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg et al., 1968), 6% sucrose and 40 mg/l 2,4-D. The culture modifications were: orientation of explants (adaxial and abaxial side of cotyledon in contact with the medium), adjustment of the medium pH (5.7 to 7.0), wounding the explants with the scalpel blades, inclusion of ethylene modulators and use of noble agar or gelrite as the solidifying agent. From this research the embryo initiation was highest when the induction medium was adjusted to pH 7.0, and solidified with gelrite, and when the explants were cultured with abaxial side facing the medium. Among the cultivars, Jack showed the highest embryogenic induction after 21 d in culture. The frequency of responding cotyledons was 100% and an average of 44.2 embryos per explant was obtained. The first somatic embryos emerged from the cotyledons after 14 d, and after 21 d the whole surface of the explant was covered with embryos. These results suggest a synergistic effect among pH, explant orientation, and gelling agent. Previous experiments using pH 8.0 for induction of somatic embryogenesis in soybean, obtained only four embryos per cotyledon (Tomatsuda and Ko, 1990). However this lower number of embryos could be due to the use of NAA rather than 2,4-D for induction. In maize coleoptiles the reduction of pH 7.0 to 5.8 caused a decrease in auxin uptake (Edwards and Goldsmith, 1980).

For soybean, the enhancement of embryo induction at pH 7.0 may result from a slower and more gradual uptake of 2,4-D when using medium containing relatively high levels of 2,4-D (Santarem et al., 1997).

The explant orientation has already been tested in soybean (Hartweck et al., 1988; Buckheim et al., 1989). However, the highest average in these studies of 11 somatic embryos per explant was obtained when the explants were cultured with abaxial side in contact with medium containing 25 mg/l 2,4-D and solidified with Phytagar. However, the use or effect of other solidifying agents, wounding, or ethylene modulators was not reported.

Some publications have reported the use of gelrite as gelling agent replacing agar, e.g. with *Magnifica indica* (De Wald et al., 1989) and *Oryza sativa* (Koety et al., 1989). Santarem et al. (1997) observed that 2,4-D concentration, gelrite and pH 7.0 increased the efficiency of somatic embryo formation. At this moment, wounding did not increase the number of embryos formed and no differences were observed between the cultivars tested, but in the wounded plants the embryos were induced earlier (Santarem et al., 1997).

Wounding is related to ethylene production (Yang and Hoffman, 1984), but the effect of ethylene modulators has not been studied in soybean somatic embryogenesis. Santarem et al. (1997) examined the effect of AVG, ACC, $AgNO_3$ $CoCl_2$ and SA, and found these to increase the number of induced embryos. Probably more research needs to be done with each tissue and step using ethylene as a growth regulator for soybean somatic embryogenesis.

Histochemical analysis showed that in wounded cotyledons, the first divisions in the tissue adjacent to the wound site occurred by 4d in culture (Santarem et al., 1997) and cell proliferation was later observed throughout the cotyledonal tissue. However, in the unwounded tissue, cell divisions were

observed after 7d in culture. At this point, in wounded tissue, rapid cell divisions were observed in adaxial parts of the cotyledon and some meristematic areas were already formed. Some degree of organization was also found in nonwounded tissue after 14d of culture. The histological observations of this study suggest that embryo induction in soybean may be from a small group of subepidermal cells and that wounding of the tissue results in earlier and more controlled production of somatic embryos (Santarem et al., 1997).

Recently Rajasekaran and Pellow (1997) were successful in regenerating soybean from epicotyls and primary leaves of soybean by somatic embryogenesis. For the embryo induction from epycotyls and primary leaves, the cotyledon halves with intact zygotic embryo axes were cultured on Murashige and Skoog (1962) medium supplemented with 10 mgl^{-1}(45.2μM)2,4-D for 4 to 6 weeks. One important finding is that no embryogenesis was observed from isolated axes, epycotyls or primary leaves. It seems that the cotyledon serves as a buffer against the adverse effects of high concentrations of 2,4-D on the growth of epicotyls and primary leaves, in addition to providing other unknown positive effects possibly hormonal and nutritive. The authors suggested that the somatic embryos originated directly from the epicotyl axis on the lamina of the primary leaves, although this observation needs to be supported by histological studies (Rajasekaran and Pellow, 1997).

In this same study, around 50% of the primary leaf explants produced from 1 to 4 globular somatic embryos per explant (Rajasekaran and Pellow, 1997). In experimental lines IJ2037 and 3305, more than 70% of the primary leaf explants produced somatic embryos compared to an average of 50% in other varieties. For the initiation of continuously proliferating cultures, the globular embryos were transferred to MS medium containing 20 mg l^{-1} (90.5μM)2,4-D,

where they continued to multiply without producing callus. Around 700 globular embryos were obtained in a clump of $10mm^3$ and this amount was doubled every 4 weeks of culture. The cultures were maintained for about 2 years without the loss of plant regeneration. The globular embryos cultured in this study were similar in growth and morphology to the cultures of immature zygotic embryo origin as described before by Finer (1988). Based on microscopic observation, the origin of secondary embryos from epidermal layers of primary somatic embryos was similar to that of Finer (1988).

For the germination step, the single globular embryos or small clumps were transferred to a liquid medium containing $2mgl^{-1}$ (11.0μM) NAA for 10 d (Rajasekaran and Pellow, 1997). The hypocotyl of the embryos elongated and the somatic embryos turned green. The germination rate was around 40%. In order to further develop the root and shoot system, the embryos were transferred to a solidified White's medium (Singh and Krikorian, 1981) containing casein hydrolysate ($2.0g\ l^{-1}$) and coconut milk (15% vol/vol). In the later stage the plantlets were transferred to soil where they flowered and set seed (Rajasekaran and Pellow, 1997).

Some transgenic soybean plants have been obtained using somatic embryogenesis (Finer and McMullen, 1991; Parrott et al., 1994; Stewart et al., 1996; Champrame, 1997) and this methodology has been improved over the last five years, although problems with embryo germination and plant fertility still remain unsolved. This latter problem could be circumvented or at least minimized by using cultures not older than one year (Stewart et al., 1996; Champrame, 1997; Santarem et al., 1997). In relation to the former problems, further basic research needs to be done to understand the effects of antibiotics on embryo germination, since there is the low percentage of germination when the

embryos had been subjected to selection pressure. Actually, this detail seems to be the limiting step for soybean transformation via somatic embryogenesis.

4 RESULTS AND DISCUSSION

The 606 ASA2 promoter was compared with its larger fragment, 1356bp, and with the CaMV 35S promoter for transient GUS expression with the soybean embryogenic suspension cultured cells. As shown in Figure 1a 606 gave higher histochemical transient expression than 35S and 1356 ASA2, using the Helium PDS-1000 gun and the enzymatic assay followed the same tendency as the histochemical assay (Figure 1b). In preliminary experiments in this laboratory the 606 ASA2 was better than the 1356bp promoter in tobacco leaves and suspension cultures in transient assays (data not shown). Apparently in the region from 606 to 1356bp some sequence negatively modulates the gene expression. Studies using promoter deletion and gel retardation assays are being conducted in order to further characterize this region. During the optimization steps in this research, the histochemical and fluorimetric GUS assay showed good correlation (Table 1).

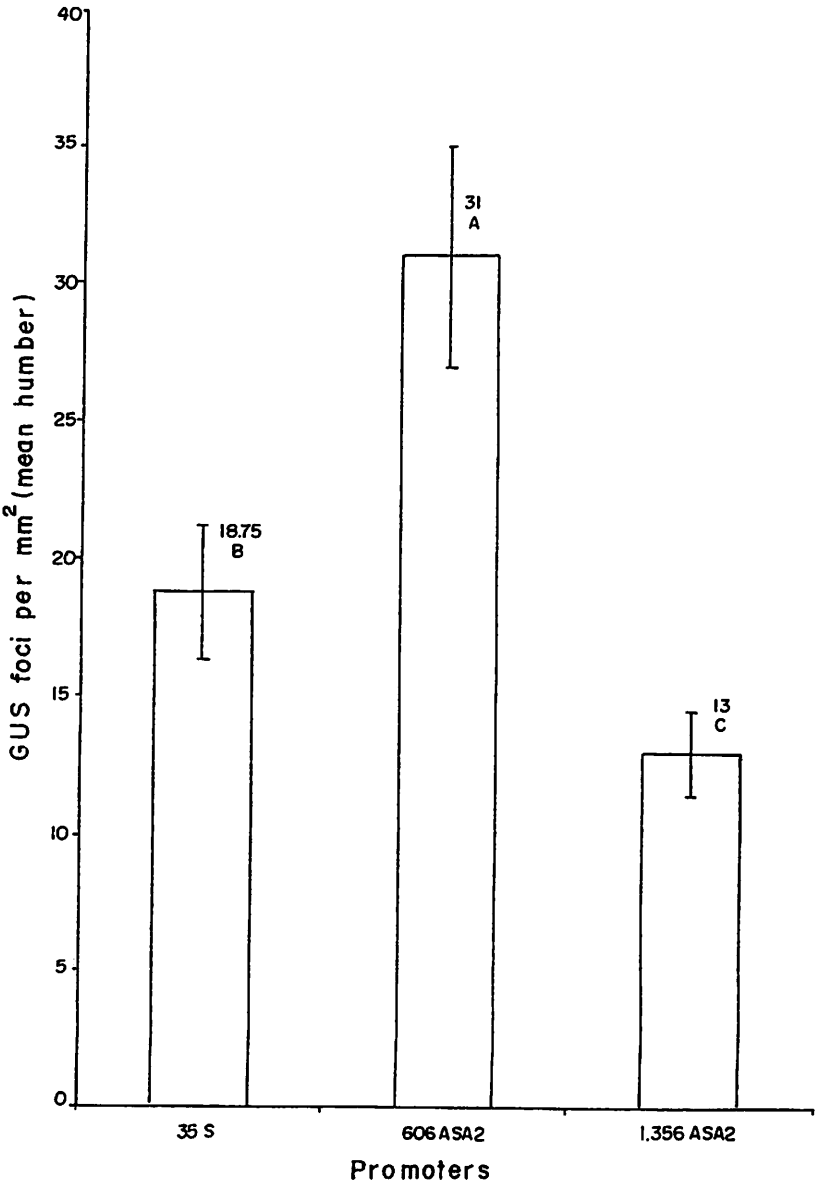

Figure 1a. Comparison of ASA2 promoter strength and length on transient GUS expression in soybean embryogenic clumps (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

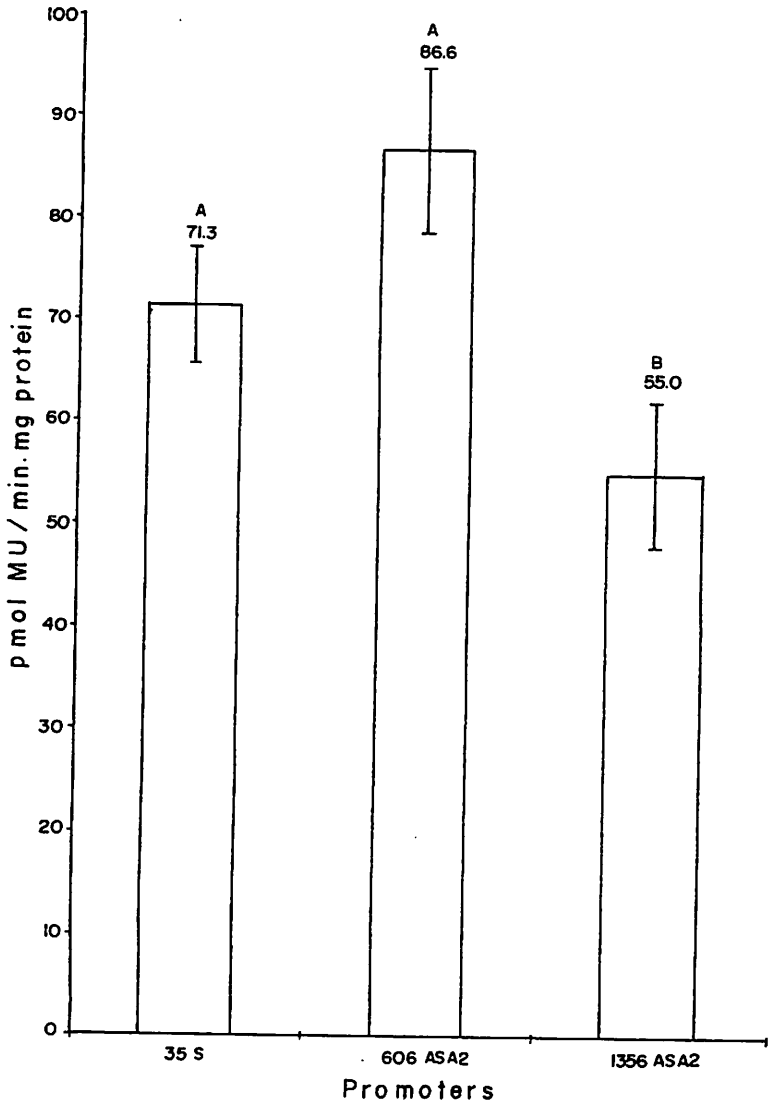

Figure 1b. Comparison of ASA2 promoter strength and length on transient GUS expression in soybean embryogenic clumps (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

TABLE 1 - Correlation coefficient between histochemical GUS assay and enzymatic GUS assay (MUG) during particle bombardment optimization steps using soybean embryogenic suspension cv. Jack.

Figures	Correlation coefficient (r)
1 A* and B**	0.72
2 A* and B**	0.89
3 A* and B**	0.90
4 A* and B**	0.94***
5 A* and B**	0.91
6 A* and B**	0.98
7 A* and B**	0.99
8 A* and B**	0.73
9 A* and B**	0.95
10 A* and B**	0.78
11 A* and B**	0.93

A* - histochemical GUS assay

B** - enzymatic GUS assay (MUG)

*** - the concentration of 5 μg DNA was not considered.

Basically the best conditions found for pressure (Figures 2a and b) and target distance (Figures 3a and b) are similar to those described by Chanprame (1997). This author observed that 1100 psi gave the highest number of stable transformants. In our research, like that of Chanprame (1997), more damage to tissue was observed with short distances and high pressures. Instead of 1100 psi,

some publications recommend using 900 psi to obtain more stable transformants (Stewart et al., 1996). The results obtained with 900 and 1100 psi are similar and this range seems to be optimal (Figures 2a and b).

In the next step the DNA amount chosen was 1 μg per shot, although in other recent publications higher amounts are recommended by Chanprame (1997) and Stewart et al. (1996) who used 5 μg per shot. In this latter publication gold particles were also used as in this paper. When we used 5 μg of DNA a diffuse blue pattern was observed and low activity was detected using the fluorimetric assay (Figure 4b). In treatments with 1 or 2 μg of DNA per shot dark and well defined blue spots were observed. In the treatment with 5 μg DNA there were not many gold particles on the embryo surface which could indicate a problem during the DNA precipitation procedure. Another consideration is that transgenic plants were obtained using 5 μg of DNA per shot (Stewart et al., 1996; Chanprame, 1997), so this higher concentration could be somehow associated with stable transformation but not transient expression. Further work needs to be done with this DNA concentration to further correlate transient expression and stable transformation.

Gold amounts (Figures 5a and b), higher than 1 mg per shot, caused tissue damage and yellow particles was seen on the embryo surface. During DNA precipitation, some clumps were observed in the samples containing more than 1 mg of gold per shot. In previous successful work, the amount used was 0.6 mg per shot (Stewart et al., 1996). We found that, the lower concentration used, 0.5 mg per shot, was still enough to obtain transient expression (Figures 5a and b). It could be worth testing 0.6 and 1.0 mg of gold per shot in stable transformation experiments using the 606 ASA2 promoter.

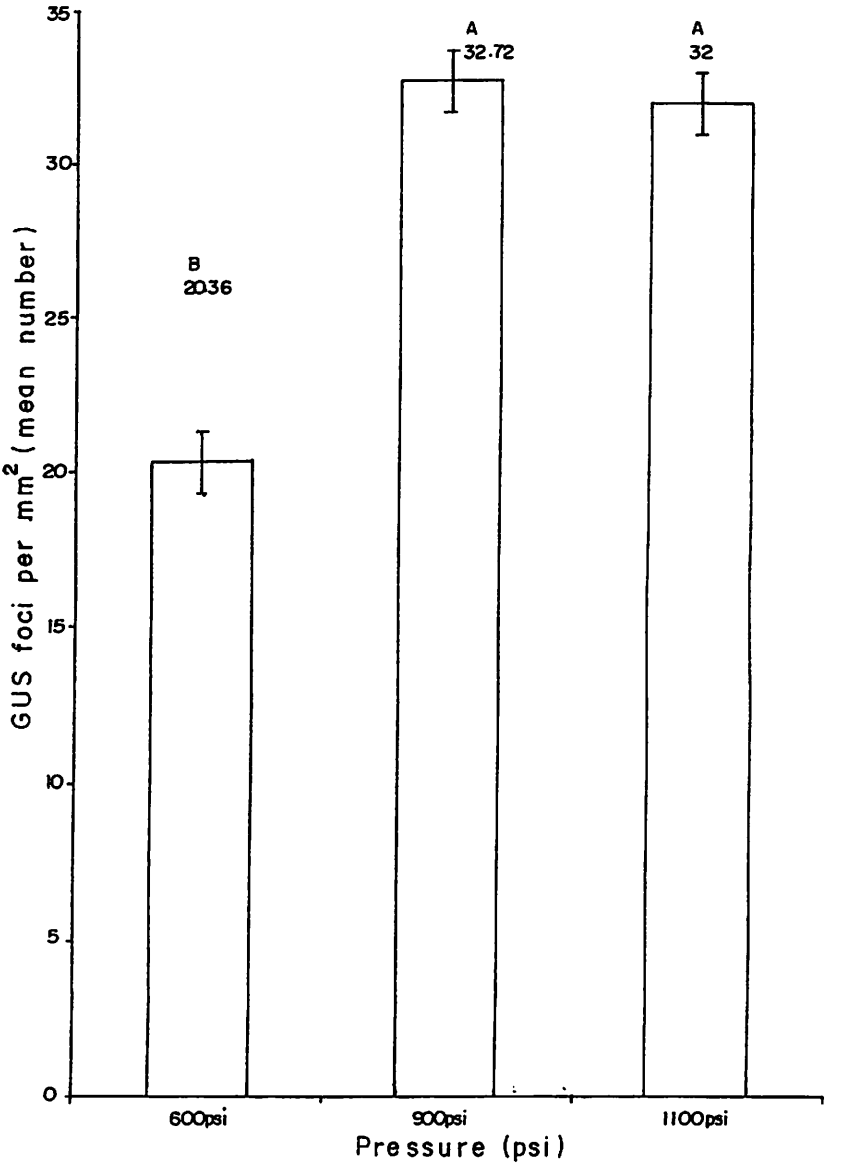

Figure 2a. Effect of Helium gas pressure on transient GUS expression in soybean embryogenic clumps (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

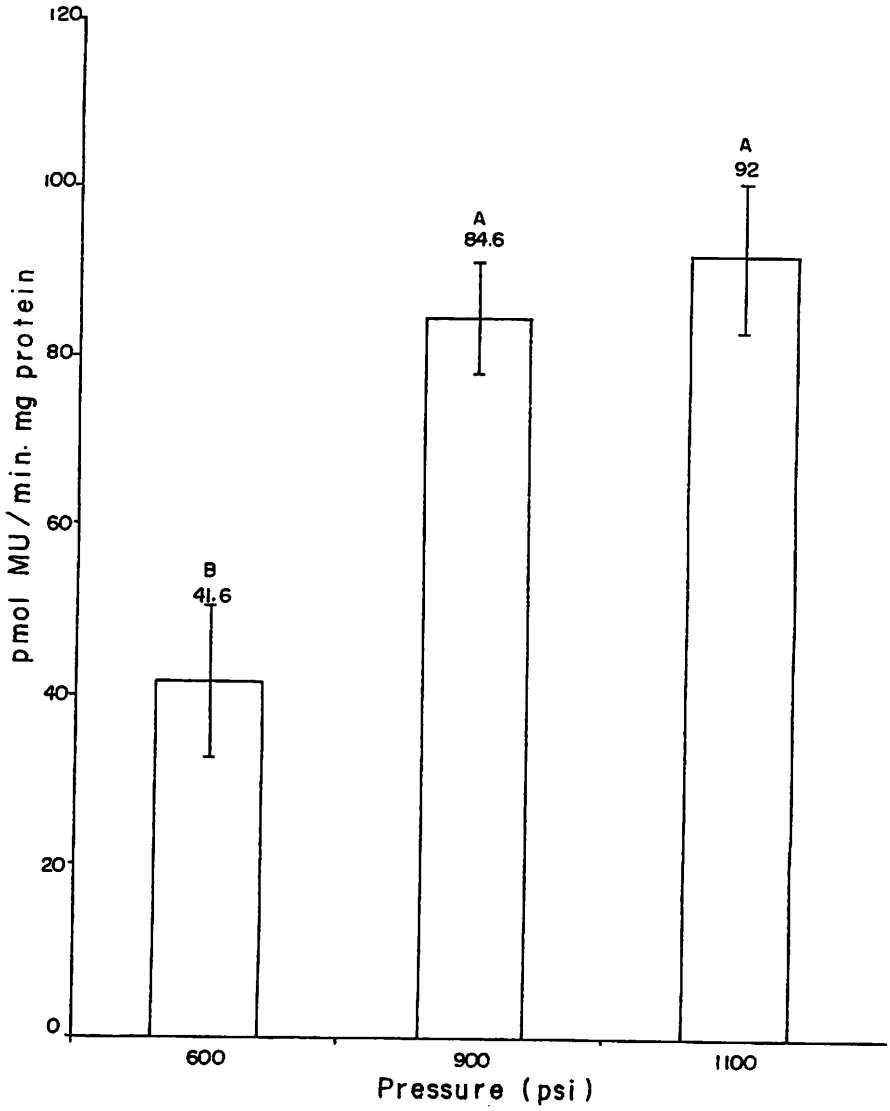

Figure 2b. Effect of Helium gas pressure on transient GUS expression in soybean embryogenic clumps (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

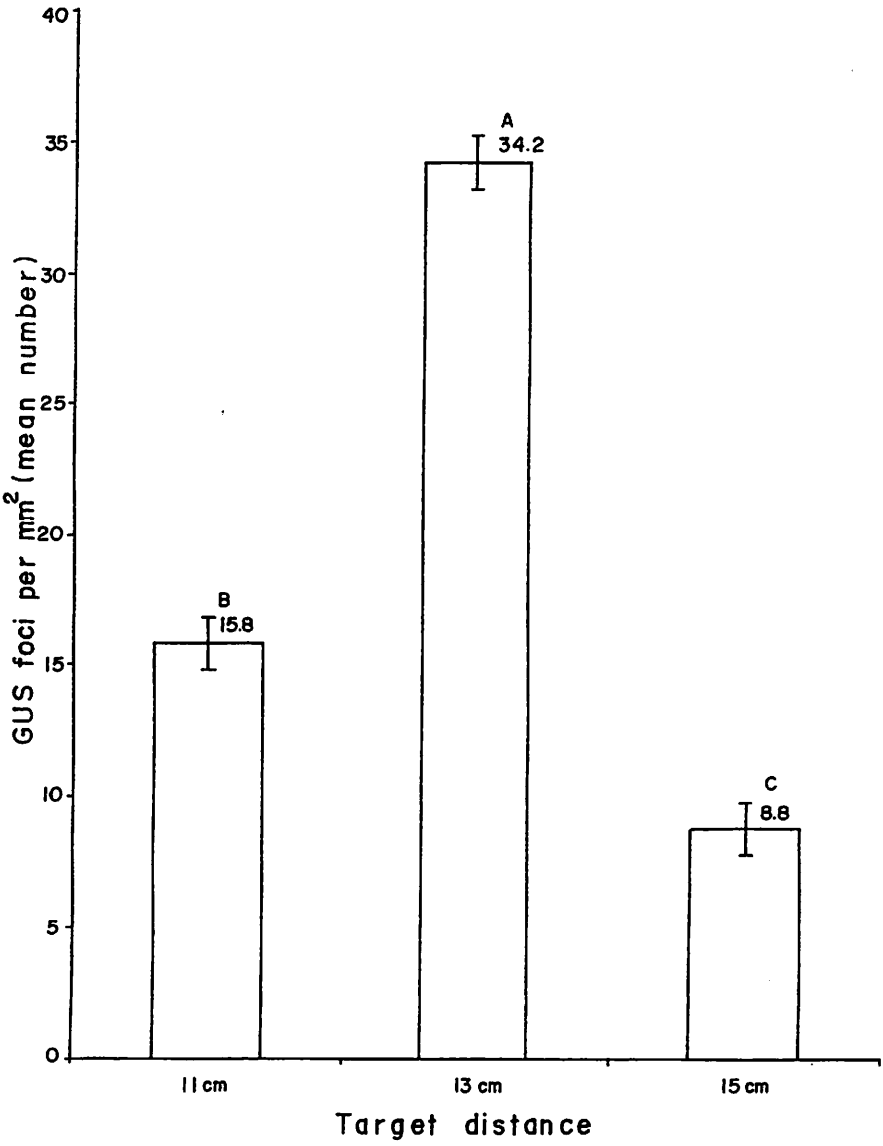

Figure 3a. Effect of target distance on transient GUS expression in soybean embryogenic clumps (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

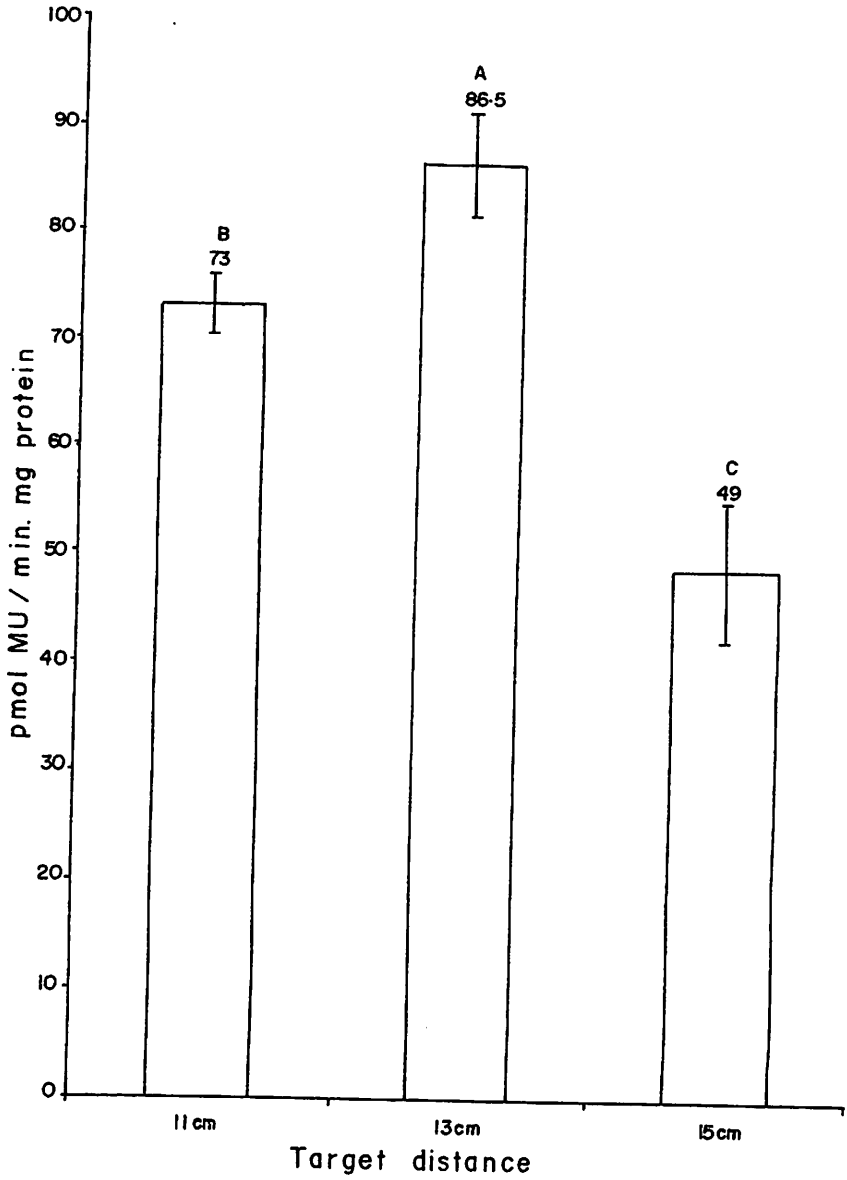

Figure 3b. Effect of target distance on transient GUS expression in soybean embryogenic clumps (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

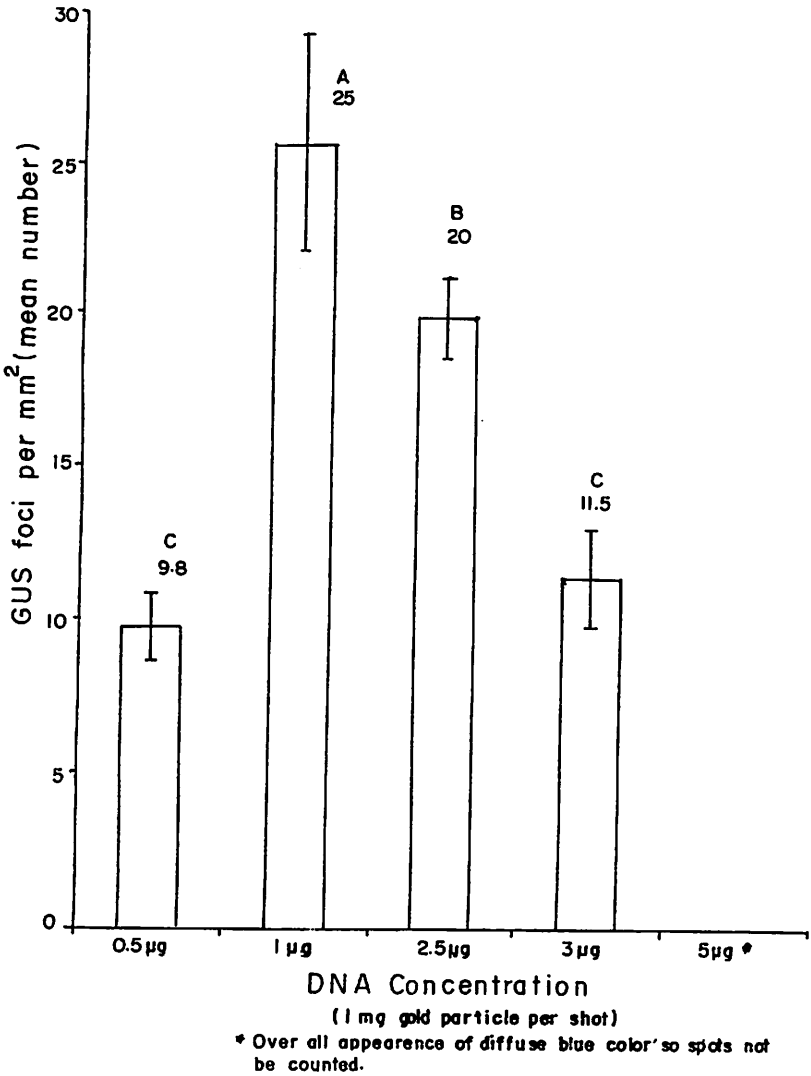

Figure 4a. Effect of DNA amount on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (Histochemical assay). Bar values shown in different letters are significantly different.

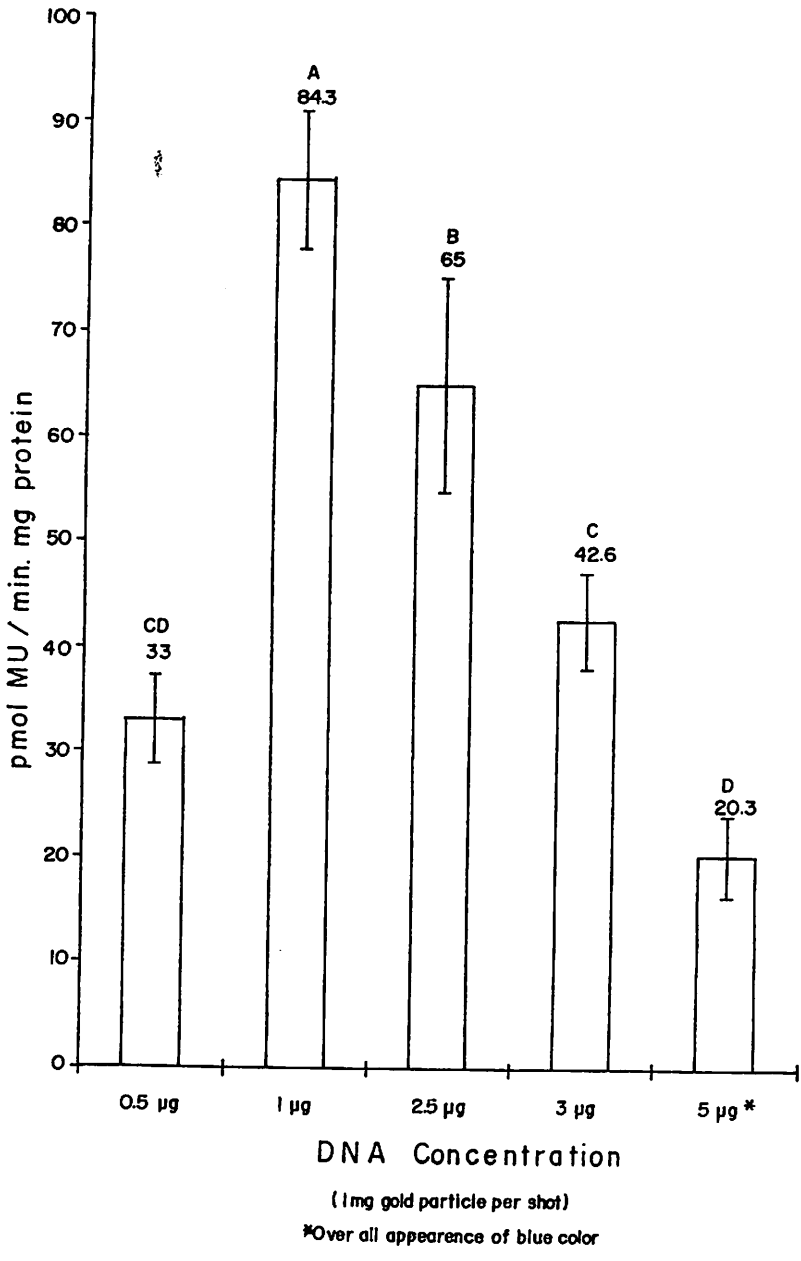

Figure 4b. Effect of DNA amount on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (MUG assay). Bar values shown in different letters are significantly different.

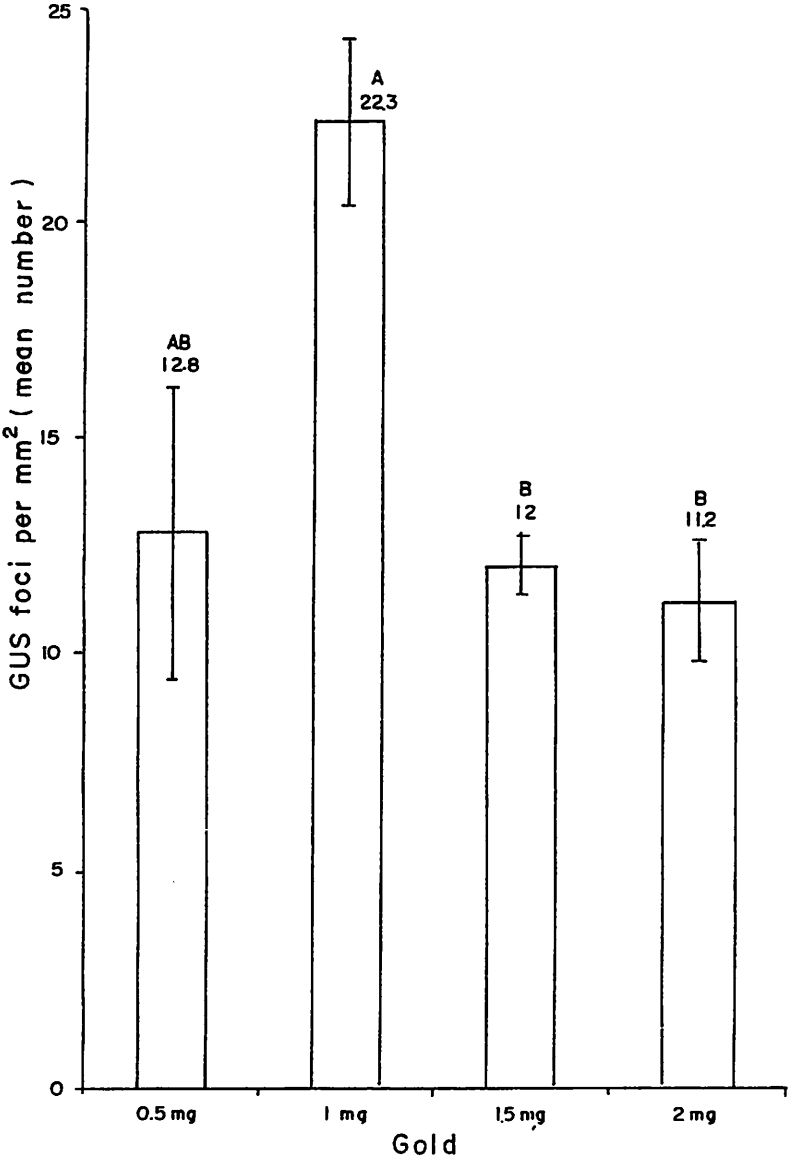

Figure 5a. Effect of gold amount on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

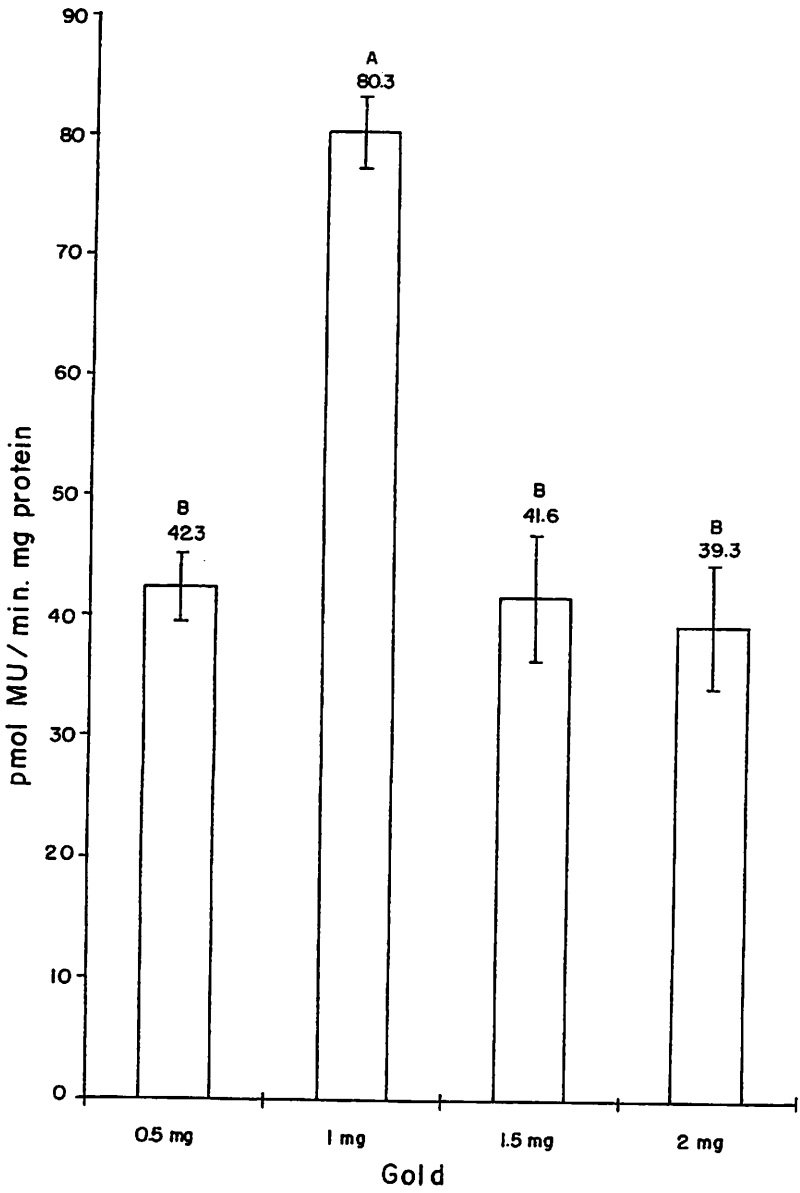

Figure 5b. Effect of gold amount on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

In order to compare DNA precipitation systems, two methodologies were used: $CaCl_2$/spermidine (Klein et al., 1988) and sodium acetate (Morikawa et al., 1989). In the former protocol spermidine seems to improve DNA precipitation in some systems (Walt et al., 1994). However in another system, the omission of spermidine produced high transient expression (Perl et al., 1992). For soybeans spermidine has been used in several experiments (Parrot et al., 1994; Stewart et al., 1996; Chanprame, 1997). Champrame (1997) found that spermidine free base or spermidine trihydrochloride resulted in similar transient gus-expression levels (Chanprame, 1997). According to the manufacturer's protocol, spermidine free base is recommended. However this form of spermidine is difficult to handle because it is very hygroscopic and oxygen sensitive, becoming unstable once the container is opened. On the other hand, spermidine trihydrochloride is more stable and easier to handle. In our research the sodium acetate precipitation methodology (Morikawa et al., 1989) associated with sonication gave gus-transient expression results similar to those obtained with $CaCl_2$/spermidine methodology (Klein et al., 1988) as seen in Figures 6a and b. The sonication seems to disrupt the aggregates and helps to form a fine and homogeneous suspension to be loaded onto the macrocarrier. Sonication has been recommended earlier by Sanford et al. (1993) and recently has been used in soybean (Stewart et al., 1996). But in relation to this latter publication, we are using sonication only in the final step before pipetting onto the macrocarrier. Sonication at earlier stages is time consuming and when done later the same fine suspension was obtained. The sodium acetate methodology (Morikawa et al., 1989) showed the same transient expression levels as $CaCl_2$/spermidine (Figures 6a and b). The DNA precipitation using sodium acetate is also quicker and easier than $CaCl_2$/spermidine and the particles also formed a fine and regular suspension

like those obtained with $CaCl_2$/spermidine and sonication before pipetting onto the macrocarrier. This methodology could be an excellent option for soybean transformation in the future.

The PIG device has been used with soybean embryogenic suspension cultures to study the effect of osmotic treatments (Finer and McMullen, 1991). Although information about physical parameters related to bombardment are scarce, the results from our research suggest that the best conditions are 80 psi (Figures 7a and b), target distance of 11 cm (Figures 8a and b), and gold amount of 1 mg per shot (Figures 9a and b). The precipitation with sodium acetate gave results similar to that of $CaCl_2$/spermidine (Figures 10a and b). These results support an important advantage, since the Particle Inflow Gun accelerates the particles in a stream of low pressure helium (Takecuchi et al., 1992) and does not need a macrocarrier to carry the particles so the force necessary to accelerate the particles is reduced. This absence of the microcarrier reduces consumables, clean up time and cycle time (Vain et al., 1993). The data out of this research, using the optimal condition (Figures 10a and b) suggest that the PIG transient expression results are close to that of the PDS/1000 (Figures 11a and b) using the same cultures at the same time. This could be a good option because the PIG is cheaper and faster. During the bombardment a baffle was used in order to reduce tissue damage and tissue displacement as observed before (Vain et al., 1993). In relation to the Particle Inflow Gun, the PDS-1000/He is safer, cleaner, and allows better control over bombardment power, distributes microcarriers more uniformly over the target cells, is more consistent from bombardment to bombardment and yields from 4 to 300-fold more transformants in the species tested (Sanford et al., 1991). However, recent publications using the PIG reported similar results in transient expression with the scutela of immature zygotic embryos of barley

(Koprek et al., 1996). The PIG may be used to recover transformants even from those genotypes with a low tendency for somatic embryogenesis. Possibly due to the lower gas blast and acoustic shock caused by the PIG, the tissue retained its viability and formed embryogenic structures in frequencies comparable to non-transformed tissue (Koprek et al., 1996). Recently, transient GUS expression was achieved using the PIG with apple cotyledonary explants of mature seeds after optimizing the pre-cultivation period of the cotyledon, the three precipitation methods (water, 25% PEG and glycerol) and amount of tungsten particles (Yang et al., 1997).

When tungsten and gold particles were compared (Figures 10a and b and 11a and b), the data showed that gold with both the Helium/PDS and PIG devices is more effective than tungsten.

When comparing the gun models, higher GUS expression was obtained with the Helium PDS/1000. The gold particles are biologically inert, uniform in size and shape, but tend to agglomerate more easily than tungsten (Kikkert, 1993), although this drawback can be circumvented by brief sonication. In addition, tungsten can be toxic to the tissues (Russell et al., 1992). In apple cotyledonary explants from seeds, tungsten amounts of 2 mg/shot or higher seem to be toxic (Yang et al., 1997). In soybean embryogenic suspension cultures some researchers used tungsten (Hadi et al., 1996; Cho et al., 1997; Champrame, 1997) and another (Stewart et al., 1996) used gold. Transgenic plants have been obtained using both metal particles (Stewart et al., 1996; Chanprame, 1997).

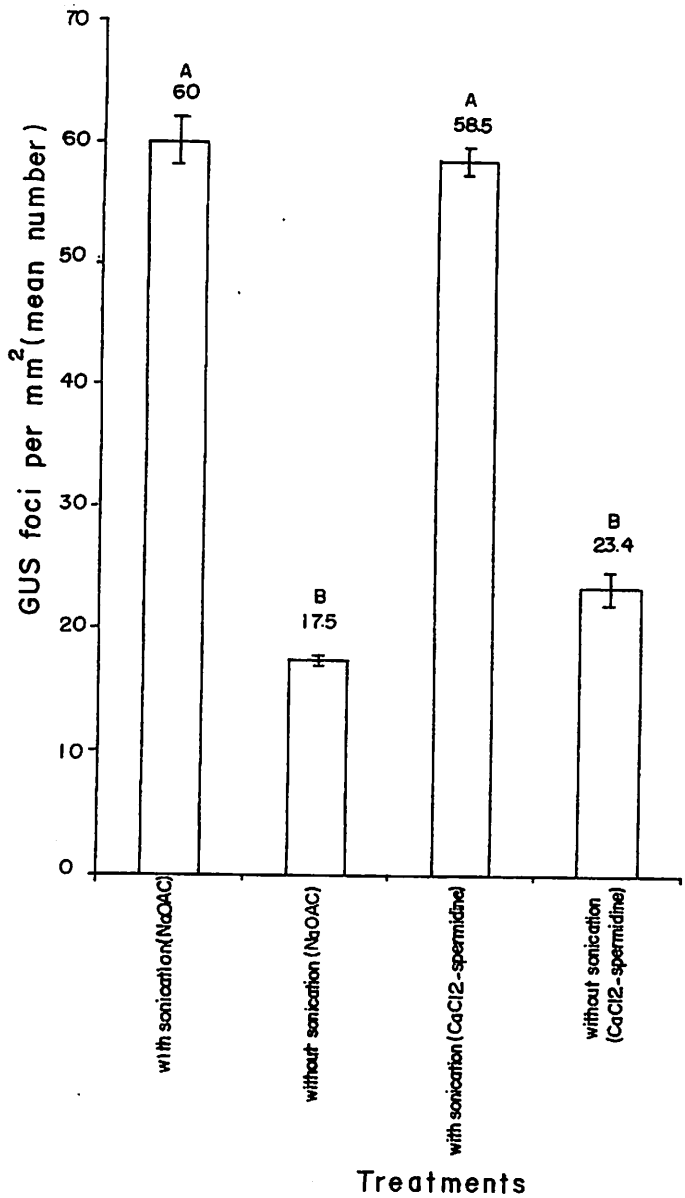

Figure 6a. Effect of using 0.3 M NaOAc or 2.5 M $CaCl_2$ + 0.1M spermidine precipitation of gold particle on transient GUS expression with soybean embryogenic suspension clumps using PDS 1000 Device (Histochemical assay). Bar values shown in different letters are significantly diffeerent at 0.05 level.

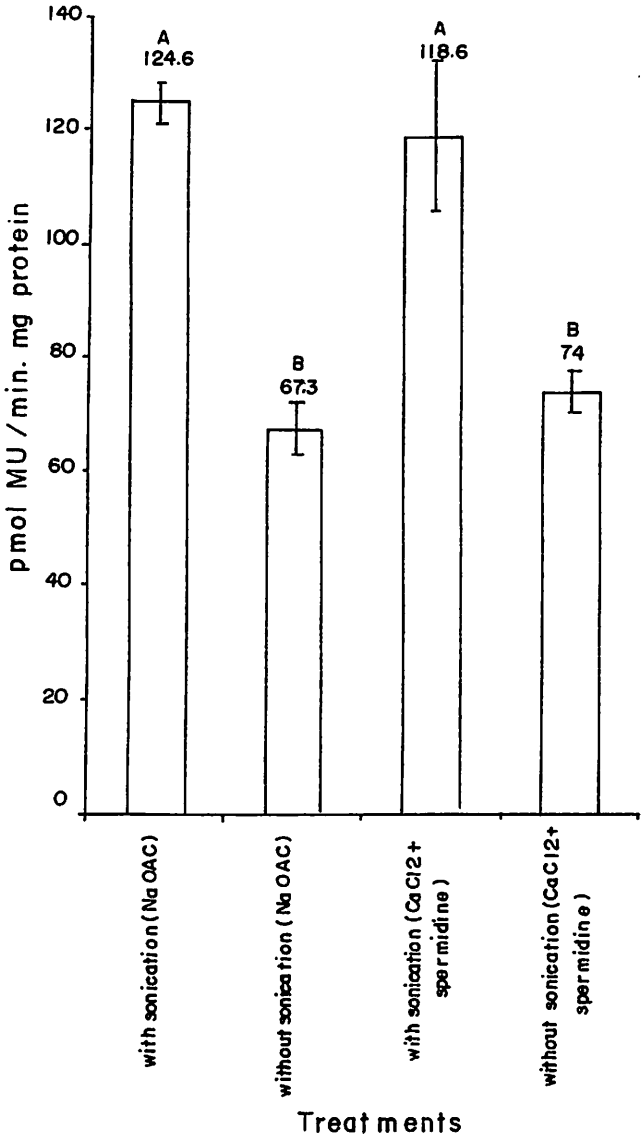

Figure 6b. Effect of using 0.3 M NaOAc or 2.5 M $CaCl_2$ + 0.1M spermidine precipitation of gold particle on transient GUS expression with soybean embryogenic suspension clumps using PDS 1000 Device (MUG assay). Bar values shown in different letters are significantly diffeerent at 0.05 level.

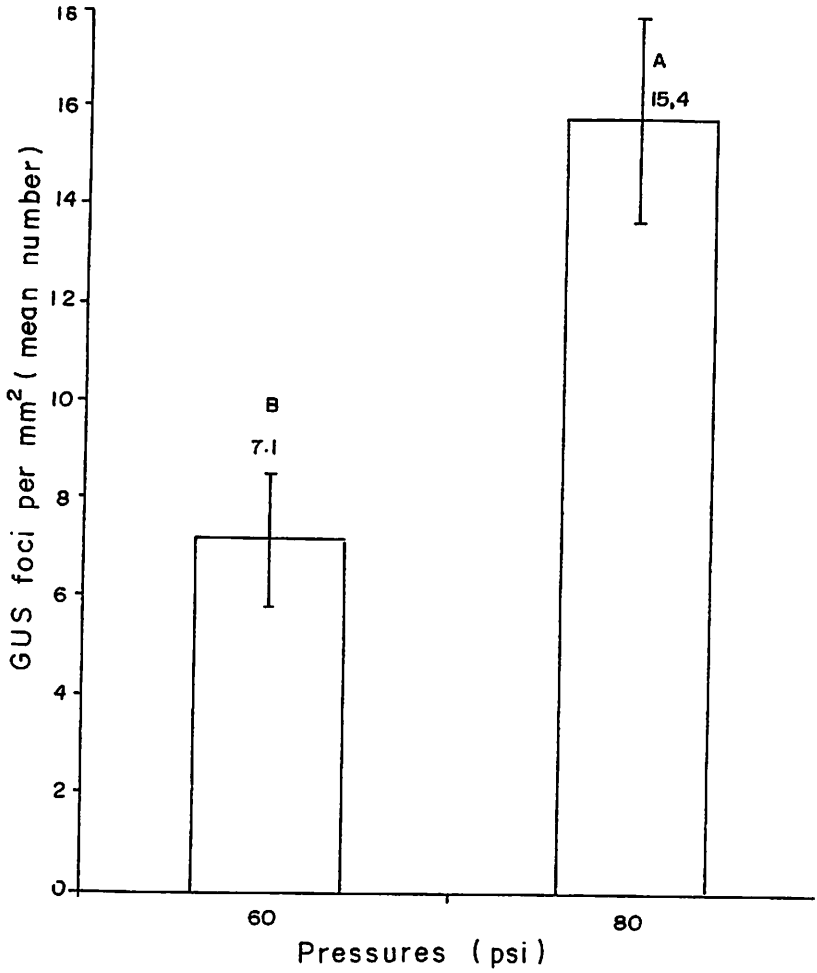

Figure 7a. Effect of various Helium gas pressures on transient GUS expression in soybean embryogenic clumps using the PIG (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

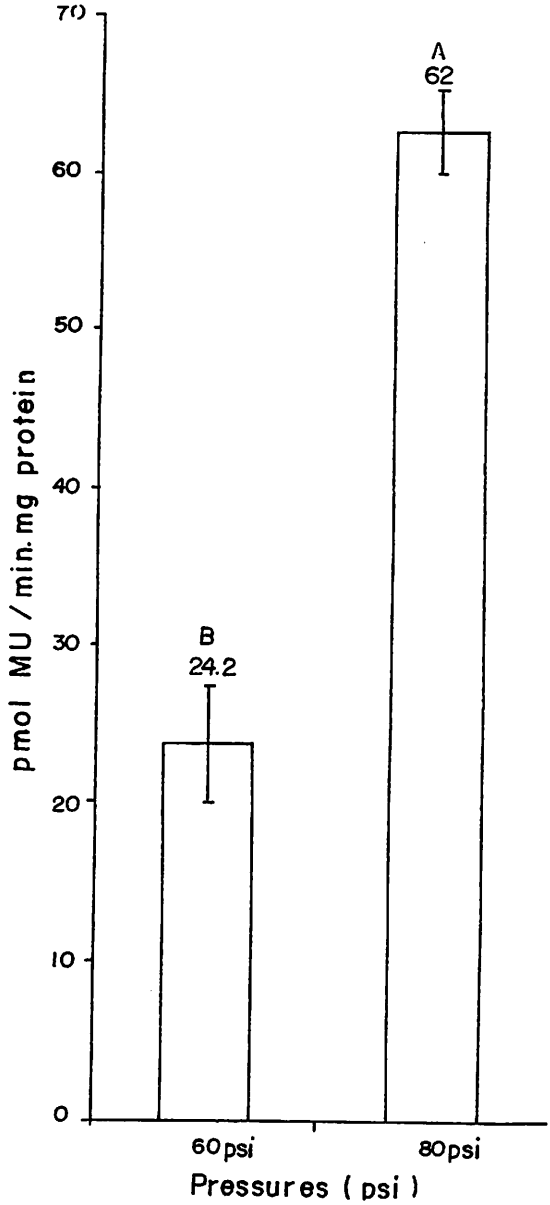

Figure 7b. Effect of various Helium gas pressures on transient GUS expression in soybean embryogenic clumps using the PIG (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

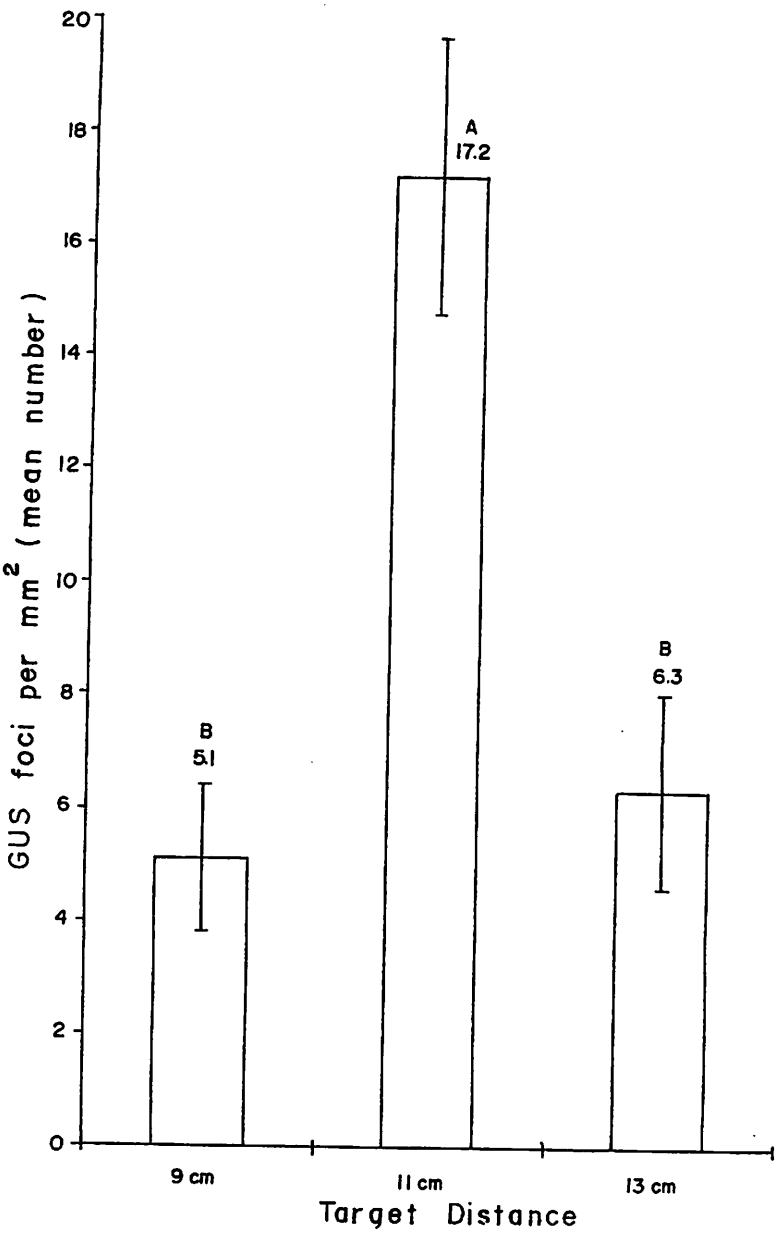

Figure 8a. Effect of different target distances on transient GUS expression in soybean embryogenic clumps using the PIG (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

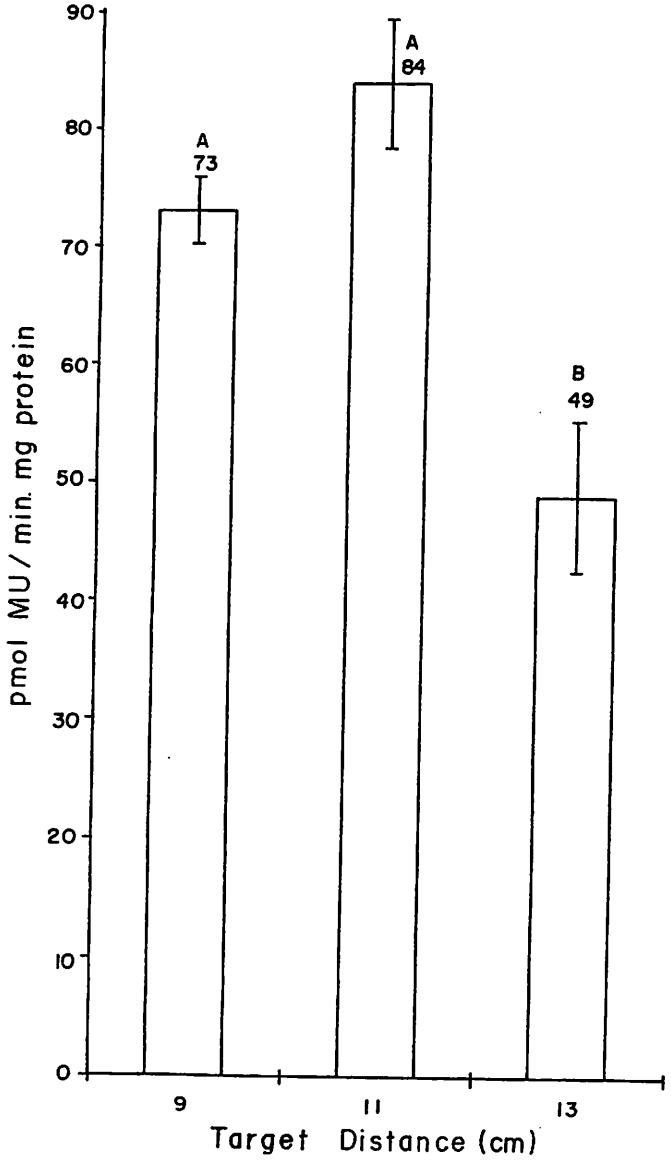

Figure 8b. Effect of different target distances on transient GUS expression in soybean embryogenic clumps using the PIG (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

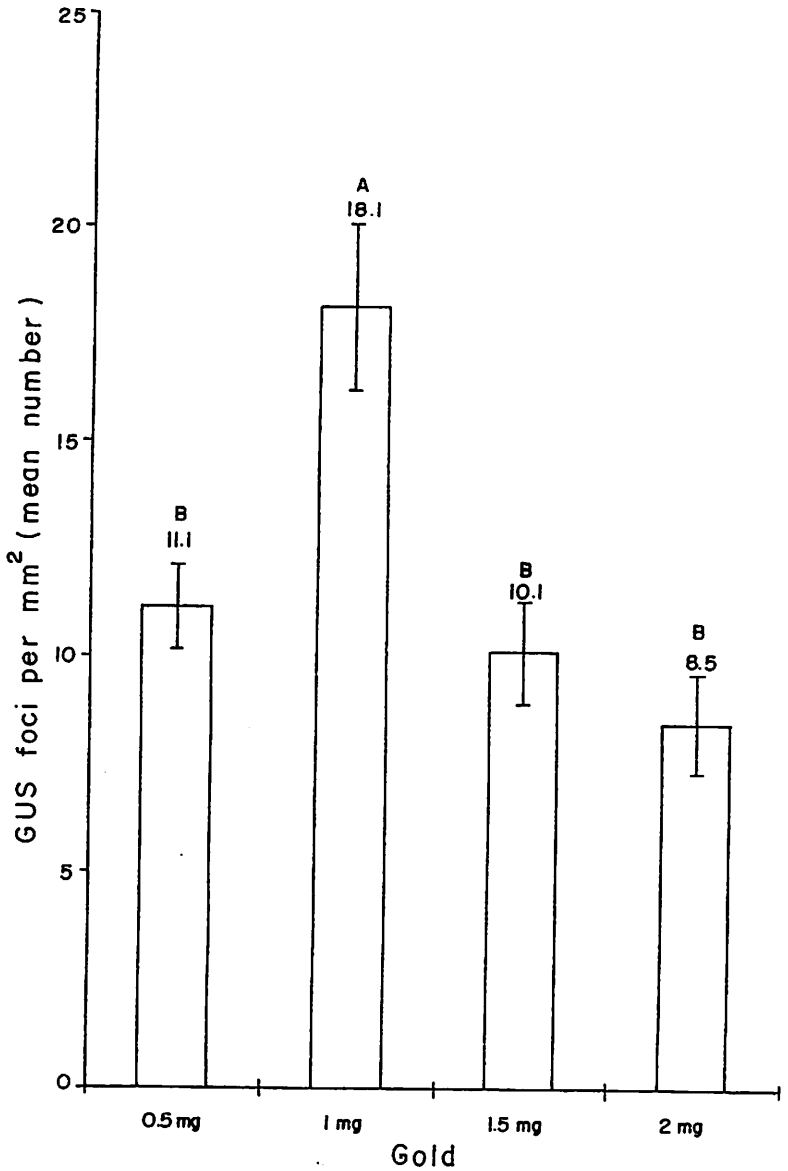

Figure 9a. Effect of gold particle amount on transient GUS expression in soybean embryogenic clumps using the PIG (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

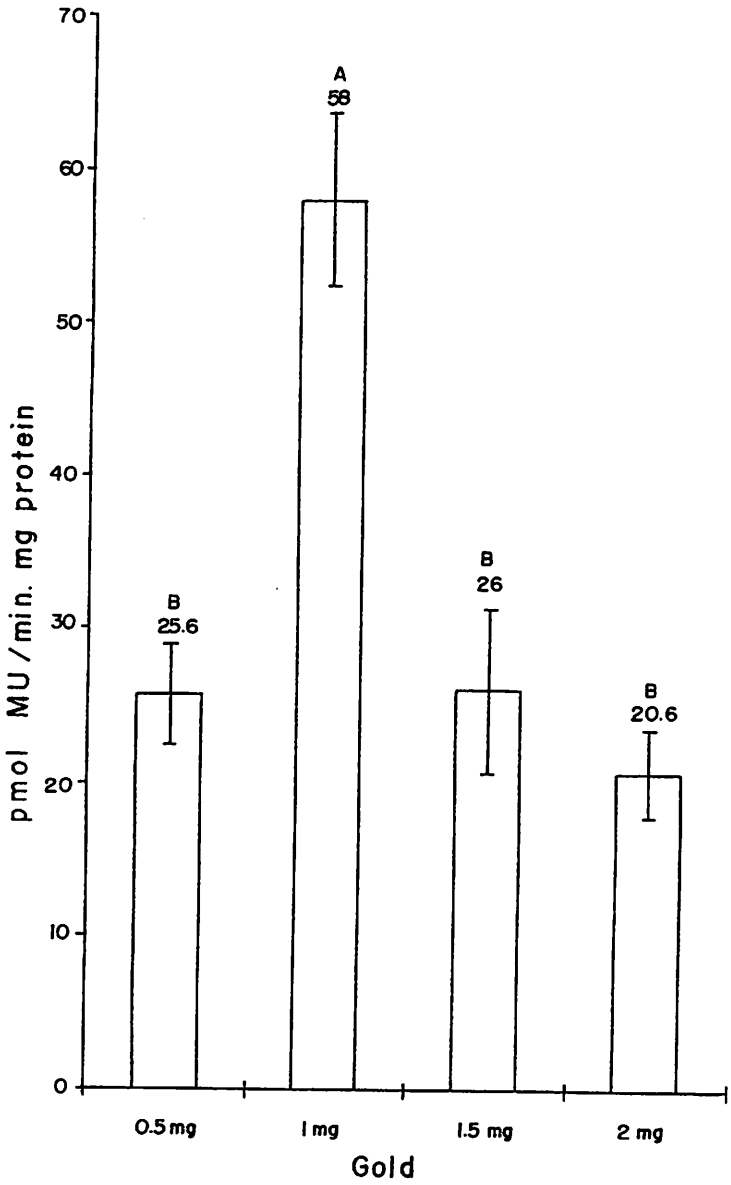

Figure 9b. Effect of gold particle amount on transient GUS expression in soybean embryogenic clumps using the PIG (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

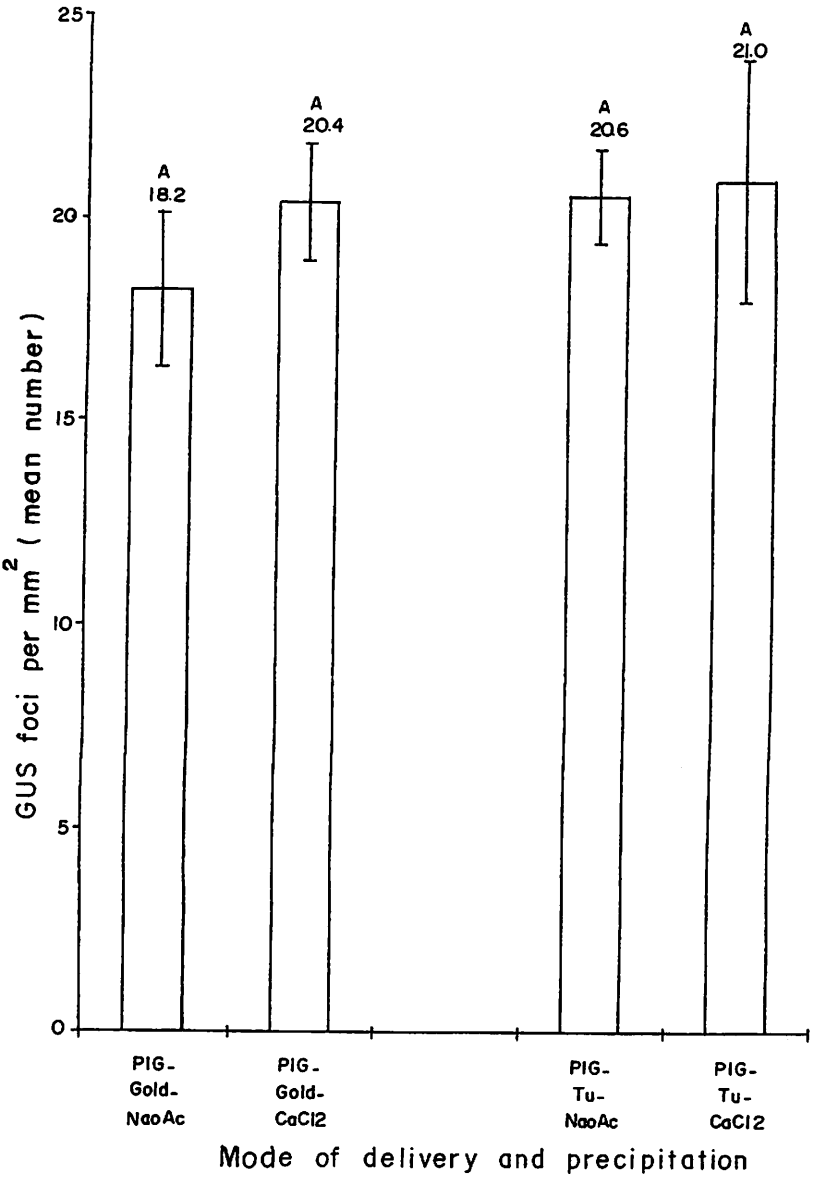

Figure 10a. Effect of mode of DNA precipitation on transient GUS expression in soybean embryogenic clumps using the PIG (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

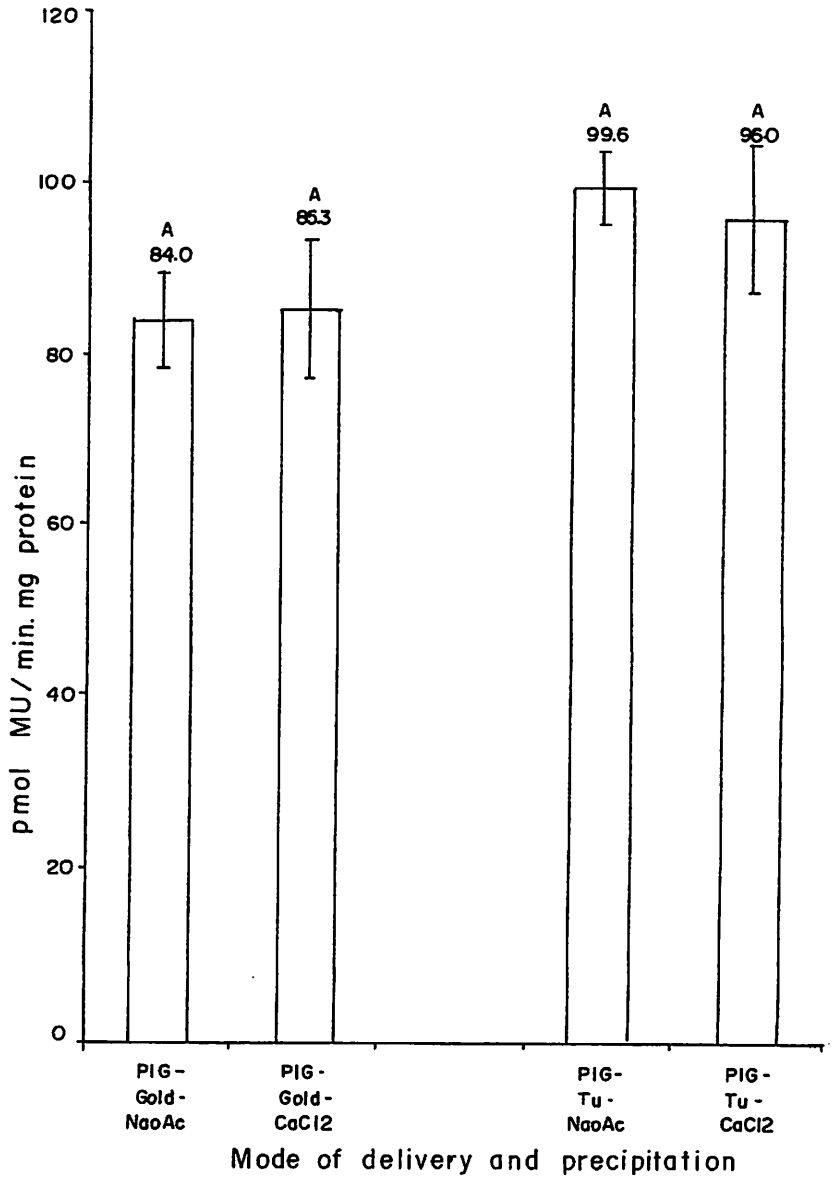

Figure 10b. Effect of mode of DNA precipitation on transient GUS expression in soybean embryogenic clumps using the PIG (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

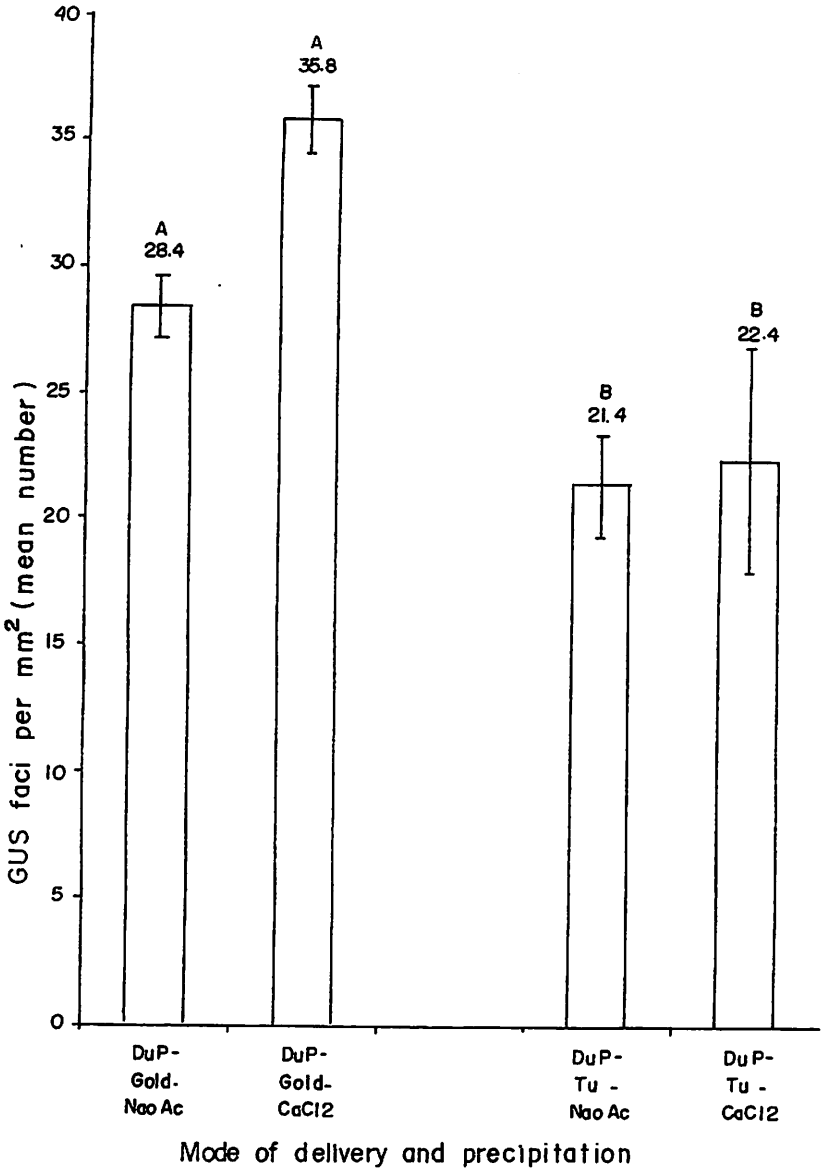

Figure 11a. Effect of mode of DNA precipitation on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (Histochemical assay). Bar values shown in different letters are significantly different at 0.05 level.

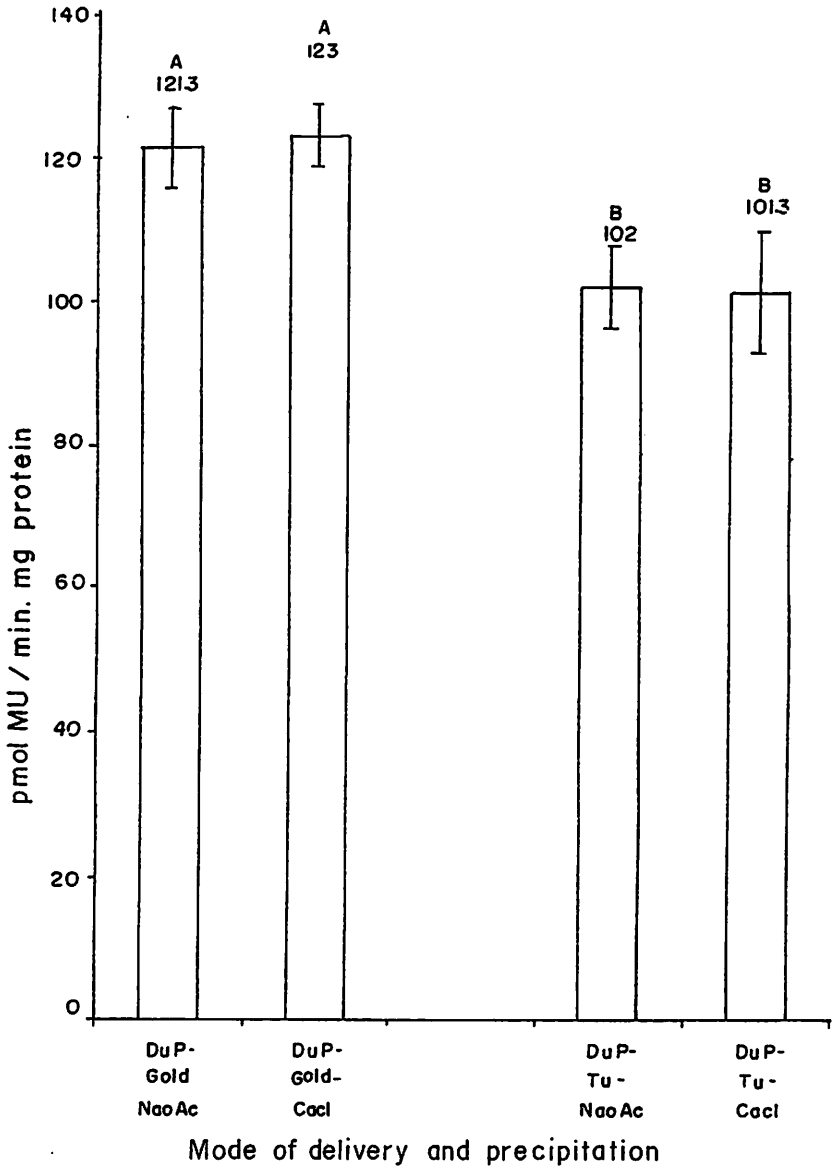

Figure 11b. Effect of mode of DNA precipitation on transient GUS expression in soybean embryogenic clumps using PDS 1000 Device (MUG assay). Bar values shown in different letters are significantly different at 0.05 level.

The transient expression assay can give a general idea of promoter strength, but it is not always an accurate way of reflecting in vivo regulation of the gene of interest (Taylor, 1997). In order to further evaluate the systems, research using the best conditions found for both guns and comparing 606 ASA2 and 35S promoters driving in separate constructs a drought tolerance gene HVA-1 (Straub et al., 1994) is being conducted. The number of stable transformed embryos will be compared as will the number of transgenic plants obtained to determine which system is better for stable transformation.

5 CONCLUSION

The 606 ASA2 GUS NOS construct gave higher transient expression in soybean embryogenic suspension cultures of cv. Jack than the larger fragment (1356 ASA2) and CaMV 35S promoters, using the Helium microprojectile bombardment device PDS/1000. The best conditions found for this gun were: 1 mg of gold, 1 μg of DNA, 1100 psi of pressure, 13 cm target distance. Sonication of the particles just before delivery increased the transient expression. The sodium acetate DNA precipitation methodology was as effective as that using $CaCl_2$/spermidine for the Helium PDS/1000 and Particle Inflow Gun (PIG). The best conditions for the PIG were: 1 mg of gold, 80 psi of pressure and 11 cm target distance.

When the two gun models were compared, using their best conditions, the Helium PDS/1000 had higher performance than the PIG. The PIG is however easier to handle, and is faster and cheaper to operate. Continuing research using 606 ASA2-HVA-1 and 35S-HVAl is being conducted to compare both promoters and bombardment devices for stable transformation.

REFERENCES

ACKERSON, R.C. Abscisic acid and precocious germination in soybeans. **Journal of Experimental Botany**, v. 35, p.414-421, 1984.

ALTEPTER, F.; VASIL, V.; SRIVASTAVA, V.; STÖGER, E.; VASIL, I. Accelerated production of transgenic wheat (*Triticum aestivum* L.) plants. **Plant Cell Reports**, v.16, p.12-17, 1996.

BAILEY, M.A.; BOERMA, H.R.; PARROTT, W.A. Genotype effects on proliferative embriogenesis and plant regeneration of soybean. **In Vitro Cell Dev. Biol.**, v.29, p.102-108, 1993.

BARWALE, V.B.; KERNS, H.R.; WIDHOLM, J.M. Plant regeneration from callus cultures of several soybean genotypes via embryogenesis and organogenesis. **Planta**, v.167, p.473-481, 1986.

BENFEY, P.N.; CHUA, N.A. Regulated genes in transgenic plant. **Science**, v.244, p.174-181, 1989.

BOYNTON, J.E.; GILHAN, N.W.; HOSLER, J.P.; JOHNSON, A.M.; JONES, A.R.; RANDOLPH-ANDERSON, B.L.; ROBERTSON, D.; KLEIN, T.M.; SHARK, K.B.; SANFORD, J.C. Chloroplast transformation in *Chlamydomonas* with high velocity microprojectile. **Science**, v.240, p.1534-1538, 1988.

BRADFORD, M.M. A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. **Annals of Biochemistry**, v.72, p.248-254, 1976.

BUCHHEIN, J.A.; COLBURN, S.M.; RANCH, J.P. Maturation of soybean somatic embryos and the transition to plantlet growth. **Plant Physiology**, v.89, p.768-775, 1989.

CHANPRAME, S. Transformation of somatic embryos of soybean with chitinase and B 1,3 glucanase genes via particle bombardment. University of Illinois, Urbana-Champaign, 1997. 120p. (Ph.D. Thesis).

CHRISTOU, P.; McCABE, D.E.; SWAIN, W.F. Stable transformation of soybean callus by DNA-coated gold particles. **Plant Physiology**, v.87, p.671-674, 1988.

CHO, MYEONG-JE; VODKIN, L.O.; WIDHOLM, J.M. Transformation of soybean embriogenic cultures by microprojectile bombardment. **Plant Biotechnology**, v.14, n.1, p.11-16, 1997.

CHO, MEYENG-JE; WIDHOLM, J.M.; VODKIN, L. Expression of lectin promoter-GUS fusions in transformed embryogenic cultures of soybean. **Plant Molecular Biology Reports**, v.13, n.3, p.255-269, 1995.

De WALD, S.G.; LITZ, R.E.; MOORE, G.A. Optimizing somatic embryo production in mango. **Journal of American Society for Horticultural Science**, v.114, p.712-716, 1989.

EDWARDS, K.L.; GOLDSMITH, M.H.M. pH dependent accumulation of indoleacetic acid by corn coleoptile sections. **Planta**, v.147, p.457-466, 1980.

FINER, J.J.; McMULLEN, M.D. Transformation of soybean via particle bombardment of embryogenic suspension culture tissue. **In Vitro Cell Dev. Biol.**, v.27, p.175-182, 1991.

FINER, J.J.; NAGASAWA, A. Development of an embryogenic suspension culture of soybean [*Glycine max* (L.) Merrill]. **Plant Cell and Organ Culture**, v.15, p.125-136, 1988.

GAMBORG, O.L.; MILLER, R.A.; OJIMA, K. Nutrient requirements of suspension cultures of soybean root cells. **Experimental Cell Research**, v.50, p.148-151, 1868.

GRAY, D.J.; FINER, J. Development and operation of five particle guns for introduction of DNA into plant cells. **Plant Cell, Tissue and Organ Culture**, v.33, p.219-257, 1993.

HADI, M.Z.; McMULLEN, P.M.; FINER, J. (1996). Transformation of 12 different plasmid into soybean via particle bombardment. **Plant Cell Reports**, v.15, p.500-505, 1996.

HARTWECK, L.M.; LAZZERI, P.A.; CUI, D.; COLLINS, G.B.; WILLIAMS, E.G. Auxin-orientation effects on somatic embryogenesis from immature soybean cotyledons. **In Vitro Cellular and Developmental Biology**, v.24, n.8, p.821-829, 1988.

HINCHEE, M.A.; CONNOR-WARD, D.V.; NEWELL, C.A.; McDONNELL, R.F.; SATO, S.; GASSER, C.S.; FISCHHOFF, D.A.; RE, D.B.; FRALEY, R.T.; HORSCH, R.B. Production of transgenic soybean plants using Agrobacterium mediated gene transfer. **Biotechnology**, v.6, p.915-922, 1988.

ILDA, A.; NAGASAWA, A.; OEDA, K. Soybean glycinin promoter identified by quantitative transient gene-expression. **Plant Cell Reports**, v.14, p.539-544, 1995.

JAIN, R.A.; JAIN, S.; WANG, B.; WU, R. Optimization of biolistic method for transient gene expression and production of agronomically useful transgenic Basmati rice plants. **Plant Cell Reports**, v.17, p.964-968, 1996.

JEFFERSON, R.A. Assaying chimeric genes in plants: The GUS gene fusion. **Plant Mol. Biol. Rep.**, v.5, n.4, p.387-405, 1987.

KIKKERT, J.R. The biolistic PDS-1000/He device. **Plant Cell and Organ Culture**, v.33, p.221-226, 1993.

KLEIN, T.M.; FROMM, M.; WEISSINGER, A.; TOMES, D.; SLETTEN, M.; SANFORD, J.C. Transfer of foreign genes into intact maize cells with high velocity microprojectiles. **Proceedings of National Academy of Science**, v.85, p.4305-4309, 1988.

KOETJ, D.S.; GRIMES, H.D.; WANG, Y.C. Regeneration of indica rice (*Oryza sativa*) from primary callus derived from immature embryos. **Journal of Plant Physiology**, v.135, p.184-190, 1989.

KOMATSUDA, T.; KO, S.W. Screening soybean [*Glycine max* (L.) Merrill] genotypes for somatic embryo production from immature embryo. **Japanese Journal of Breeding**, v.40, p.371-375, 1990.

LAZZERI, P.A.; HILDEBRAND, D.F.; COLLINS, G.B. Soybean somatic embryogenesis: effect of hormones and culture manipulations. **Plant Cell Tissue Organ Culture**, v.10, p.197-208, 1987.

LIU, J.R.; CANTLIFE, D.J. Somatic embryogenesis and plant regeneration in tissue cultures of sweet potato (*Ipomea batatas* Poir). **Plant Cell Reports**, v.3, p.112-115, 1984.

McCABE, D.; SWAIN, W.F.; MARTINELL, B.J.; CHRISOU, P. Stable transformation of soybean (*Glycine max*) by particle acceleration. **Biotechnology**, v.6, p.923-926, 1988.

MERKLE, S.A.; WETZSTEIN, H.V.; SOMMER, H.E. Somatic embryogenesis in tissue cultures of pecan. **Hort Sci.**, v.22, p.128-130, 1986.

MORIKAWA, H.; ILDA, A.; YAMADA, Y. Transient expression of foreign genes in plant cells and tissues obtained by a simple biolistic device (particle-gun). **Appl. Microbiol. Biotechnol.**, v.31, p.320-322, 1989.

MURASHIGE, T.; SKOOG, F.A. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Physiol. Plant**. V.15, p.473-497, 1962.

PARROTT, W.A.; ALL, J.N.; ADANG, M.J.; BAILEY, M.A.; BOERMA, H.R.; STEWART, J.R. Recovery and evaluation of soybean plants transgenic for a *Bacillus thuringiensis* var. Kurstaki inseticidal gene. **In Vitro Cell Dev. Biol.**, v.30, p.144-149, 1994.

PERL, A., KLESS, H., GALLI, G. and GALUN, E. Improvement of plant regeneration and GUS expression in scutellar wheat calli by optimization of culture conditions and DNA-microprojectile delivery procedures. **Mol. Gen. Genet.**, v.235, p.279-284, 1992.

PIERSON, E. S., van LAMMEREN, A. A., SCHEL, J. H. N. In vitro development of embroids from punched leaf discs of *Coffea canephora*. **Protoplasma**, v.115, p.208-216, 1983.

RAJAREKAN, KANNIAH and PELLOW, J. W. Somatic embryogenesis from cultured epicotyls and primary leaves of soybean [glycine max (L.) Merill]. **In Vitro Cell Dev. biol. Plant**, v.33, p.88-91, 1997.

RANCH, J.P.; OGLESBY, L.; ZIELINSKY, A.C. Plant regeneration from embryo-derived tissue cultures of soybeans. **In Vitro Cellular and Developmental Biology**, v.11, n.21, p.653-658, 1985.

RUSSELL, J.A.; ROY, M.K.; SANFORD, J.C. Physical trauma and tungsten toxicity reduce the efficiency of biolistic transformation. **Plant Physiology**, v.98, p.1050-1056, 1992.

SANFORD, J.C.; De CIT, M.J.; RUSSELL, J.A.; SMITH, F.D.; HARDPENDING, P.R.; ROY, M.K.; JOHNSTON, S.A. An improved helium-driven biolistic. **Technique**, v.3, p.3-16, 1991.

SANFORD, J. C., SMITA, F. D. and RUSSELL, J. A. (1993). Optimizing the biolistic process for different biological applications. Methods in Enzymology, 217:483-509.

SANFORD, J. C. Biolistic plant transformation. **Physiologia Plantarum**, v.79, p.206-209, 1990.

SANTAREM, E. R., PELISSIER, B. and FINER, J. J. Effect of explant orientation, pH, solidifying agent and wounding on initiation of soybean somatic embryos. In Vitro Cell Dev. on initiation of soybean somatic embryos. **In Vitro Cell Dev. Biol.**, v.33, p.13-19, 1997.

SATO, S., NEWELL, C., KOLACZ, K., TREDO, L., FINER, J. J., HINCHEE, M. Stable transformation via particle bombardment in two different soybean regeneration systems. **Plant Cell Reports,** v.12, p.408-413, 1993.

SINGH, M., KRIKORIAN, A. D. White's standard nutrient solution. **Ann. Bot.**, v.47, p.133-139, 1981.

SIVAMANI, E., SHEN, P., OPALKA, N., BEACHY, R. N. and FAUQET, C. M. Selection of large quantities of embryogenic calli from indica rice seeds for production of fertile transgenic plants using the biolistic method. **Plant Cell Reports**, v.15, p.322-327, 1996.

SONG, H.S.; BROTHERTON, J.E.; GONZALES, R.A.; WIDHOLM, J.M. Tissue culture specific expression of a naturally-occuring *Nicotiana tabacum* feedback-insensitive anthranilate synthase. **Plant Physiology**. In press.

STEWART, C. N., ADANG, M. J., ALL, J. N., BOERMA, H. R., CARDINEAU, G., TUCKER, D. and PARROTT, W. A. Genetic transformation, recovery and characterization of fertile soybean transgenic for a synthetic *Bacillus thuringienssis* cryiac gene. **Plant Physiology**, v.112, p.121-129, 1996.

STIFF, C.M.; KILIAN, A.; ZHOU, HUAPING; KUDRNA, D.A.; KLEINAOFS, A. Stable transformation of barley callus using biolistic particle bombardment and the phosphinothricin acetyltransferase (bar) gene. **Plant Cell, Tissue and Organ Culture**, v.40, p.243-248, 1995.

STRAUB, P. F., SHEN, Q., DAVID, HO, T. H. Structure and promoter analysis of an ABA-and-stress-regulated barley gene, HVAI. **Plant Molecular Biology**, v.26, p.617-630, 1994.

TAKEVCHI, Y., DOTSON, M. and KEEN, N. T. Plant transformation: A simple particle bombardment device based on flowing helium. **Plant Molec. Biol.**, v.18, p.835-839, 1992.

VAIN, P., KEEN, N., MURILLO, J., RATHUS, C., NEMES, C. and FINER, J. Development of particle inflow gun. **Plant Cell, Tissue and Organ Culture**, v.33, p.237-246, 1993.

VAN ECK, J.M.; BLOWERS, A.D.; EARLE, E.D. Stable transformation of tomato cell cultures after bombardment with plasmid and YAC DNA. **Plant Cell Reports**, v.14, p.299-304, 1995.

WALTER, C., SMITH, D. R., CONNETT, M. B., GRACE, L. and WHITE, D. W. R. A biolistic approach for the transfer and expression of a gus A reporter gene in embryogenic cultures of *Pimis radiata*. Plant Cell Reports, 14:69-74, 1994.

WIDHOLM, J. M. In "Transformation of Plants and Soil Microorganisms, Series of Plant and Microbiol. Biotechnology," eds. by Wang, K., Herrera-Estrella, A., Montagu, M.), p.101-124, Cambridge University Press, Cambridge, 1995.

WRIGHT, M. S., KOEHLER, S. M., HINCHEE, M. Plant regeneration by organogenesis in *glycine max*. **Plant Cell Reports**, v.5, p.150-154, 1986.

YANG, H. Y., MENEGUCI, J. L. P. and KORBAN, S. S. Transient gene expression in apple (Malus X domestica L.) following particle bombardment. Submitted to Journal of Horticultural Science, 1997.

YANG, S. F., HOFFMAN, N. E. Ethylene biosynthesis and its regulation in higher plants. **Ann. Rev. Plant Physiol.**, v.35, p.155-189, 1984.

ZELENIN, A. V., TITOMIROV, A. V., KOLESNIKOV, V. A. Genetic transformation of mouse cultured cells with the help of high-velocity DNA injection. FEBS Lett. 244, 65-267, 1989.