



# GENETICS AND CYTOLOGY

INTERNATIONAL JOURNAL DEVOTED TO GENETICAL  
AND CYTOLOGICAL SCIENCES

Published by  
THE EGYPTIAN SOCIETY OF GENETICS

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Volume 42

January 2013

No. 1

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GENETIC TRANSFORMATION OF COMMON BEANS (*Phaseolus vulgaris* L.) CALLI BY BIOLISTIC GUN AND VIA *Agrobacterium tumefaciens*

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Common bean (*Phaseolus vulgaris* L.) is a vegetable crop and the most cultivated species of the genus *Phaseolus*. Common beans are the major source of protein and important food in the developing world (Gatica Arias *et al.*, 2010). The insertion of genes encoding useful traits such as drought stress tolerance and herbicide resistance into beans is potentially of great agricultural importance (Kwapata *et al.*, 2012). Transformation requires the insertion of DNA into plant cells, incorporation into chromosomes and expression in cells that could be regenerate into transgenic plants. However, despite considerable interest there are only few publications describing successful transformation of common beans. The main hindrance for transformation is that common

bean is recalcitrance in this respect, and success has been very limited (Kelly, 2010). Researchers in Brazil, used particle bombardment protocol to insert *bar* gene for herbicide resistance and virus resistance (RNAi) into the pinto bean *V. olathe* (Aragão and Faria, 2009). Researchers in Japan inserted the *lea* gene for drought tolerance in kidney beans using sonication and vacuum infiltration (Liu *et al.*, 2005).

A great attention is paid for the development of common beans which are resistant to stress. Although attempts have long been made on *in vitro* culture, organogenesis, embryogenesis and regeneration of beans (Mok *et al.*, 1986; Mohamed *et al.*, 1993; Gémesné Juhász *et al.*, 1995;

Eissa *et al.*, 1999; Gatica Arias *et al.*, 2010). The genetic transformation of common bean has been difficult till now. Somatic embryos of *Vicia faba* L. were enhanced on MS medium supplemented with 2,4-dichlorophenoxy acetic acid (2,4-D) (Hamdy and Hattori, 2006). *Mucuna pruriens* explants cultured on MS medium supplemented with 2,4-D produced embryogenic callus (Harini and Sathyanarayana, 2009). Mongomaké *et al.* (2009) developed regeneration system from non meristematic explants in *Fabaceae*. Direct regeneration ability from different explants of *Cicer arietinum* L. (Naz *et al.*, 2007) reported the response of nodal explant was better than shoot apex.

The use of genetic engineering is viewed as a logical approach to improve legume crops (Morginski and Kartha, 1985; Hildebrandt *et al.*, 1986). Common bean has been transformed using the biolistic DNA delivery system (Aragáo *et al.*, 1996; Eissa *et al.*, 2001; Kwapata *et al.*, 2012). Genes were also introduced into beans using *Agrobacterium* system (McClellan *et al.*, 1991). However, research to make the *Agrobacterium*-based transformation method amendable to common beans has continued as the system is perceived to possess several advantages over other forms of transformation including: the ability to transfer large segments of DNA with minimal rearrangement; the precise insertion of transgenes resulting in fewer copies of inserted genes; and it is a simple technology with lower cost. Moreover, it allows for the stable integration of

a defined segment of DNA into the plant genome and generally results in an improved stability of expression over generations than the direct DNA delivery methods. The members of genus *Phaseolus* have been shown to be susceptible to *Agrobacterium* and transgenic calli have been reported by (Allavena, 1985; McClellan *et al.*, 1988). Chickpea kanamycin resistance via *Agrobacterium* system was obtained (Ignacimuthu and Prakash, 2006). Transformed calli were obtained using *Vigna unguiculata* leaf disc cocultivated with *Agrobacterium* (Garcia *et al.*, 1986). Transient GUS expression in the lentil embryo transformed by *Agrobacterium* was reported by Sarker *et al.* (2003) and Hassan *et al.* (2007). An efficient *Agrobacterium*-mediated transformation of banana was also suggested by Subramanyam *et al.* (2011).

Although common beans transformation utilizing microparticle bombardment technology and *Agrobacterium*-based techniques have been reported, there is no convincing report on an efficient transformation method for this important crop. For that purpose transformation of two commercialized bean varieties with economically important genes were planned, and common beans calli transformation were started. The objectives in the present investigation were: (1) produce transgenic beans calli with the neomycin phosphotransferase II (*npt II*) and mannitol-1-phosphate dehydrogenase (*mtlD*) genes by biolistic gun method, and (2) produce stable transgenic beans calli via *A. tumefaciens* method containing

phosphinothricin acetyl transferase (*bar*) and  $\beta$ -glucuronidase (*gusA*) genes.

## MATERIALS AND METHODS

### *Plant material and sterilization method*

In these studies, two commercial common bean varieties Fönix and Maxidor (*Phaseolus vulgaris*) were used as source of explants. The seeds are kindly supplied by the Genetics and Horticultural Plant Breeding Department, Faculty of Horticultural Sciences, Corvinus University, Budapest, Hungary, to whom my thanks are due. Seeds were surface-sterilized by soaking in 95% ethanol for two min, and then in 15% solution of commercial bleaches containing (5.25% sodium hypochlorite) for 15 min, followed by four times rinses with sterile double-distilled water. Sterilized seeds were then germinated on MS basal salts medium (Murashige and Skoog, 1962) pH 5.7 without plant growth regulators for 7-10 days before being used for callus induction and transformation experiments.

### *Agrobacterium strain and binary vector*

Disarmed *Agrobacterium tumefaciens* strain A281 [kindly provided by Agricultural Biotechnology Center (ABC), Gödöllő, Hungary] was used in the present work for indirect transformation method. The strain contained the standard binary vector system pRGG *bar* H1, contains a *bar* selectable marker gene (phosphinothricin acetyltransferase gene) conferring the herbicide resistance and

*gusA* reporter gene ( $\beta$ -glucuronidase GUS gene). This study was carried out at the Tissue Culture Laboratory and Genetic Modified Organisms Laboratory, Genetics and Horticultural Plant Breeding Department, Faculty of Horticultural Sciences, Corvinus University, Budapest, Hungary.

### *Tissue culture medium and conditions*

For callus induction, hypocotyls segments from 7-10 days old aseptically grown seedlings of common beans were dissected and incubated on B5 medium (Gamborg *et al.*, 1968) supplemented with 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup> 2,4-dichlorophenoxy acetic acid (2,4-D) as the auxin source. The pH of the medium was adjusted to 5.7. Medium contained 30 g l<sup>-1</sup> sucrose and solidified with 7 g l<sup>-1</sup> agar, autoclaved at 120°C for 20 min. Cultures were kept at 24-26°C under cool white fluorescent lights with a 16 h photoperiod. The growing friable calli were transferred to fresh medium after 15 days, and sub-cultured every three to four weeks for calli developing and maintenance. The experiment was repeated three times, and the procedure and conditions of callus induction were followed as described by Eissa *et al.* (2001).

### *Transformation methods*

In order to optimize and develop a reproducible genetic transformation protocols of common beans (*P. vulgaris* L.), two transformation methods have been used, namely direct microparticle bom-

bardment (biolistic gun) and indirect (*Agrobacterium tumefaciens*)-based technique. Both biolistics and *A. tumefaciens* as gene delivery systems have made extensive use of both *npt II* and *gusA* genes as markers to monitor transformation.

### ***Sensitivity of common beans calli to kanamycin, mannitol and phosphinotricin***

Fönix and Maxidor control calli were tested for kanamycin sulfate, osmosis protector drought stress mannitol, and the herbicide phosphinotricin (PPT) sensitivities using three replicates per each treatment. Calli were tested on B5 medium containing (0, 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 mg l<sup>-1</sup>) kanamycin; (0, 0.2, 0.4, 0.6, 0.8, 1.0, 1.2, 1.4, 1.6, 1.8 and 2 mol l<sup>-1</sup>) mannitol; and (0, 0.5, 1, 2, 3, 4, and 5 mg l<sup>-1</sup>) phosphinotricin. Each experiment was repeated three times independently and calli were weighed by mg after 57 days from culture initial.

### ***Growth of Agrobacterium strain and effect of augmentin***

*Agrobacterium* strain A281, harboring the helper plasmid pRGG *bar* H1 was grown in Luria Bertani (LB) agar medium with 50 mg l<sup>-1</sup> kanamycin at 28°C overnight. The plasmid contains phosphinotricin acetyl transferase (*bar*) gene as selectable marker and  $\beta$ -glucuronidase (*gusA*) gene as reporter. To determine the optimum augmentin concentration that eliminates the *Agrobacterium* after co-cultivation, the strain was grown on medium containing (0, 300, 400,

500, 600, 700, 800, 900 and 1000 mg l<sup>-1</sup>) augmentin. The experiment was repeated three times to define the concentration of augmentin that was effective to eliminate and prevent the over growth of *Agrobacterium*.

### ***Plasmids DNA and gene shooting***

Two plasmids were mixed in 1:1 ratio and used together for common beans calli co-transformation. Plasmid pFF19K contains neomycin phosphotransferase II (*npt II*) gene as selectable marker conferring the kanamycin resistance and plasmid pFF19 *mtlD* contains mannitol-1-phosphate dehydrogenase (*mtlD*) gene as drought stress mannitol tolerance. The two plasmids (kindly provided by the ABC, Gödöllő, Hungary) were used in the present work. The standard physical factors were used for gene shooting, as it was described by Jenes *et al.* (1996 and 1997) i.e., the appropriate pressure of N<sub>2</sub> gas, the shooting distance and the size of the tungsten particles used as microprojectiles. Tungsten particles 0.7-1.6  $\mu$ m diameter on average were used as microprojectiles to bind the actual DNA molecules on their surface. Plasmids DNA binding on the surface of tungsten particles were carried out with the Ca(NO<sub>3</sub>)<sub>2</sub> precipitation of DNA.

### ***Calli proliferation and selection***

Kanamycin useful selective agent, indicating *npt II* gene. Shot parts of common beans calli cells were removed from the original calli bombarded with the two plasmids' DNA three days after gene

shooting and cultured on a selective medium containing only 50 mg l<sup>-1</sup> kanamycin for calli proliferation for one month. Kanamycin were sterilized by filtration through disposable filter and incorporated into pre-cooled autoclaved medium. Selection of resistant calli was carried out by transferring calli to three selective media containing one marker selection kanamycin or mannitol, and two markers selection (kanamycin and mannitol).

#### ***Co-cultivation, calli proliferation and genetic selection***

*Agrobacterium* strain was grown overnight in LB medium and freshly streaked bacteria were suspended in sterile distilled water. The fresh calli were infected by 1-2 ml of *Agrobacterium* suspension and co-cultivated for three days at 24-26°C under light on B5 medium (pH 5). Addition of 300-375 mg l<sup>-1</sup> augmentin to B5 medium controlled *Agrobacterium* growth. The calli were transferred two times at three days intervals to fresh B5 medium containing 300-375 mg l<sup>-1</sup> augmentin (pH 5.7) for three days. Secondary calli cells (E<sub>1</sub> generation) were removed from the original inoculated calli (E<sub>0</sub>) at three months after inoculation. To screen for transformants, calli (E<sub>2-10</sub> generation) of each sub-clone were transferred to the B5 medium containing 2-5 mg l<sup>-1</sup> phosphinothricin, the concentration was then gradually increased over an eight months period to 5 mg l<sup>-1</sup>, and three to four weeks transferred schedule.

#### ***Transformation tests***

##### ***Kanamycin and mannitol selection tests***

For transformation tests, the bombarded common beans calli were first placed on selective medium containing only 50 mg l<sup>-1</sup> kanamycin. Selective tests were made by transferring resistant healthy green white calli into three selective media containing one marker selection 50 mg l<sup>-1</sup> kanamycin, or 0.8 mol l<sup>-1</sup> mannitol, and two markers selection 50 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol for calli selections for three months, sub-cultured every three to four weeks. To screen for transformed cells, calli were cultured on selective media containing 60, 70, 100 and 150 mg l<sup>-1</sup> kanamycin, 0.8 mol l<sup>-1</sup> mannitol, and 70 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol for three months, and later on selective medium containing 150 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol. The selective tests were repeated four or more times using two markers 150 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol for four months. It was necessary to keep the calli cells in direct contact with selective medium to develop transformed calli cells.

##### ***Phosphinothricin selection test***

Calli derived from 3 mg l<sup>-1</sup> phosphinothricin selective medium were cultured on B5 medium containing 4 mg l<sup>-1</sup> phosphinothricin and later on medium containing 5 mg l<sup>-1</sup> phosphinothricin to test their ability to form an actively growing calli in the presence of high concentration of phosphinothricin. It was necessary to keep calli in direct contact with selective

medium to develop transformed common beans calli cells.

### ***Assay of GUS reporter activity***

Histochemical staining of GUS activity arising from *gusA* gene expression in transformed common beans calli was assayed using a histochemical assay as described by Jefferson (1987). Histochemical GUS assay was used to assess the transient expression of the *gusA* (GUS) gene in common beans calli after selection test. Calli grown on selective medium containing 3 mg l<sup>-1</sup> phosphinothricin were checked and determined for the presence of GUS reporter activity by staining a small portion of calli cells incubated at 37°C over night in a thermostat with GUS buffer. GUS buffer containing 1 mM X-GLUC (5-Bromo-4-chloro-3-indolyl β-D-glucuronide), 100 mM sodium phosphate buffer pH 7.0, 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide and 0.1% (v/v) Triton X-100. X-GLUC is the best substrate for histochemical localization of the β-glucuronidase activity in the cells and tissues this substrate produces a blue precipitate at the site of enzyme activity. Blue spots were detected with the aid of a stereomicroscope. No GUS activity was detected in control calli.

## **RESULTS AND DISCUSSION**

### ***Callus induction and maintenance***

Calli were induced from hypocotyl segment cultured on B5 medium supplemented with 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup>

2,4-D within one week in bean varieties Fönix and Maxidor. Pieces of the most friable calli were transferred to B5 medium in order to establish a calli culture. B5 medium containing 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup> 2,4-D produced much calli proliferation from hypocotyl segment, and callus should be sub-cultured every three to four weeks.

Callus formation was observed in immature cotyledons of bean cultured on medium supplemented with 0.4 mg l<sup>-1</sup> 2,4-D and 1 mg l<sup>-1</sup> BAP (Giovinazzo *et al.*, 1993). Similar callus induction systems were reported in beans by Mohamed *et al.* (1993). Callus was induced from bean hypocotyl (Velich *et al.*, 1994). B5 medium supplemented with 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup> 2,4-D proved to be optimal in callus induction from hypocotyl and epicotyl in *P. vulgaris* L. (Gémesné Juhász *et al.*, 1995). Primary leaf explants from 7 days old seedlings of horsegram produced calluses on 2,4-D containing medium (Mohamed *et al.*, 2004).

The results obtained in the present study indicated that the B5 medium supplemented with 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup> 2,4-D induced much and good calluses, and are in agreement with previous studies. The author can recommend getting a rapidly growing calli which is a good starting material for introducing foreign DNA into common bean cells (Fig. 1).

### ***Effect of kanamycin sulfate and drought stress mannitol***

Since selection test is one of the major steps of any transformation experi-

ments the present results suggest that selection tests can be used for the development of transgenic common beans calli. The kanamycin and mannitol sensitivities of calli growth were studied in the two bean varieties to investigate the suitability of kanamycin resistance ( $Km^r$ ) and mannitol tolerance ( $Man^t$ ) as selectable markers for calli transformations. The results indicated that 50 mg l<sup>-1</sup> kanamycin, and 0.8 mol l<sup>-1</sup> mannitol caused almost total inhibition of control calli growth and were sufficient to kill the non transgenic control calli cells. Calli cells were dehydrated because of high osmotic stress treatment. After repeating each experiment three times with three replicates per each treatment, the results showed that 50 mg l<sup>-1</sup> kanamycin sulfate, and 0.8 mol l<sup>-1</sup> mannitol concentrations were effective to select transformed common beans calli and to kill the control and enough to differentiate the transgenic from non transgenic calli (Figs 2 and 3). The side of calli sample faced with the medium was damaged extremely and necrosis was observed. The use of higher concentration to differentiate the transgenic calli with *npt II* gene from non transgenic ones was recommended.

### **Transformation and selection**

Mannitol is the main chemical preferred as PEG 6000 for the *in vitro* osmotic stress treatments compared to other osmolites such as, NaCl and sugars. The toxic levels of kanamycin and mannitol to control calli tissue were determined at first. For genetic transformations the standard physical factors described by

Jenes *et al.* (1996 and 1997) and used by Eissa *et al.* (2001) were used. Recalcitrant common beans were started to improve and introduce economically important genes such as *mtlD* drought tolerance gene. When *mtlD* gene is activate in transgenic beans, the mannitol content of the cytoplasm is higher than in normal cells and these tissues can better tolerate dehydration stress. The bombarded common beans calli were incubated for three days without selection to allow expression of the resistance genes. After selection on one marker selection kanamycin containing media 50, 60, 70, 100 and 150 mg l<sup>-1</sup>, several kanamycin resistance calli had been obtained, where they survived and grew, while control calli turned brown and died (Fig. 4). After selection on one marker selection 0.8 mol l<sup>-1</sup> mannitol containing medium, mannitol tolerance calli had been obtained. After double selection on 150 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol containing medium, kanamycin resistance and mannitol tolerance beans calli had been obtained, while control calli were not able to grow and died (Fig. 5). The growth of putative transgenic calli was rapid, doubled in size during two weeks and appeared white and green friable after double selection. Resistance of the double selected common beans calli were verified and confirmed by their ability to grow on selective medium containing two markers repeatedly.

These results showed that kanamycin is an effective selection marker for common beans. Stable kanamycin re-

sistance and mannitol tolerance transgenic calli lines were obtained after co-transformation with genes encoding the enzymes neomycin phosphotransferase and mannitol-1-phosphate dehydrogenase. The presence of 50 mg l<sup>-1</sup> kanamycin inhibits callus growth and causes a rapid browning of the bean tissues (Mariotti *et al.*, 1989). For selection kanamycin resistance of soybean Christou *et al.* (1988) used 50 and 100 mg l<sup>-1</sup> kanamycin. Selection resistant bean calli could be distinguished by adding 75 mg l<sup>-1</sup> kanamycin to the medium (Eapen *et al.*, 1987). Transformed callus was further cultured in the presence of 100 mg l<sup>-1</sup> kanamycin. After direct gene transfer Köhler *et al.* (1987) detected kanamycin resistance calli of moth bean on a medium with 75 mg l<sup>-1</sup> kanamycin. Similar results are reported by Genga *et al.* (1990), i.e., kanamycin resistance bean calli was obtained after selection on 100 mg l<sup>-1</sup> kanamycin containing medium. Kanamycin resistance calli were maintained by successive subcultures on medium with kanamycin, to verify callus transformation. Transformed bean callus was capable of growing in the presence of 500 µg ml<sup>-1</sup> kanamycin whereas control ceased growing at 200 µg ml<sup>-1</sup> (McClellan *et al.*, 1991). Callus production of transformed dry bean explants continued on the MS medium while control did not produce callus at 20 mg l<sup>-1</sup> kanamycin. The kanamycin was increased to 50 mg l<sup>-1</sup> over a two month period (McClellan *et al.*,

1988). 50 mg l<sup>-1</sup> kanamycin was chosen by Franklin *et al.* (1993) for selecting transformed callus from hypocotyls explant of *P. vulgaris*. The callus proliferated rapidly on a kanamycin selection medium. Co-cultivated bud explant of *P. acutifolius* selected on a medium with 300 mg l<sup>-1</sup> of kanamycin as a selective agent (Dillen *et al.*, 1997) while that concentration completely inhibited callus formation on non transformed leaf discs explant. Common bean varieties were genetically transformed *via* the Biolistic bombardment of the apical shoot meristem primordium. Transgenes included *gus* color marker, the *bar* herbicide resistance selectable marker used for *in vitro* selection of transgenic cultures, and the barley (*Hordeum vulgare*) late embryogenesis abundant protein (*HVA1*) which conferred drought tolerance (Kwapata *et al.*, 2012). According to the present results, it is possible to introduce osmosis protective genes into common beans calli by a special biolistic gun mediated DNA transfer.

### ***Effect of phosphinothricin and augmentin***

The phosphinothricin sensitivity of calli growth in two commercial bean varieties was studied to investigate the suitability of phosphinothricin resistance (PPT<sup>r</sup>) as a selectable marker for calli transformation. 0.5 mg l<sup>-1</sup> phosphinothricin caused almost total inhibition of the growing of calli, which changed brown and died. After repeating the experiment three



times with three replicates, the results showed that 0.5 mg l<sup>-1</sup> phosphinothricin concentration was effective to kill the control callus and it was enough to differentiate the transgenic calli with *bar* gene from the non transgenic ones (Fig. 6). *Agrobacterium* strain 281 was grown on LB agar medium with 300, 400, 500, 600, 700, 800, 900 and 1000 mg l<sup>-1</sup> augmentin under normal conditions. This experiment was repeated two times to define the minimum concentration that was effective to kill and eliminate the *Agrobacterium*. The results showed that 300 mg l<sup>-1</sup> augmentin was enough to kill and eliminate the *Agrobacterium*, so higher concentration 375 mg l<sup>-1</sup> was used to prevent over growth and to kill the bacteria.

### ***Transformation and selection***

A great attention is paid for the development of common beans which are resistant to biotic or abiotic stresses. In the present study, development of stresses resistant transgenic calli was obtained by microprojectile gun and *Agrobacterium* systems. The *bar* gene encodes the enzyme phosphinothricin acetyl transferase, which catalyses and detoxify the phosphinothricin and yielding an inactive product, N acetyl phosphinothricin (De Block *et al.*, 1987). PPT (phosphinothricin, active ingredient of the herbicide bialaphos) inhibits glutamine synthetase, an important enzyme during photosynthetic electron transport. This detrimental effect of the herbicide causes cell death due to the accumulation of ammonia in non transformed cells. The prob-

lem with this irreversible non selective effect of the herbicide is that the tissue is chimeric with transformed and non transformed cells. Ammonia tends to be diffused across the cell membrane from the non transformed cells into the neighbouring transformed cell, which leads to the low recovery rate of transformants. Calli were screened for *bar* gene expression by selecting for growth on high concentration 5 mg l<sup>-1</sup> phosphinothricin containing medium. At first most of the non transformed calli turned brown and died within several weeks on low concentration 0.5, 1 or 2 mg l<sup>-1</sup> phosphinothricin. However, several transformed calli remained white and healthy on very high concentration 5 mg l<sup>-1</sup> phosphinothricin.

Hanafy (2002) showed that 2 mg l<sup>-1</sup> phosphinothricin totally suppressed callus development from faba bean explants and all the explants died, while resistant callus started to proliferate from the explants inoculated with *Agrobacterium*. The present results are in agreement with that obtained by Toldi *et al.* (2000) in rice tolerant to the phosphinothricin selection, and by Iser *et al.* (1999) and Wu *et al.* (2003). They used 5 and 2-4 mg l<sup>-1</sup> phosphinothricin for selection in wheat and suggested that the late phosphinothricin selection combined with GUS expression proved effective to identify transgenic plants. Pauk *et al.* (2002) selected transformed wheat calli on medium with 10 mg l<sup>-1</sup> phosphinothricin. Wheat embryos transformed by *bar* gene germinated onto 5 mg l<sup>-1</sup> phosphinothricin medium expressed resistance, while control

showed sensitivity (Mihály *et al.*, 2002). Callus culture media containing 5 and 10 mg l<sup>-1</sup> phosphinothricin were used for selection of transgenic embryogenic cultures of bentgrass (Chai *et al.*, 2002). Differences were observed between the present results and the other researchers, may be due to different genotypes used in other plant families.

In the present study the phosphinothricin was added to the medium at 2, 3, 4 and 5 mg l<sup>-1</sup>, and their effects were tested on growth and color over a period of six months and three to four week transfer schedule to screen for transformant calli. At all concentrations of phosphinothricin, the transformed calli survived and grew while control turned brown and died (Fig. 7). Resistance of the selected calli was verified by their ability to grow on a selective medium. The initial experiments focused on the demonstration and optimization of transient expression of phosphinothricin resistance and *gusA* genes (*bar* and *gusA*) in callus cultures using the expression plasmid A281 pRGG. Using the optimal conditions established for transient gene expression, a series of subculture steps directed towards the recovery of stable transformants common beans calli were conducted.

#### **Confirmation of transformants (GUS reporter assay)**

Transformed common beans calli were assayed for GUS reporter activity to confirm the expression of the *gusA* gene using the histochemical assay test. The *gusA* gene correctly expressed in calli

cultures grown on 4 mg l<sup>-1</sup> phosphinothricin selective medium. Transgenic calli were grown on selective medium stained blue when stained with X *GLUC*, while control, non transformed were not able to grow in the presence of phosphinothricin, neither showed any positive reaction in the *in vitro* assays. Similar results were obtained by Mariotti *et al.* (1989); Genga *et al.* (1990); Becker *et al.* (1994) and Lewis and Bliss (1994) in *Phaseolus*, Hinchee *et al.* (1988) in soybean, and De Kathen and Jacobsen (1990) in *Pisum*. The GUS assay of *Beta vulgaris* explant co-cultivated with *Agrobacterium* reported by Molnár *et al.* (2002). DNA delivery in *Thlaspi* via *Agrobacterium* resulted in kanamycin resistance transgenic tissues proven by GUS assay (Safavi and Asgari, 2011). Selection for phosphinothricin resistance and assays for GUS reporter activity indicated the expression of *bar* gene as phosphinothricin selectable marker and *gusA* reporter gene in the transgenic common beans calli. Using the method described in the present experiment, rapidly growing calli cultures of transgenic common beans obtained to transform it for transient and stable gene expression by using *Agrobacterium* method. The results are considered to be significant steps in the development of commercially transgenic common bean plants.

#### **Concluding remarks**

In the present investigation, protocols for biolistic gun and *Agrobacterium*-mediated transformations in commercialized bean varieties are demonstrated. Both

have powerful techniques that permit transformations of the recalcitrant common beans. These transformation systems will be valuable tool for breeding and genetically engineer useful traits, e.g., drought stress and herbicide resistance. Selection tests and assay analysis would confirm the transgenic nature of common beans calli. Optimization of protocols for the development of transgenic beans calli with appropriate plasmids and vectors should enable the production of transgenics in common beans with improved economically treats like drought stress and herbicide resistance. These transformations systems could be useful for future studies on transferring these economically important genes into commercialized bean varieties plants.

### SUMMARY

Common bean is one of the most recalcitrant species for *in vitro* manipulation and transformation. In this study efficient genetic transformation methods were developed and performed in commercialized bean varieties calli by utilized microparticle bombardment (direct biolistic gun) and indirect *A. tumefaciens*-based technique. Hypocotyl segments cultured on Gamborgs medium supplemented with 1 mg l<sup>-1</sup> kinetin and 2 mg l<sup>-1</sup> 2,4-dichlorophenoxy acetic acid proved the best for callus induction and maintenance. Co-transformed calli were double selected on selective medium using 150 mg l<sup>-1</sup> kanamycin and 0.8 mol l<sup>-1</sup> mannitol. Transient genes expressions were obtained in calli cells and the results showed that the cells

shot by biolistic DNA delivery method can be transformed to get kanamycin resistant and mannitol tolerant. The presence and integration of the neomycin phosphotransferase II (*npt II*) and osmosis protector mannitol-1-phosphate dehydrogenase (*mtlD*) genes into *P. vulgaris* L. genome were confirmed and verified by double selection tests. Transgenic calli were selected after co-cultivated with *Agrobacterium* method on selective medium containing 5 mg l<sup>-1</sup> phosphinotricin (PPT). Transient genes expressions were obtained in the calli and the results showed that the common beans calli co-cultivated with *Agrobacterium* can be transformed to get phosphinotricin resistant and  $\beta$ -glucuronidase reporter positive tissues. The presence and integration of the phosphinotricin acetyl transferase and  $\beta$ -glucuronidase genes into *P. vulgaris* L. genome were confirmed and verified by marker selection and histochemical assay tests. These results suggest an efficient biolistic gun, and *Agrobacterium*-mediated transformation methods for stable integration of economically important genes into common beans calli, and that these transformations systems could be useful for future studies on transferring economically important genes into common bean plants.

### ACKNOWLEDGEMENT

The author wishes to thank Prof. István Velich, Department of Genetics and Horticultural Plant Breeding, Faculty of Horticultural Sciences, Corvinus University, Budapest, Hungary, for the laboratory

facilities. The Author is also grateful to Mrs. Gyurcsáné Millei Ágnes for skilful technical assistance at the same Department. Special thanks go to Prof. István Nagy, Agricultural Biotechnology Center (ABC), Gödöllő, Hungary, for providing the two plasmids and *Agrobacterium* strain.

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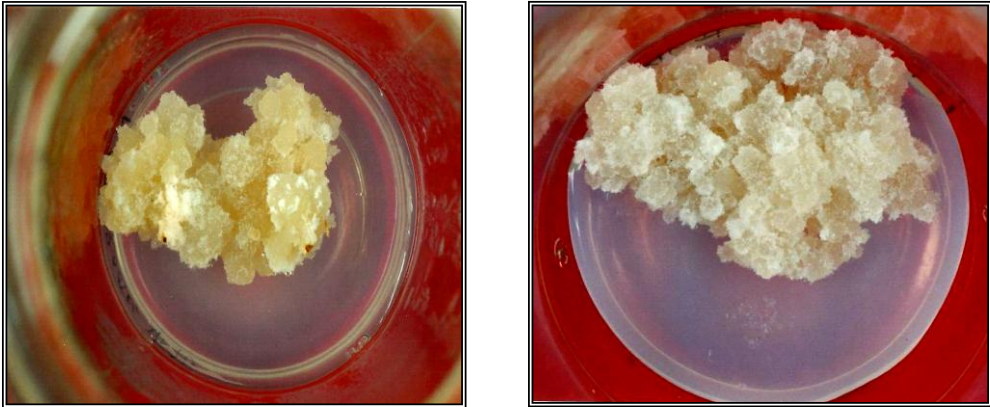


Fig. (1): Calli developing and maintenance from hypocotyls explant sub-cultured on Gamborgs medium containing  $1 \text{ mg l}^{-1}$  kinetin and  $2 \text{ mg l}^{-1}$  2,4-D, (A) Fönix calli and (B) Maxidor calli.

Fig. (2): Effect of different concentrations of kanamycin sulfate on growth of Fönix and Maxidor calli cultured on Gamborgs medium.

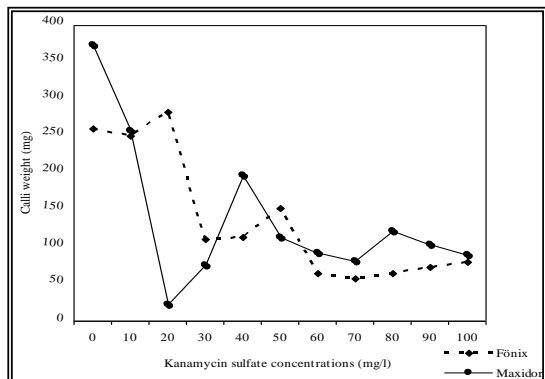
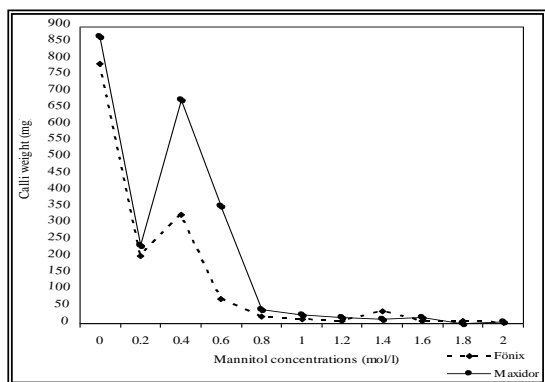


Fig. (3): Effect of different concentrations of drought stress mannitol on growth of Fönix and Maxidor calli cultured on Gamborgs medium.



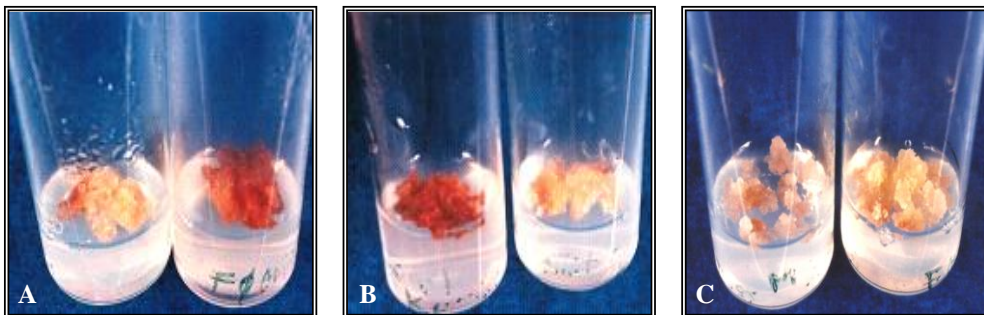


Fig. (4): Transformed calli by biolistic gun method survived and grew on one selective Gamborg's media containing 50 and 150 mg l<sup>-1</sup> kanamycin as selectable marker, (A) Fönix calli growing on selective medium containing 50 mg l<sup>-1</sup> kanamycin showing kanamycin resistance, while control turned brown and died, (B) Maxidor calli growing on selective medium containing 50 mg l<sup>-1</sup> kanamycin showing kanamycin resistance, while control turned brown and died, and (C) transgenic Fönix and Maxidor calli resistant, survived and grew on selective medium containing 150 mg l<sup>-1</sup> kanamycin.

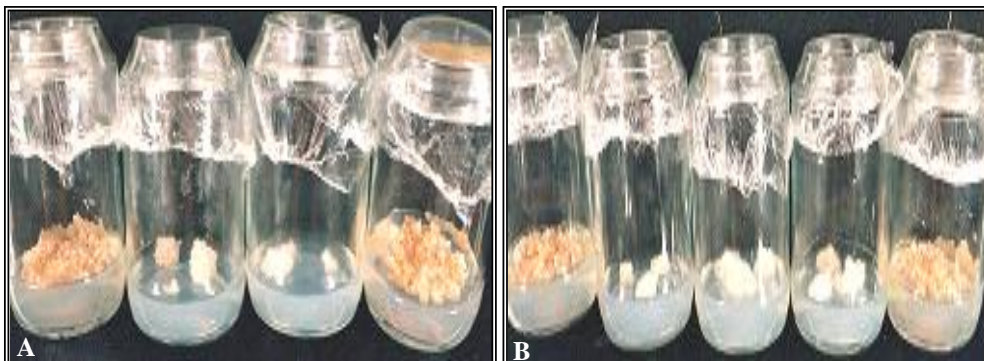


Fig. (5): Transformed calli by biolistic gun method survived and grew on one selective Gamborg's media containing drought stress mannitol and double selective (kanamycin + mannitol), (A) Fönix calli growing on one selective medium containing 0.8 mol l<sup>-1</sup> mannitol showing mannitol tolerance, while control turned brown and died, (B) Maxidor calli growing on one selective medium containing 0.8 mol l<sup>-1</sup> mannitol showing mannitol tolerance, while control turned brown and died, and (C) transgenic Fönix calli resistant, survived and grew on double selective medium containing (150 mg l<sup>-1</sup> kanamycin + 0.8 mol l<sup>-1</sup> mannitol) showing kanamycin resistance and mannitol tolerance, while control turned brown and died.

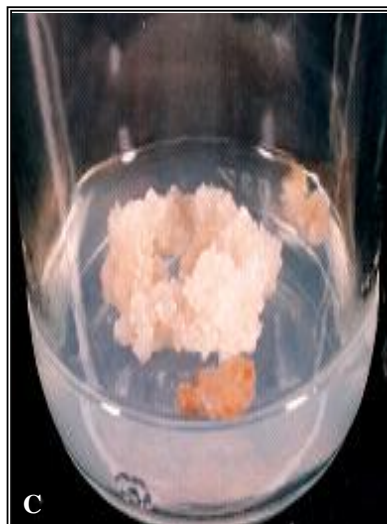


Fig. (6): Effect of different concentrations of herbicide phosphinothricin on growth of Fönix and Maxidor calli cultured on Gamborgs medium.

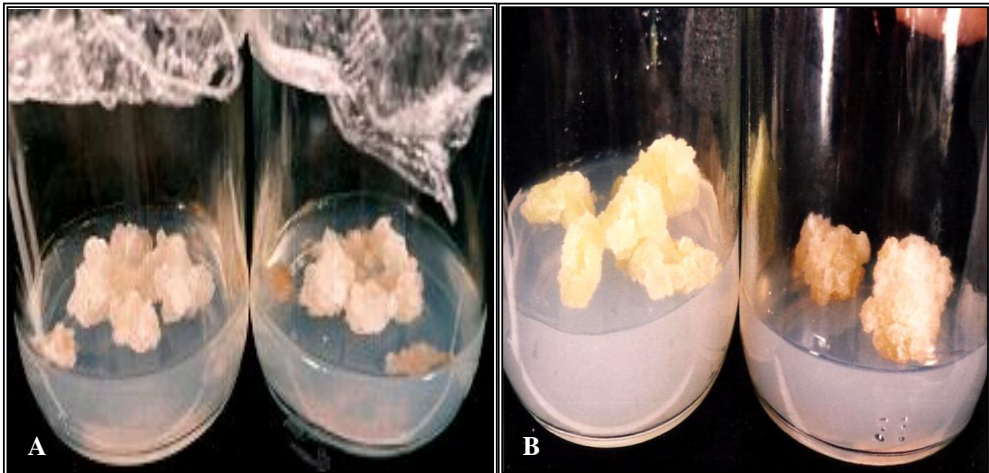
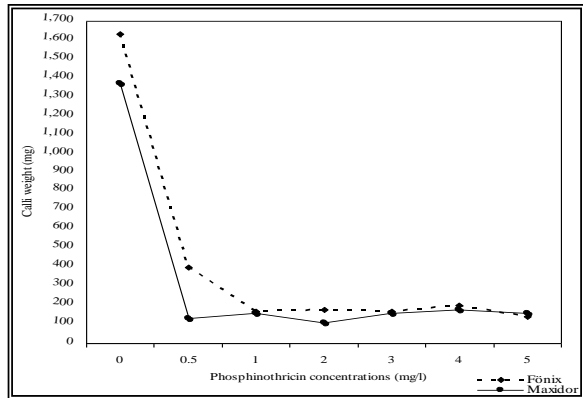


Fig. (7): Transformed calli by Agrobacterium based technique survived and grew on selective Gamborgs media containing 2 and 4 mg l<sup>-1</sup> herbicide, phosphinothricin (PPT), (A) Fönix calli showing herbicide resistance, growing on selective medium containing 2 mg l<sup>-1</sup> PPT while control turned brown and died, (B) Fönix and Maxidor calli growing on selective medium containing 4 mg l<sup>-1</sup> PPT and (C) stable expression of the gusA gene in transgenic Fönix calli stained blue, resistant, survived and grew on selective medium containing 4 mg l<sup>-1</sup> herbicide PPT using a stereomicroscope.

