# GENETIC TRANSFORMATION *VIA* BIOLISTIC GENE GUN METHOD AND REGENERATION OF COMMON BEAN

**E. A. Eissa**

Genetics Department, Faculty of Agriculture, Fayoum University, Fayoum, Egypt.

**ABSTRACT**

*The objective of the present research was to utilize biotechnology to improve agronomical traits such as drought stress tolerance in common bean (Phaseolus vulgaris* L.*) as important recalcitrant crop species via a particle gun "Genebooster™" for the bombardment of plant tissues with DNA. The process involves the high velocity acceleration of microprojectiles carrying foreign DNA, penetration of the cell wall and membrane by microprojectiles, and delivery of the DNA into plant cells. The initial phase of the two common bean varieties "Fönix" and "Maxidor" focused on the development of a highly regenerable tissue culture procedure amenable to the particle gun physical method. The results showed that MS medium supplemented with 1 mg/l BA and 0.1 mg/l NAA were the optimal for shoot induction from meristematic ring of cotyledonary node explants in the both bean varieties. The author has developed a tissue culture protocol that enables the "meristematic ring" that develops below axillary shoots on nodal bean explants to produce shoot buds, and developed an electric-discharge particle acceleration procedure to produce transgenic plants in the two common bean varieties. The plasmid pFF19K, harboring the kanamycin resistance gene nptII as a selectable marker and pFF19-mtlD harboring the mtlD gene which confers mannitol (drought stress) tolerance were used for adapting transformation in the two common bean varieties. The results indicated that the successful delivery of plasmids DNA and efficient gene transfer into common bean meristematic tissues included 20 bar nitrogen pressure, 135 mm shooting distance, 1.1 µm diameter size of microprojectiles, and twice bombarding of the target tissues. Multiple shoots are then generated and screened to recover transgenic plants at a rate of 7.6% and 8.4% in "Fönix" and "Maxidor" bean varieties, respectively. Transgenic plants were recovered using both kanamycin (100 mg/l) and mannitol (1.2 M/l) screening to introduce the neomycin phosphotransferase II (nptII) and the mannitol-1-phosphate dehydrogenase (mtlD) genes into the two common bean varieties. The introduced nptII and mtlD genes have been shown to confer strong resistance and tolerance in transgenic beans to kanamycin and mannitol stress applications. The transgenic bean plants were healthy and the method has proven to be applicable to common bean crop. To my knowledge, this is the first report on the production of common bean transgenic plants with mtlD gene using biolistic gun-mediated gene transfer system.*

Key Words: ***Common bean, Transformation, Regeneration, Biolistic gun, Drought tolerance, mtlD Gene.***

## INTRODUCTION

Common bean (*Phaseolus vulgaris* L.) is the most important food legume for human nutrition in the developing world, and provides 22% of the total protein requirement worldwide (Delgado-Sánchez *et al* 2006 and Kwapata *et al* 2012). In addition, *P. vulgaris*, as a member of *Leguminosae*

possesses the valuable property of the enriching soil fertility by its capacity to fix atmospheric nitrogen. Santalla *et al* (1998) reported that regeneration ability from *P. vulgaris* and *P. coccineus* explants containing a cotyledon and embryonic axis varied between species and genotypes. Indirect regeneration of different genotypes of *P. vulgaris* was reported also by Arellano *et al* (2009). In another study, Kwapata *et al* (2010) reported that common bean apical shoot meristem primordium culture could produce multiple shoots. Callus cultures only were developed from various explants of French bean (Mahamune *et al* 2011), and common bean (Eissa 2013a). In another experiments and yet, regeneration from *in vitro* cultures of common bean cotyledonary node explants was reported, and its potential applications were discussed by Eissa *et al* (2002) and Eissa (2013b). Efficient *in vitro* regeneration systems are prerequisite for using recent advances in biotechnology to improve such important crop plants.

Genetic improvement by conventional breeding cannot add certain genes that do not exist in the *P. vulgaris* gene pool. Transformation is one of the interesting areas of biotechnology because characteristics can be added with minimal alteration of the target plants genome, and gene transfer technology need to be utilized in this important crop species (Aragão *et al* 1992, 1998, 1999 and 2002). Drought stress is a major factor limiting the crop productivity in the world and genetic transformation has become a tool for plant improvement. Among the requirements for successful transformation of plants are target tissues competent for regeneration, an efficient DNA delivery method, agents to select for transgenic tissues, and a simple efficient reproducible genotype. The major success in legume transformation was achieved by methods based on transformation of the pre-existing meristems on the embryo axes, cotyledonary nodes, and shoot tips as explants (Hanafy 2002, Hanafy *et al* 2005 and Eissa 2013b). Since a long time, several types of shooting device have been improved to transform living plant cells of different plant species with foreign DNA like onion, wheat, tobacco, soybean, maize and cowpea (Klein *et al* 1988, Sautter *et al* 1991, Sautter 1993, Gray and Finer 1993, McCabe and Christou 1993, Kikkert 1993, Vain *et al* 1993, Oard 1993, Gisel *et al* 1996 and Balázs, 2000). Transformation using the direct gene transfer system successes with recalcitrant common bean (Allavena and Bernacchia 1991, Aragão *et al* 1992, 1993 and 1996, Russell *et al* 1993, Aragão and Rech 1997 and Kim and Minamikawa 1997). *Agrobacterium*-mediated transformation of *P. vulgaris* has been reported on the use of sonication and vacuum infiltration for transfer of a group of 3 *lea* genes, and transgenic plants exhibited growth under drought stress (Liu *et al* 2005). A recent report (Amugune *et al* 2011) on transformation of *P. vulgaris* revealed the importance of specificity of *Agrobacterium* strains. Using biolistic gun, Aragão *et al* (2002) developed transgenic *P. vulgaris* resistant to herbicide, and only one plant was

genetically engineered (Bonfim *et al* 2007), also Rech *et al* (2008) published a protocol on an efficient genetic transformation of common bean. Zhu (2002) reported that drought stress cause plants to lose cellular turgidity, followed by the aggregation of proteins and yield losses. Tobacco, a plant that normally does not contain detectable concentrations of mannitol, was engineered to produce mannitol, by introducing a single gene from *E. coli* (Tarczynski *et al* 1992 and 1993). Plants transformed with the mannitol-1-phosphate dehydrogenase (*mtlD*) gene expressed the mannitol-1-phosphate dehydrogenase enzyme and accumulated mannitol throughout the plant. For the transformation of *P. vulgaris* it is essential to define an *in vitro* culture system that allows successful plant regeneration. Efforts to produce transgenic beans have suffered from a lack of efficient DNA delivery and regeneration systems. A critical step in the development of a plant transformation procedure is to deliver DNA to tissues that can regenerate plants, and transgenic plants are then recovered through de novo shoot formation. The efficiency of genetic engineering of *P. vulgaris* has remained a challenge till now.

Transformation of common beans as an economic important crop can improve the useful agronomical traits such as drought stress tolerance. Therefore, the primary objective of this work was to establish a stable protocol for highly efficient regeneration system of common beans as a base of genetic transformation. The ultimate goal was to introduce *mtlD* gene encoding drought stress tolerance as a useful agronomic trait in two commercial varieties of common bean by biolistic gene gun method. The microprojectiles carried the selectable marker gene neomycin phosphotransferase II (*nptII*) encoding kanamycin resistance as well as *mtlD* gene delivering it into the target living tissues. The transfer of *mtlD* gene as osmoprotectant to both varieties of common bean and the development of drought stress tolerance of transgenic shoots and plants were reported.

## MATERIALS AND METHODS

### Plant materials, seeds sterilization method, and explants preparation

Dry seeds of the commercial common bean varieties “Fönix” and “Maxidor” were evaluated for their regeneration and transformation efficiency. Seeds were kindly supplied by the Department of Genetics and Horticultural Plant Breeding, Faculty of Horticultural Sciences, Corvinus University, Budapest (formerly: Szent István University), Hungary, to whom my thanks are due. For surface-sterilization method, dry seeds were selected based on a healthy and similar size, and rinsed in tap water for 1 min, then rinsed three times with distilled water, soaked in 75% ethanol for 4 min, and again rinsed three times in distilled water. Then, the seeds were soaked in 20% commercial Clorox for 15 min, and germinated in test tubes on MS (Murashige and Skoog 1962) medium. Cotyledonary node

meristematic tissues containing a portion of the hypocotyl, epicotyl and cotyledons cultures were prepared from excised three- to four-days-old seedlings. Approximately, 3/4 of each cotyledon was removed from the explant, leaving the meristem of the cotyledon fragments as an intact attached to the nodal region. For shoot induction, the meristematic cotyledonary node explants were cultured on MS medium containing 1 mg/l 6-benzyladenine (BA) and 0.1 mg/l α-naphthalene acetic acid (NAA), 30 g/l sucrose and 7 g/l agar. The pH of the media was adjusted to 5.7 before autoclaving at 120°C for 20 min. Cultures were maintained in a growth chamber at 24-25°C with a 16/8 h light/dark cycle at white light. The cotyledonary node meristematic tissues of the two common bean varieties "Fönix" and "Maxidor" were chosen as explants for the genetic transformation experiment because it produces high number of shoot regeneration (Eissa 2013b).

**Plasmids DNA and particle gun**

Two plasmids were introduced into the target meristematic living tissues which are able to regenerate full plants *via* organogenesis, kindly donated by the Agricultural Biotechnology Center (ABC), Gödöllő, Hungary. Plasmids used include pFF19K harboring the *nptII* gene as selectable marker conferring the kanamycin resistance, and pFF19-mtlD harboring the *mtlD* gene isolated from *E. coli*, which confers mannitol drought stress tolerance. The two plasmids were used for co-transformation of the two common bean varieties "Fönix" and "Maxidor". When the *mtlD* gene is active in transgenic plant tissues, the mannitol content of the cytoplasm is higher than non-transgenic, and transgenic plant tissues can better tolerate dehydration stress. Direct gene transfer *via* particle bombardment using the Genebooster$^{TM}$ system produced by ELAK Ltd. Co., Budapest, Hungary was established in common beans. It consists of a barrel, a vacuum chamber for bombardment which is a steel box covered by plastic coat and an electronic control unit includes the vacuum and pressure sensors, electric valves, the controlling microprocessors and a stainless steel secondary gas container. A vacuum pump is connected to the device to provide vacuum in the vacuum chamber. The macroprojectiles are made of plastic "bonomit", and between the stopping plate is made of steel and the target tissue there is usually a stainless steel screen of various mesh size mounted (Fig. 1/A and 1/B).

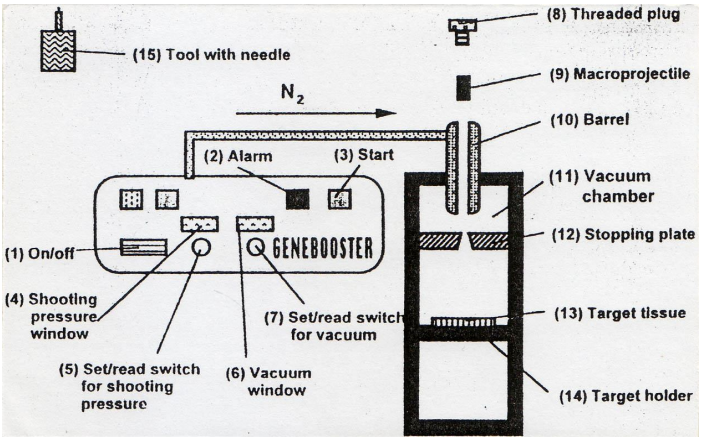


**Fig. (1/A): The mean functional parts of the Genebooster [TM].**

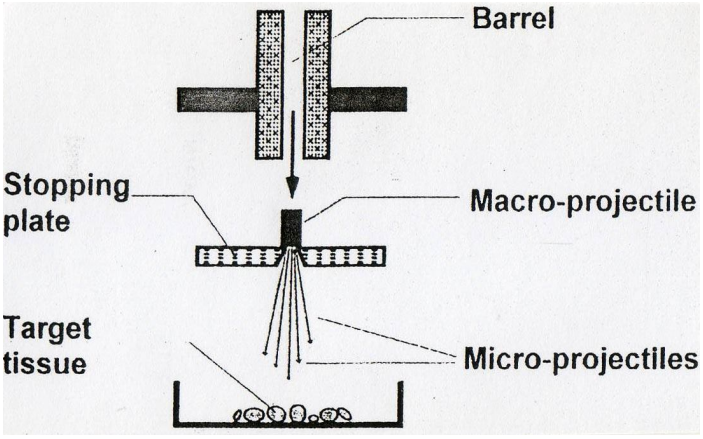


**Fig. (1/B): The principle of gene delivery with the Genebooster [TM].**

**Biolistic-mediated transformation and bombardment conditions**

Before gene shooting the two plasmids were mixed in 1:1 ratio and used for the two varieties co-transformation. Cotyledonary node meristematic tissues from germinated seedlings were prepared and cultured in a circle into the middle of plates on top of the MS regeneration medium immediately before the bombardment with the mixed plasmids. The Genebooster[TM] was used as a particle accelerating device to deliver plasmids DNA into *in vitro* cultured node meristematic tissues of both varieties. The total number of explants in each variety was 500 and the

number of explants was 10 node tissues/plate. In this experiment the optimization of some physical factors, the appropriate pressure of $N_2$ gas for the bombardment of the tissue, the shooting distance and the size of the tungsten particles used as microprojectiles effecting the use of this device, and the production of transgenic was used as reported by Jenes *et al* (1996 and 1997) and Balázs (2000). The explants were bombarded under high-pressure of 20 bar $N_2$ gas at a distance of 135 mm, and the macrocarriers made of plastic were accelerated by it. Tungsten particles 1.1 µm diameters were used as microcarriers to bind the actual DNA molecules on their surface, and provide the penetration through the hard plant cell wall, then the nucleic acid molecules are released and can be integrated into the genome. With the help of this method plant cells and tissues can be transformed with high efficacy and transgenic plants can be regenerated from them. The vacuum chamber included the stopping plate and shelves for the target tissues (Fig. 1/A and 1/B). The two mixed plasmids binding on the surface of tungsten particles was carried out with the $Ca(NO_3)_2$ precipitation of DNA. The coated plasmids DNA were bombarded into the explants using two time of bombardment frequencies. Stable genetic transformation of both common bean varieties was performed using the biolistic delivery for bombardment of the mixed plasmids into the node meristematic tissues. The bombarded tissues were cultured in regeneration media without the use of any chemical selections for 24 h, to allow expression of the two genes.

**Sensitivity of cotyledonary node explants to kanamycin, and drought stress mannitol**

An effective concentrations of kanamycin, and mannitol for the selection of transformants were assessed by preliminary tests. Cotyledonary node meristematic tissues for both bean varieties were cultured on shoot regeneration MS medium, plus 0, 50, 75, 100, 150 and 200 mg/l kanamycin, and 0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2.0 M/l mannitol as drought stress conditions, in order to determine the concentrations which inhibited the growth of wild type cotyledonary node tissues. The inhibitory concentration, thus, determined was used for the selection of transgenic cotyledonary node tissues resistant to this concentration of kanamycin, and tolerant to this concentration of mannitol drought stress. Kanamycin at a concentration of 100 mg/l, and mannitol at a concentration of 1 M/l were used for the selection of transformed shoots and plants in both common bean varieties.

**Selection and regeneration of transformed tissues**

To establish the selection protocol, it was necessary to study kanamycin, and mannitol sensitivities on the growth of non-transformed common bean

cotyledonary node meristematic tissues. For transformation and regeneration tests, the bombarded common bean meristematic tissues were transferred at first to selective MS regeneration medium containing only 50 mg/l kanamycin, or 0.6 M/l mannitol for one week. Cotyledonary node explants were then transferred to selection medium plus 75 mg/l kanamycin, or 0.8 M/l mannitol for two weeks. Selective tests were then made by transferring resistant healthy green meristematic explant tissues into three selective media containing 100 mg/l kanamycin or 1 M/l mannitol, and 100 mg/l kanamycin and 1 M/l mannitol for transformed buds and shoots selections for five months, sub-cultured every two weeks. To screen for transformed buds and shoots regenerated from the explants of both two varieties, 150 mg/l kanamycin and 1.2 M/l mannitol were added to regeneration medium as selection agents. The selection of stable transgenic shoots for both bean varieties was based on the use of 1.2 M/l mannitol selection for the *mtlD* drought stress tolerance gene expression, and stable transgenic green shoots continued on this medium. Subsequently putative transformed and surviving green shoots were transferred onto kanamycin, or mannitol and 0.1 mg/l indole-3-butyric acid (IBA) containing MS medium to verify their ability to root prior transfer to greenhouse.

## RESULTS AND DISCUSSION

### Establishment of a regeneration system in common beans

The aim was to develop a highly efficient particle bombardment-based transformation method for *P. vulgaris*. Therefore, a procedure for regeneration of shoots from cotyledonary nodes explants in *P. vulgaris* should be of great help to attain genetic transformation of this important recalcitrant species. Regeneration from cotyledonary nodes as explants in two common bean varieties “Fönix” and “Maxidor” was studied in the present investigation. The results showed that MS medium supplemented with 1 mg/l BA and 0.1 mg/l NAA was the optimal for shoot induction from meristematic ring of cotyledonary node explants in both bean varieties. A similar finding was reported by Eissa *et al* (2002) and Eissa (2013b), who showed that MS medium containing 1 mg/l BA and 0.1 mg/l NAA was the optimal for shoot regeneration in the two varieties. This *in vitro* culture system carries a high potential for regeneration and transformation of *P. vulgaris* to improve this important recalcitrant species. The development of highly regenerable tissue culture procedure from nodal explants showed organogenesis and bud formation from the meristematic cells in the nodal tissues. The present results indicated that the shoots were formed from actively dividing cells located at the axillaries bud regions, and the method was designed to optimize rapid and high frequency direct shoot regeneration of the commercial important common beans. Cotyledonary node explants exhibited higher regeneration efficiency than other several explants tested.

Kwapata *et al* (2010) reported that the optimum medium for regeneration of multiple shoots from the embryonic axis of common bean was MS containing 2.5 mg/l BA and 0.1 mg/l IAA. Callus cultures only were initiated from the different explants of French bean on MS medium with 1.5 mg/l BAP, and three levels of IAA (Mahamune *et al* 2011), and common bean on B5 (Gamborg *et al* 1968) medium with 1 mg/l kinetin and 2 mg/l 2,4-D (Eissa 2013a). The highly efficient regeneration system is considered a new addition that will open the door for improving common beans by *in vitro* techniques. A well defined, preferably simple, shoot regeneration protocol is a prerequisite for the production of transgenic plants. Microprojectile bombardment is the only procedure capable of delivering DNA into cells in virtually any tissue of any plant and, thus, is widely used for transformation of any species. Therefore, this method for regeneration was suitable for direct transformation particle gun protocol and used in transformation and regeneration experiments in both common bean varieties. The bean transformation system was based upon the development of a tissue culture protocol of multiple shoots from cotyledonary node meristematic tissues.

**Establishment of a transformation system and production of drought tolerant plants in common beans**

The lack of a transformation system for common bean can be attributed to the lack of a tissue culture system with many meristems to serve as transformation targets. The results of this investigation indicate that the cotyledonary nodes meristematic tissue contains undifferentiated cells, and might be a good explant for genetic transformation. Therefore, it is the subepidermal cell layer that needs to be targeted *via* the biolistic gun for transformation, and the researcher tried to standardize the biolistic delivery bombardment to hit these layers. The microprojectile bombardment induced fine pores in target cells for easy transformation subsequently. The optimal physical factors affecting the successful delivery of plasmids DNA into the living cells of common bean meristematic tissues included nitrogen pressure applying for the biolistic device was 20 bars to achieve the penetration of the particles into the target cells; the suitable shooting distance was 135 mm between the gun barrel and target explants to penetrate the cell wall and membrane of the target tissues, and the optimal size of microprojectiles was 1.1 µm diameter for efficient gene transfer. Bombarding the target tissues twice, yielded the highest efficiency of regeneration and transformation in both bean commercial varieties “Fönix” and “Maxidor”. The results of De Guglielmo-Cróquer *et al* (2010) showed that the transformed coffee plantlets with the *B. thuringiensis* (*cry1ac*) gene without reporter (*gusA*) gene is feasible by biolistic gun. Kanamycin a widely used marker for plant transformation that can be phytotoxic and inhibit untransformed tissues, and

the *nptII* gene can be used as a selectable marker in plants. The development of transgenic plants of two common bean varieties that are able to grow in the presence of high concentrations of kanamycin and mannitol, and that express the gene product required, for resistance to the drug and for tolerance to drought stress without reporter *gusA* gene were reported. The selectable marker *nptII* gene encodes the protein product phosphorrylates and inactivates kanamycin, and renders the drug non toxic to plant cells as reported and discussed in details by Eissa (2013a and b).

The development of efficient transformation protocols for common beans will open up the possibility of transferring useful genes into this crop, such as gene for mannitol drought stress tolerance. The bombarded cotyledonary node explants were incubated for 24 hours without kanamycin selection to allow expression of the resistance gene. To study the suitable selection conditions for the common bean varieties, the cotyledonary node tissues were incubated on regeneration medium containing different concentrations of kanamycin for 3 weeks. Cotyledonary nodes were cultured on regeneration media with 50 and 75 mg/l kanamycin as selection agent for regeneration. Stable transformants were then selected by culturing the explants on medium containing 100 mg/l kanamycin. Results showed that kanamycin at a concentration of 100 mg/l, totally suppressed the growth of the cotyledonary node tissues as reported by Eissa (2013b). These results provide strong confirmation for a kanamycin resistant gene transferred to common bean plant cells. The availability of simple selection assay becomes extremely useful such as selection method that may facilitate the identification of the transgenic plants. Following several transfers on MS medium containing kanamycin and mannitol, most of the shoots obtained from biolistic gun bombarded turned yellow, browned and died. The percentage of co-transformed cotyledonary node tissues by the coated plasmids DNA bombarded with biolistic gun, cultured on double selective MS regeneration medium containing 100 mg/l kanamycin and 1.2 M/l mannitol, and supplemented with 1 mg/l BA and 0.1 mg/l NAA was 7.6% and 8.4% in “Fönix” and “Maxidor” varieties, respectively (Table 1 and Fig. 2). Selection pressure was applied when excised shoots were rooted on MS medium containing 50 mg/l kanamycin and 1 M/l mannitol (Fig. 3). Subsequent selection on media with kanamycin and mannitol resulted in the recovery of shoots and plants resistant to the kanamycin and tolerant to the mannitol. Kanamycin-resistant and mannitol-tolerant shoots and plants appeared 2-3 weeks following planting on solid medium. The presence of the DNA insert in the bean genome was shown by its resistance to kanamycin and tolerance to mannitol. Drought and salinity abiotic stresses are a major factor limiting the crop productivity in all over the world. The main goal of this study was to improve the drought stress tolerance of common bean through *mtlD* drought stress tolerance gene transformation

based on application of genetic engineering to introduce this important trait into this important legume. When *mtlD* gene activates in transgenic beans, the mannitol content of the cytoplasm is higher than in normal cells and these tissues can better tolerate dehydration stress. Since selection of explants is one of the major steps of any transformation experiments the results presented here of transient *nptII* and *mtlD* expressions, suggests that cotyledonary node meristematic tissues can be used as explant for the development of transgenic varieties of common bean without reporter *gusA* gene.

**Table 1. Co-transformation efficiency from cotyledonary node tissues of two common bean varieties bombarded by pFF19K and pFF19-mtlD plasmids, selected on double selective MS regeneration medium containing 1 mg/l BA and 0.1 mg/l NAA.**

| Variety | Kanamycin (mg/l) and Mannitol (M/l) double selection | Number of cotyledonary nodes explant | Number of kanamycin resistant and mannitol tolerant nodes | Percentage of co-transformed node explants |
|---|---|---|---|---|
| Fönix | 100 + 1.2 | 500 | 38 | 7.6 |
| Maxidor | 100 + 1.2 | 500 | 42 | 8.4 |

**Percentage of co-transformed explants (explants giving rise to one or more kanamycin resistant and mannitol tolerant shoot compared to the total number of explants).**

**Fig. (2): Selection of stable kanamycin resistant and mannitol stress tolerant transgenic green shoots of bean Fönix variety growing on double selective MS regeneration medium containing 100 mg/l kanamycin and 1.2 M/l mannitol.**

**Fig. (3): Transgenic green shoots of bean Fönix variety growing and rooting very well on double selective MS rooting medium containing 50 mg/l kanamycin and 1 M/l mannitol.**

Drought tolerance is a very complex character involving the structure and activity of all the plant organs, and there are, however, only few reports on osmotically stressed cell cultures. The present paper describes the selection of an osmotically mannitol tolerant cotyledonary nodes meristematic tissues of two common bean varieties. This system gives the author the chance of using it to introduce useful genes into common beans. Tarczynski *et al* (1993) demonstrated that the mannitol-accumulating, transformed tobacco exhibited greater salt tolerance than non-transformed, and mannitol can promote salt tolerance in higher plants. Mannitol, a six carbon noncyclic sugar alcohol, occurring in bacteria, fungi, algae, and in vascular plants (Pharr *et al* 1995). Roles for mannitol in these organisms, including, free radical scavenging and osmoregulation, are important in stress tolerance, which causes osmostress in plants. The *mtlD* gene was used to screen for transgenic shoots and to confer strong tolerance to mannitol application. A similar finding was reported by Bordas *et al* (1997), who stated that a halotolerance (*hal1*) gene isolated from yeast, *nptII* as marker gene, and the construct was introduced into melon; transformants were selected for their ability to grow on media containing kanamycin. Also, Kwapata *et al* (2012) reported that common beans were genetically transformed with the *Hordeum vulgare* (*HVA1*) drought tolerance gene *via* the biolistic bombardment of the apical shoot meristem primordium, and transgenes conferred drought tolerance. Results of Lal *et al* (2008) indicated that the production of the *H. vulgare* late embryogenesis abundant (HVA1) proteins encodes by the *HVA1* gene helps in better performance of transgenic mulberry by protecting membrane stability of plasma membrane from injury under drought stress. Effort to engineer maize for improved abiotic stress (Assem *et al* 2008 and 2010) immature embryo were bombarded with the Bax-Inhibiting glutathione S-transferase (*BI-GST*) gene, and the *HVA1* gene, for abiotic stress tolerance, transgenic events have been tested by the *bar* gene for herbicide resistance, and suggest that the stress tolerance may be improved in *BI-GST*, and *HVA1* transgenic plants. Also, Fahmy *et al* (2012) used immature wheat embryos as explants for the biolistic mediated transformation, and 0.4 M sorbitol was the best for GUS assays from wheat calli.

Russell *et al* (1993) reported that transgenic navy bean plants produced *via* particle gun to introduce DNA into seed meristems were recovered at a rate of only 0.03% using the *gusA* and *bar* genes. Aragão *et al* (1992 and 1993) developed a simple protocol to introduce the Brazil nut methionine-rich albumin (2S) and *gusA* gene in common bean mature embryos based on biolistic method, and reported that the parameters influencing transient expression of *gusA* gene in bean embryonic axes, cotyledons, apical meristems and leaves were evaluated after gene delivery with particle gun. Also, Aragão *et al* (1996 and 1999) reported that by exploiting the biolistic

process to generate transgenic bean plants with *gusA*, *neo* and the 2S genes, the average frequency of transgenic plants from bombarded embryonic axes was only 0.9%, and the methionine content was increased 14% over untransformed. The bombardment/*Agrobacterium* system appears to be a promising method to stably transform bean through the regeneration of plants directly from transformed apical meristems (Brasileiro *et al* 1996). Aragão and Rech (1997) obtained transgenic bean plants from a Carioca cultivar by particle bombardment of the shoot apex. Shoot apices of French bean seeds were bombarded with gold particles coated with a plasmid carrying *gusA* gene; the tissue expression of the gene was observed histochemically in transgenic tissues (Kim and Minamikawa 1997). Vianna *et al* (2004) demonstrated that the transformation of bean plants through particle gun frequencies were 0.8% and 0.7% for the variety Carioca, and 0.2% and 0.3% for the variety Olathe. Bonfim *et al* (2007) reported that immediately after bombardment embryonic axes mature seed of common bean cv. Olathe Pinto were transferred to MS medium containing BA and the herbicide was used as a selective agent to generate transgenic plants highly resistant to bean golden mosaic virus. Rech *et al* (2008) reported a protocol of particle gun in common bean apical meristems and selection of transgenic plants; the average frequency of producing transgenic bean plants resistant to the herbicide was 2.7%. This has strongly limited the application of genetic engineering techniques to improve this important crop. Efficient genetic transformation was performed with the *nptII* and osmosis protector (*mtlD*) genes in calli of "Fönix" and "Maxidor" bean varieties through biolistic gun (Eissa 2013a). Gulati and Jaiwal (1993) reported that an osmotically mannitol tolerant callus line of *Vigna radiata* has been isolated from callus cultures grown on medium with mannitol. Öktem *et al* (1999) performed a transformation experiment with cotyledonary nodes of lentil for the expression of *gusA* gene through particle gun. Particle gun has been used to transfer the plasmid DNA to the mature embryo axes, cotyledonary nodes and cotyledons of faba bean (Hanafy 2002). Metry *et al* (2007) demonstrated that the transformation frequency of faba bean using microprojectile bombardment protocol was as high as 2% for one cultivar using the mature embryo as the explant. The results of the present research are much better and agree with literature data.

In conclusions, the results show that particle gun can be used for the introduction of useful *mtlD* gene into the common bean genome and indicate the technique useful in the recovery of engineered plants by transformation of regenerable meristematic tissues. The bombardments were carried out using Genebooster$^{TM}$ driven by compressed nitrogen gas. The protocol used in the experiments gave promising results for the efficient regeneration and subsequent genetic transformation of common bean plants without reporter *gusA* gene. The transformation events were selected by two

different approaches, selection for only one marker kanamycin or mannitol, and double selection using kanamycin and mannitol at the same time. Tolerant cotyledonary node shoots, and plants showed greater osmotically tolerance to mannitol osmotic stress in the two commercial varieties tested. This may be due to the cellular accumulation of metabolic products of mannitol which contribute to osmotic adjustment. This research describes a method for high-efficient recovery of transgenic common bean plants, by combining resistance to the kanamycin as a selectable marker, tolerance to the mannitol drought stress, multiple shoot induction from cotyledonary node meristematic tissues and biolistics techniques. Theoretically, the particle gun can be used to deliver DNA sequences into any kind of tissues but transgenic plants can be produced only from tissues capable of regenerating buds and shoots.

## REFERENCES


**Allavena, A. and G. Bernacchia (1991).** Attempting genetic transformation of bean by high velocity microprojectiles. Ann. Rep. Bean Impr. Coop. 34: 137-138.

**Amugune, N.O., B. Anyango and T.K. Mukiama (2011).** *Agrobacterium*-mediated transformation of common bean. Afri. Crop Sci. J. 19(3): 137-147.

**Aragão, F.J.L., M.-F. Grossi de Sa, E.R. Almeida, E.S. Gander and E.L. Rech (1992).** Particle bombardment-mediated transient expression of a Brazil nut methionine-rich albumin in bean (*Phaseolus vulgaris* L.). Plant Mol. Bio. 20: 357-359.

**Aragão, F.J.L., M.-F. Grossi de Sa, M.R. Davey, A.C.M. Brasileiro, J.C. Faria and E.L. Rech (1993).** Factors influencing transient gene expression in bean (*Phaseolus vulgaris* L.) using an electrical particle acceleration device. Plant Cell Rep. 12: 483-490.

**Aragão, F.J.L., L.M.G. Barros, A.C.M. Brasileiro, S.G. Ribeiro, F.D. Smith, J.C. Sanford, J.C. Faria and E.L. Rech (1996).** Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed *via* particle bombardment. Theor. Appl. Gen. 93(1-2): 142-150.

**Aragão, F.J.L. and E.L. Rech (1997).** Morphological factors influencing recovery of transgenic bean plants (*Phaseolus vulgaris* L.) of a Carioca cultivar. Int. J. Plant Sci. 158(2): 157-163.

**Aragão, F.J.L., S.G. Ribeiro, M.G. Barros, A.C.M. Brasileiro, D.P. Maxwell, E.L. Rech and J.C. Faria (1998).** Transgenic beans (*Phaseolus vulgaris* L.) engineered to express viral antisence RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. Mol. Breed. 4(6): 491-499.

**Aragão, F.J.L., L.M.G. Barros, M.V. de Sousa, M.F. Grossi de Sá, E.R.P. Almeida, E.S. Gander and E.L. Rech (1999).** Expression of a methionine-rich storage albumin from the Brazil nut (*Bertholletia excelsa* H.B.K., *Lecythidaceae*) in transgenic bean plants (*Phaseolus vulgaris* L., *Fabaceae*). Gen. Mol. Bio. 22(3): 445-449.

**Aragão, F.J.L., G.R. Vianna, M.M.C. Albino and E.L. Rech (2002).** Transgenic dry bean tolerant to the herbicide glufosinate ammonium. Crop Sci. 42(4): 1298-1302.

**Arellano, J., S.I. Fuentes, P. Castillo-España and G. Hernández (2009).** Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) *via* indirect organogenesis. Plant Cell Tiss. Org. Cul. 96(1): 11-18.

**Assem, S.K., E.H.A. Hussein and T.A. El-Akkad (2008).** Genetic transformation of Egyptian maize lines using the late embryogenesis abundant protein gene, *HVA1*, from barley. Arab J. Biotech. 11(1): 59-70.

**Assem, S.K., E.H.A. Hussein, H.A. Hussein and S.B. Awaly (2010).** Transformation of the salt-tolerance gene *BI-GST* into Egyptian maize inbred lines. Arab J. Biotech. 13(1): 99-114.

**Balázs, E. (2000).** Agricultural Biotechnology Center for Improved Agriculture. Hung. Agri. Res. 2: 14-17.

**Bordas, M., C. Montesinos, M. Dabauza, A. Salvador, L.A. Roig, R. Serrano and V. Moreno (1997).** Transfer of the yeast salt tolerance gene *HAL1* to *Cucumis melo* L. cultivars and *in vitro* evaluation of salt tolerance. Trans. Res. 6: 41-50.

**Bonfim, K., J.C. Faria, E.O.P.L. Nogueira, É.A. Mendes and F.J.L. Aragão (2007).** RNAi-mediated resistance to bean golden mosaic virus in genetically engineered common bean (*Phaseolus vulgaris*). Mol. Plant-Mic. Int. 20(6): 717-726.

**Brasileiro, A.C.M., F.J.L. Aragão, S. Rossi, D.M.A. Dusi, L.M.G. Barros and E.L. Rech (1996).** Susceptibility of common and tepary beans to *Agrobacterium* spp. strains and improvement of *Agrobacterium*-mediated transformation using microprojectile bombardment. J. Amer. Soc. Hor. Sci. 121(5): 810-815.

**De Guglielmo-Cróquer, Z., I. Altosaar, M. Zaidi and A. Menéndez-Yuffá (2010).** Transformation of coffee (*Coffea arabica* L. cv. Catimor) with the *cry1ac* gene by biolistic, without the use of markers. Braz. J. Bio. Bio. 70(2): 387-393.

**Delgado-Sánchez, P., M. Saucedo-Ruiz, S.H. Guzmán-Maldonado, E. Villordo-Pineda, M. González-Chavira, S. Fraire-VelÁzquez, J.A. Acosta-Gallegos and A. Mora-Avilés (2006).** An organogenic plant regeneration system for common bean (*Phaseolus vulgaris* L.). Plant Sci. 170(4): 822-827.

**Eissa, A.E., Gy. Bisztray and I. Velich (2002).** Plant regeneration from seedling explants of common bean (*Phaseolus vulgaris* L.). Acta Bio. Szeged. 46(3-4): 27-28.

**Eissa, A.E. (2013a).** Genetic transformation of common beans (*Phaseolus vulgaris* L.) calli by biolistic gun and *via Agrobacterium tumefaciens*. Egyp. J. Gen. Cytol. 42(1): 1-20.

**Eissa, A.E. (2013b).** Genetic transformation and regeneration of common bean (*Phaseolus vulgaris* L.) using *Agrobacterium* system. Egyp. J. Gen. Cytol. 42(1): 127-150.

**Fahmy, A.H., J. Li, W. Abou El-Wafa and S.E. El-Khodary (2012).** Optimization of biolistic-mediated transformation conditions in immature embryos of two commercial wheat cultivars. Arab J. Biotech. 15(2): 267-276.

**Gamborg, O.L., R.A. Miller and K. Ojima (1968).** Nutrient requirements of suspension cultures of soybean root cells. J. Exp. Res. 50: 151-158.

**Gisel, A., V.A. Iglesias and C. Sautter (1996).** Ballistic microtargeting of DNA and biologically active substances to plant tissue. Plant Tiss. Cul. Bio. 2(1): 33-41.

**Gray, D.J. and J.J. Finer (1993).** Development and operation of five particle guns for introduction of DNA into plant cells. Plant Cell Tiss. Org. Cul. 33: 219.

**Gulati, A. and P.K. Jaiwal (1993).** Selection and characterization of mannitol-tolerant callus lines of *Vigna radiata* (L.) Wilczek. Plant Cell Tiss. Org. Cul. 34: 35-41.

**Hanafy, M.S. (2002).** Development of an efficient transformation system to field bean (*Vicia faba*), manipulation of the sulphur-rich protein content *via* genetic engineering. PhD. Diss. p. 46-51. (Von dem Fachbereich Biologie Der Universität Hannover zur Erlangung des Grades eines).

**Hanafy, M., T. Pickardt, H. Kiesecker and J. Jacobsen (2005).** *Agrobacterium*-mediated transformation of faba bean (*Vicia faba* L.) using embryo axes. Euphytica 142: 227-236.

**Jenes, B., P. Bittencourt, Á. Csányi, J. Pauk, I. Nagy, O. Toldi, and E. Balázs (1996).** The GENEBOOSTER–a new microparticle bombardment device–for genetic transformation of plants. Plant Tiss. Cul. Bio. 2(1): 42-51.

**Jenes, B., O. Toldi, P. Bittencourt, I. Nagy, Á. Csányi and E. Balázs (1997).** The GENEBOOSTER$^{TM}$–designed and developed by the Agricultural Biotechnology Center, Gödöllő. Hung. Agri. Res. 3: 14-17.

**Kikkert, J.R. (1993).** The Biolistic® PDS-1000/He device. Plant Cell Tiss. Org. Cul. 33: 221-226.

**Kim, J.W. and T. Minamikawa (1997).** Stable delivery of a canavalin promoter-*β*-glucuronidase gene fusion into French bean by particle bombardment. Plant Cell Physio. 38(1): 70-75.

**Klein, T.M., M. Fromm, A. Weissinger, D. Tomes, S. Schaaf, M. Sletten and J.C. Sanford (1988).** Transfer of foreign genes into intact maize cells with high-velocity microprojectiles. Proc. Nat. Acad. Sci. USA 85: 4305-4309.

**Kwapata, K., R. Sabzikar, M.B. Sticklen and J.D. Kelly (2010).** *In vitro* regeneration and morphogenesis studies in common bean. Plant Cell Tiss. Org. Cul. 100: 97-105.

**Kwapata, K., T. Nguyen and M. Sticklen (2012).** Genetic transformation of common bean (*Phaseolus vulgaris* L.) with the *Gus* color marker, the *Bar* herbicide resistance, and the barley (*Hordeum vulgare*) *HVA1* drought tolerance genes. Int. J. Agri. 2012: 1-8.

**Lal, S., V. Gulyani and P. Khurana (2008).** Overexpression of *HVA1* gene from barley generates tolerance to salinity and water stress in transgenic mulberry (*Morus indica*). Trans. Res. 17(4): 651-663.

**Liu, Z.C., B.-J. Park, A. Kanno and T. Kameya (2005).** The novel use of a combination of sonication and vacuum infiltration in *Agrobacterium*-mediated transformation of kidney bean (*Phaseolus vulgaris* L.) with *lea* gene. Mol. Breed. 16(3): 189-197.

**Mahamune, S.E., R.P. Bansode, S.M. Sangle, V.A. Waghmare, N.B. Pandhure and V.S. Kothekar (2011).** Callus induction from various explants of French bean (*Phaseolus vulgaris* L.). J. Exp. Sci. 2(4): 15-16.

**McCabe, D. and P. Christou (1993).** Direct DNA transfer using electric discharge particle acceleration (ACCELL$^{TM}$ technology). Plant Cell Tiss. Org. Cul. 33: 227-236.

**Metry, E.A., R.M. Ismail, G.M. Hussien, T.M. Nasr El-Din and H.A. El-Itriby (2007).** Regeneration and microprojectile–mediated transformation in *Vicia faba* L.. Arab J. Biotech. 10(1): 23-36.

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

**Oard, J. (1993).** Development of an airgun device for particle bombardment. Plant Cell Tiss. Org. Cul. 33: 247-250.

**□ktem, H.A., M. Mahmoudian, F. Eyidooan and M. Yücel (1999).** GUS gene delivery and expression in lentil cotyledonary nodes using particle bombardment. Lens News. 26(1 and 2): 3-6.

**Pharr, D.M., J.M.H. Stoop, J.D. Williamson, M.E. Studer Feusi, M.O. Massel and M.A. Conkling (1995).** The dual role of mannitol as osmoprotectant and photoassimilate in celery. HortSci. 30(6): 1182-1188.

**Rech, E.L., G.R. Vianna and F.J.L. Aragão (2008).** High-efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. Nat. Prot. 3(3): 410-418.

**Russell, D.R., K.M. Wallace, J.H. Bathe, B.J. Martinell and D.E. McCabe (1993).** Stable transformation of *Phaseolus vulgaris via* electric-discharge mediated particle acceleration. Plant Cell Rep. 12: 165-169.

**Santalla, M., J.B. Power and M.R. Davey (1998).** Efficient *in vitro* shoot regeneration responses of *Phaseolus vulgaris* and *P. coccineus*. Euphytica 102(2): 195-202.

**Sautter, C., H. Waldner, G. Neuhaus-Url, A. Galli, G. Neuhaus and I. Potrykus (1991).** Micro-targeting: high efficiency gene transfer using a novel approach for the acceleration of micro-projectiles. Bio/Tech. 9: 1080-1085.

**Sautter, C. (1993).** Development of a microtargeting device for particle bombardment of plant meristems. Plant Cell Tiss. Org. Cul. 33: 251-257.

**Tarczynski, M.C., R.G. Jensen and H.J. Bohnert (1992).** Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Nat. Acad. Sci. USA 89: 2600-2604.

**Tarczynski, M.C., R.G. Jensen and H.J. Bohnert (1993).** Stress protection of transgenic tobacco by production of the osmolyte mannitol. Sci. 259: 508-510.

**Vain, P., N. Keen, J. Murillo, C. Rathus, C. Nemes and J.J. Finer (1993).** Development of the particle inflow gun. Plant Cell Tiss. Org. Cul. 33: 237-246.

**Vianna, G.R., M.M.C. Albino, B.B.A. Dias, L.D.M. Silva, E.L. Rech and F.J.L. Aragão (2004).** Fragment DNA as vector for genetic transformation of bean (*Phaseolus vulgaris* L.). Sci. Hor. 99(3-4): 371-378.

**Zhu, J.K. (2002).** Salt and drought stress signal transduction in plants. Ann. Rev. Plant Bio. 53: 247-273.

# النقل الوراثي باستخدام جهاز الدفع المباشر لإطلاق الجينات والاستيلاد لصنفين من الفاصوليا

**عيسى احمد عيسى**

قسم الوراثة – كلية الزراعة – جامعة الفيوم – الفيوم – مصر

الهدف من الدراسة هو استخدام تكنيك زراعة الأنسجة والهندسة الوراثية لتحسين صفة تحمل الجفاف التي تعتبر من الصفات الاقتصادية الهامة في إنتاجية محصول الفاصوليا المستعصي للنقل الوراثي والاستيلاد وذلك بإدخال جين نيومايسين فوسفوترانسفيريز *II* (*nptII*) كجين انتخابي وجين مانيتول-١- فوسفات ديهيدروجينيز (*mtlD*) كجين هام اقتصادي لتحمل الجفاف بواسطة جهاز الدفع المباشر لإطلاق الجينات (جينبوستر) في النسيج القادر على الاستيلاد واستحداث نموات خضرية. وهذا التكنيك يشتمل على استخدام جزيئات صغيرة لحمل ال DNA بقوة دفع عالية لكي ينفذ من الجدار الخلوي والغشاء البلازمي ويصل إلى داخل الخلايا النباتية. ولكي يتم التحول الوراثي في الصنفين المستخدمين من الفاصوليا في هذه التجربة وهما "فونكس" و"ماكسيدور" كان لابد بداية من التوصل للاستيلاد بطريقة من طرق زراعة الأنسجة والتي تكون مناسبة لجهاز الدفع المباشر. وقد بينت النتائج أن البيئة المغذية MS المضاف إليها ١ مليجرام/لتر BA وأيضا 0.1 مليجرام/لتر NAA كانت مناسبة جدا للاستيلاد وتكوين نموات خضرية من المنطقة المرستيمية في الجزء النباتي المقطوع من البادرة والمحتوى على أجزاء صغيرة من الفلقتين في كلا من الصنفين. ولإتمام التحول الوراثي ونقل الجينات في كلا من الصنفين محل الدراسة فقد تم إجراء الانتخاب لإنتاج نباتات محولة وراثيا بجين تحمل اجهاد الجفاف. وقد دلت النتائج على أن نجاح إدخال ال DNA في الأنسجة المرستيمية باستعمال جهاز الدفع المباشر لنقل الجينات باستخدام البلازميد pFF19K الذي يحتوى على الجين الانتخابي *nptII* و البلازميد pFF19-mtlD الذي يحتوى على الجين الهام اقتصاديا *mtlD* - قد كان تحت ضغط نيتروجين ٢٠ بار ومسافة إطلاق ١٣٥ مليمتر وجزيئات تنجستن قطرها ١.١ كحامل للمادة الوراثية وبإطلاق الدقائق مرتين. و تم إنتاج نباتات محولة وراثيا بمعدل ٦.٧% و ٨.٤% في الصنفين "فونكس" و"ماكسيدور" على التوالي. و تم انتخاب النباتات المحولة وراثيا بإضافة تركيزات عالية من كلا من الكانميسين (١٠٠ مليجرام/لتر) والمانيتول (١.٢ مول/لتر) في البيئة الانتخابية أعلى من التركيزات التي لا تتحملها النباتات غير المحولة وراثيا (الكنترول) وذلك للتأكد من دخول كلا من جين *nptII* وجين *mtlD* في جينوم كلا من الصنفين المستخدمين من الفاصوليا. كما أن دخول هذه الجينات في جينوم كلا من الصنفين قد اظهر مقاومة وتحمل عاليين في النباتات المحولة وراثيا في الصنفين في البيئات الانتخابية المضاف إليها الكانميسين والمانيتول. وعلى حد معلومات المؤلف يعتبر هذا البحث هو الأول لنقل جين التحمل للجفاف *mtlD* للفاصوليا واستيلاد نموات خضرية ونباتات قادرة على تحمل الظروف البيئية المعاكسة باستخدام جهاز الدفع المباشر لإطلاق الجينات.