

## AGROBACTERIUM-MEDIATED TRANSFORMATION OF COMMON BEAN

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### ABSTRACT

The common bean (*Phaseolus vulgaris* L.) is an important human dietary constituent being a rich source of protein. Genetically improved bean varieties are required as optimum yields are not realised due to constraints such as diseases and insect pests. The objective of this study was to evaluate the potential of two common bean varieties Mwitmania and Rose coco to *in vitro* *Agrobacterium tumefaciens*- mediated transformation. Mature seed embryos germinated for 1-2 days on moist filter paper, were stab inoculated with *A. tumefaciens* strains LBA 4404 (pBI 121), EHA 105 (pCAMBIA 1201) and EHA 105 (pCAMBIA 1301), harbouring  $\beta$ -glucuronidase (GUS) intron plasmids. The infected embryos were co-cultivated for 3-4 days on basal Murashige and Skoog, 1962 medium with B5 vitamins (MSB5) or medium supplemented with 10  $\mu$ M benzyl-aminopurine (BAP) and cultured on regeneration and selection medium consisting of 10  $\mu$ M BAP and 50 mg L<sup>-1</sup> kanamycin or hygromycin. Transformed shoots and roots confirmed by histochemical staining for GUS activity were obtained in 40 weeks old Mwitmania plantlets from explants infected with *A. tumefaciens* LBA 4404 (pBI 121). No GUS expression was observed in all Rose coco and Mwitmania shoots from explants infected with EHA 105 (pCAMBIA 1201) or EHA 105 (pCAMBIA 1301).

*Key Words:* *Agrobacterium tumefaciens*,  $\beta$ -glucuronidase, *Phaseolus vulgaris*

### RÉSUMÉ

Le haricot commun (*Phaseolus vulgaris* L.) est un important constituant du régime alimentaire humain riche en protéines. Des variétés génétiquement améliorées sont nécessaires, les rendements optimum n'étant pas réalisés suite aux contraintes comme maladies et pestes dues aux insectes. L'objectif de cette étude était d'évaluer le potentiel de deux variétés de haricot commun, Mwitmania et Rose coco en transformation médiée *in vitro* *Agrobacterium tumefaciens*. De grains embryonnaires matures en germination sous papier filter pendant 1-2 jours, étaient inoculés en *stab* avec des strains de LBA 4404 (pBI 121) *A. tumefaciens*, EHA 105 (pCAMBIA 1201) et EHA 105 (pCAMBIA 1301), harbouring  $\beta$ -glucuronidase (GUS) "intron plasmids". Les embryons infectés étaient co-cultivés pendant 3-4 jours sur la base de média Murashige and Skoog, 1962 avec des vitamines B5 (MSB5) ou média supplémenté avec 10mM benzyl-aminopurine (BAP) et cultivé sur le média sélectionné et régénéré composé de 10 mM BAP et 50 mg L<sup>-1</sup> kanamycin ou hygromycin. Les tiges et racines transformées, confirmées par la culture histochimique pour activité *GUS* étaient obtenues dans les plantules de Mwitmania âgées de 40 semaines issues des explants infectés avec *A. tumefaciens* LBA 4404 (pBI 121). Aucune expression *GUS* n'était observée dans toutes les tiges de Rose coco et Mwitmania shoots issues des explants infectés avec EHA 105 (pCAMBIA 1201) ou EHA 105 (pCAMBIA 1301).

*Mots Clés:* *Agrobacterium tumefaciens*,  $\beta$ -glucuronidase, *Phaseolus vulgaris*

## INTRODUCTION

The common bean (*Phaseolus vulgaris* L.) is the most important food legume worldwide and a leading source of low cost proteins (Arellano *et al.*, 2009). Despite having a potential yield of over 4000 kg ha<sup>-1</sup>, bean yields in West Asia and North America range from 1100 to 1500 kg ha<sup>-1</sup> while yield in Latin America and African countries range from 500 to 600 kg ha<sup>-1</sup> (Aragao and Rech, 2001). Some of the factors contributing to low yields are poor agronomic practices, diseases and insect pests, low soil fertility and lack of improved varieties (Aragao *et al.*, 2002). Besides the conventional breeding techniques, attempts have been made to obtain improved bean varieties through genetic transformation. Two methods are currently available for common bean transformation, indirect gene transfer using a disarmed gram negative soil bacterium such as *Agrobacterium*, or direct gene transfer techniques mainly particle bombardment or electroporation (Dillen *et al.*, 1995; Kim and Minamikawa, 1997; Aragao *et al.*, 2002; Rech *et al.*, 2008). Transformation can be monitored by use of the  $\beta$ -glucuronidase (GUS) gene of *Escherichia coli* (Jefferson, 1987). The GUS, intron gene allows expression in transformed plant tissues but not in bacterial cells and can be used for direct determination of the transformation rate as a measure of susceptibility. Histochemical GUS assays (Jefferson, 1987) and green fluorescent protein (GFP) techniques (Zambre *et al.*, 2005; Colpaert *et al.*, 2008) are available to detect specific cell type gene expression.

The common bean, like other legumes, is generally recalcitrant to *Agrobacterium*-mediated transformation due to poor regeneration in tissue culture (Svetleval *et al.*, 2003; Zambre *et al.*, 2005; Colpaert *et al.*, 2008; Arellano *et al.*, 2009). Most grain legumes transformed by *Agrobacterium*-based methods generally yield low rates of transformation, for instance, 0.1-5% (Atkins and Smith, 1997; Veltcheva *et al.*, 2005). Some leguminous species that have been transformed using *Agrobacterium* include soybean, *Glycine max.* Mer. (Chee *et al.*, 1989; Liu *et al.*, 2004; Paz *et al.*, 2005; Dang and Wei, 2007), pea nut, *Arachis hypogea* L. (Eapen and George, 1994), pea, *Pisum sativum* (Puonti-Kaelas *et al.*, 1990; Schroeder *et al.*,

1993) and cow pea, *Vigna unguiculata* L (Polpelka *et al.*, 2006). Bean explants that have been used in *Agrobacterium*-mediated transformation include cotyledonary nodes (McClellan *et al.*, 1991), protoplasts (Leon *et al.*, 1991), mature seed embryos (Aragao *et al.*, 1992), leaf discs and hypocotyls (Franklin *et al.*, 1993), seed meristems (Russell *et al.*, 1993), intact shoot tips of germinating seeds (Lewis and Bliss, 1994), shoot apex of embryogenic axis (Kim and Minamikawa, 1995), apical meristems (Brasileiro *et al.*, 1996), seedlings and shoot apices (Aragao and Rech, 1997), and embryo axis (Dillen *et al.*, 1995; 1997). Kanamycin was used in some cases as the selective agent and transformation was confirmed using GUS expression (Aragao *et al.*, 1993).

McClellan *et al.* (1991) obtained transformed callus and roots from cotyledonary nodes and hypocotyls of dry bean infected with *A. tumefaciens* strain C58Z707 and avirulent *A. rhizogenes* strain A4RS (pRiB278b), respectively. The transformed callus and roots, though capable of growing on MS medium with 5  $\mu$ M BAP and 50 mg L<sup>-1</sup> kanamycin could not regenerate plants. Franklin *et al.* (1993) produced a kanamycin resistant callus from leaf disc or hypocotyl explants of green bean infected with *A. tumefaciens* EHA 101 (pKYLX71GUS) using SH (Schenk and Hildebrandt, 1972) media with 10  $\mu$ M p-chlorophenoxy acetic acid (pCA), 2  $\mu$ M 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.5  $\mu$ M kinetin (KIN). The callus expressed  $\beta$ -glucuronidase (GUS) activity but no transgenic plants were obtained. Dillen *et al.* (1997) obtained GUS expression in regenerated bean shoots derived from seedling node explants infected with *A. tumefaciens* - A2760 (Hoekema *et al.*, 1983) and EHA 105 (Hood *et al.*, 1993) using an intron-containing GUS gene as a reporter system. However, no transgenic common bean plants were recovered. Zambre *et al.* (1998; 2005) using *Agrobacterium* obtained transgenic plants in Adzuki bean, *Phaseolus acutifolius* but not in *P. vulgaris*.

The axillary bud (Franklin *et al.*, 1993; Zhang *et al.*, 1997) and embryonic axis (Gatica-Arias *et al.*, 2010) tissue culture protocols for whole plant regeneration described in the common bean are yet to be adopted successfully for use in an

*Agrobacterium* based transformation system, hence the need to explore other methods. Lewis and Bliss (1994) obtained tumours and GUS expression when intact shoot tips of germinating *P. vulgaris* seeds were stab inoculated *in planta* with *A. tumefaciens* C58, but no transgenic plants were recovered. Such *in planta* techniques overcome the complications of regenerating plants from tissue-cultured material, as regeneration does not rely on shoot production from callus or from suspensions of cells or protoplasts. In soybean (*Glycine max*) Chee *et al.* (1989) produced transgenic plants, and seed-derived progeny *in planta* by infecting plumules, cotyledonary nodes and surrounding regions of axenic soy bean seedlings with *A. tumefaciens* carrying binary vectors. Colpaert *et al.* (2008) obtained transgenic roots expressing GUS and GFP fluorescence from de-rooted *P. vulgaris* seedlings infected *in vitro* with *Agrobacterium rhizogenes*.

Stable transformation has been realised in common bean using the direct gene delivery systems. Transgenic common bean plants have been obtained by particle bombardment of DNA into mature seed meristems (Russell *et al.*, 1993; Aragao *et al.*, 1996). Transgenic bean plants expressing resistance to bean golden mosaic geminivirus (Aragao *et al.*, 1998) and tolerance to the herbicides glufosinate ammonium and imazapyr (Aragao *et al.*, 2002; Rech *et al.*, 2008) have been developed through particle bombardment. The development of an efficient gene transfer system in the bean using *A. tumefaciens* would greatly benefit breeding programmes by introducing novel genes into the crop.

The objective of the study was to determine whether mature zygotic embryos of two common bean varieties grown in Kenya could be transformed *in vitro* by *Agrobacterium tumefaciens*.

## MATERIALS AND METHODS

**Plant material and explant preparations.** Mature seeds of two popular common bean varieties grown in Kenya, Rose coco (GLP-2) and Mwitmania (GLP-92), were surface sterilised in 70% (v/v) ethanol for 2 minutes and 1% (v/v)

sodium hypochlorite for 20 minutes. The sterilised seeds were rinsed four times with sterile water and germinated on moist sterile filter paper for 1 to 2 days or until the radicle emerged. The cotyledons of the germinating seeds were split open to expose the embryo, which was then detached. Half seed embryos were obtained by the removal of the root apex as described by Schroeder *et al.* (1993).

**Medium.** Murashige and Skoog (1962), medium supplemented with B5 vitamin (Gamborg *et al.*, 1968) was used in all the experiments. The medium abbreviated as MSB5, consisted of MS macro- and micro-salts plus 30 g L<sup>-1</sup> sucrose, 10 mg L<sup>-1</sup> thiamine HCl, 1 mg L<sup>-1</sup> pyridoxine HCl; 1 mg L<sup>-1</sup> nicotinic acid and 100 mg L<sup>-1</sup> myo-inositol. The pH was adjusted to 5.8 ± 0.1 using 1.0 M hydrochloric acid or sodium hydroxide and the medium solidified by adding 8 g L<sup>-1</sup> agar. The medium was sterilised by autoclaving at 1 kg cm<sup>-2</sup> and 121 °C for 20 minutes. Thereafter, the medium was dispensed into sterile bottles or petri dishes in a laminar flow cabinet.

**Preparation of *A. tumefaciens* cultures.** Solid and suspension cultures of *Agrobacterium tumefaciens* strains: LBA 4404 (pBI 121), EHA 105 (pCAMBIA 1201) and EHA 105 (pCAMBIA 1301), harbouring  $\beta$ -glucuronidase (GUS) intron plasmids were used. A colony of *A. tumefaciens* LBA 4404 (pBI 121) was streaked onto solidified Luria Bertani (LB) medium (Miller, 1972) containing 100 µgml<sup>-1</sup> rifampicin and 100 µgml<sup>-1</sup> kanamycin and incubated at 28 °C for 3 days. Liquid *A. tumefaciens* LBA 4404 (pBI 121) cultures were initiated by picking off a colony with a needle, and inoculating 125 ml LB medium containing 100 µgml<sup>-1</sup> rifampicin and 100 µg ml<sup>-1</sup> kanamycin in 250 ml conical flasks. Cell suspensions were grown overnight on a shaker at 28 °C and 180 revolutions per minute.

A colony of *A. tumefaciens*, EHA 105 (pCAMBIA 1201), was streaked onto solidified LB medium containing 100 µgml<sup>-1</sup> chloramphenicol and incubated at 28 °C for 2 - 3 days. Thereafter, liquid cultures were prepared by inoculating non-solidified LB medium containing 100 µgml<sup>-1</sup> chloramphenicol with a colony of *A. tumefaciens* EHA 105 (pCAMBIA 1201) and

incubating overnight on a shaker as already described. *A. tumefaciens* EHA 105 (pCAMBIA 1301) solid and liquid cultures were prepared in a similar manner except that 50 µgm<sup>-1</sup> kanamycin was used for bacterial selection instead of 100 µgm<sup>-1</sup> chloramphenicol. The negative control *A. tumefaciens* LBA 4404 and EHA 105 were grown on LB media without antibiotics.

***In vitro* infection of seed embryos.** Whole mature germinated embryos of the two bean genotypes were infected by injecting the embryonic axis using a 30-gauge needle attached to a 1-ml syringe containing liquid *Agrobacterium* culture as described by Lewis and Bliss (1994). Half seed embryos with the root apices removed, were inoculated by dipping in each bacterial suspension culture for 5 to 15 minutes and then dried using sterile tissue paper to remove excess bacteria. The infected explants were co-cultivated on MSB5 basal or medium supplemented with 10 µM BAP for three to four days. After co-cultivation, explants infected with *A. tumefaciens* LBA 4404 (pBI 121) were transferred onto MSB 5 medium supplemented with 10 µM BAP, 50 mg L<sup>-1</sup> kanamycin and 250 mg L<sup>-1</sup> cefotaxime for shoot growth and selection of transformed plants. Cefotaxime was used to kill excess bacteria. Those infected with *A. tumefaciens* EHA 105 (pCAMBIA 1201) and EHA 105 (pCAMBIA 1301) were transferred onto medium supplemented with 10 µM BAP, 50 mg L<sup>-1</sup> hygromycin and 250 mg L<sup>-1</sup> cefotaxime. Co-cultivated control explants infected with *A. tumefaciens* LBA 4404 and EHA 105 were transferred onto medium with 10 µM BAP and 250 mg L<sup>-1</sup> cefotaxime. All the cultures were incubated at 25 ± 2 °C, under a 16 / 8 hour dark light cycle and 3000 lux light intensity, respectively.

**β-Glucuronidase (GUS) assay.** Explants of the two bean genotypes at various stages of growth were tested for β-glucuronidase (GUS) activity using the histochemical staining method described by Jefferson *et al.* (1987). The first histochemical assay was done after 12 days using three explants removed at random from each replicate. The GUS assay was repeated after 22, 32 and 40 weeks.

## RESULTS

Mature whole seed embryo explants of *P. vulgaris* L var. Mwiternia infected with strain LBA 4404 (pBI 121) and co-cultivated on MSB5 basal medium exhibited slow growth when subcultured onto MSB5 medium supplemented with 10 µM BAP, 50 mg L<sup>-1</sup> kanamycin and 250 mg L<sup>-1</sup> cefotaxime (Table 1). Out of the 20 whole mature seed embryos infected, 17 explants (85%) died within three weeks and the remaining three (15%) died after 10 weeks. All the 10 half seed embryos also infected with strain LBA 4404 (pBI 121) died within three weeks. The control, whole and half mature seed embryo explants infected with strain LBA 4404 and cultured on medium with 10 µM BAP and 250 mg L<sup>-1</sup> cefotaxime, regenerated multiple buds after two to four weeks but these were confirmed to be negative for GUS activity when tested after three to 10 weeks.

Mature whole and half seed embryos of var. Mwiternia co-cultivated on MSB5 basal medium after infection with either *A. tumefaciens* strain EHA 105 (pCAMBIA) 1201 or EHA 105 (pCAMBIA 1301) died within six weeks of culture on to MSB5 medium with 10 µM BAP, 250 mg L<sup>-1</sup> cefotaxime and 50 mg L<sup>-1</sup> hygromycin (Table 1). The control explants infected with strain EHA 105 did not regenerate multiple buds, but gave rise to single shoot and root meristems, which were negative for GUS activity when tested after eight to ten weeks.

Table 2 shows data for whole and half mature seed embryos of *P. vulgaris* var. Rose coco, co-cultivated on MSB5 basal medium for three days after infection with *A. tumefaciens* LBA 4404 (pBI 121) and EHA 105 (pCAMBIA 1201). All the explants died within two to three weeks when cultured on MSB5 selection medium supplemented with 10 µM BAP, 50 mg L<sup>-1</sup> kanamycin or hygromycin and 250 mg L<sup>-1</sup> cefotaxime. Thirteen out of 20 whole mature seed embryos of var. Rose coco co-cultivated on MSB5 basal medium after infection with strain EHA 105 (pCAMBIA 1201) turned green within 5 days, but exhibited minimal shoot growth during the first 2 weeks of culture onto MSB5 medium supplemented with 10 µM BAP, 50 mg L<sup>-1</sup> hygromycin and 250 mg L<sup>-1</sup> cefotaxime. Root

TABLE 1. Transformation of mature seed embryos of *Phaseolus vulgaris* L var. Mwitemania (Kenyan) co-cultivated on MSB5 basal medium

Agrobacterium strain	Explant	Replicates	Number of explants	Remarks
LBA 4404 (PBI 121)	Whole seed embryo	4	20	Slow growth, 17 explants died within 3 weeks
	Half seed embryo	2	10	Slow growth, all explants died within 3 weeks
LBA 4404	Whole seed embryo	2	10	Multiple bud regeneration within 2-4 weeks. No GUS activity
	Half seed embryo	2	10	Multiple bud regeneration after 2-4 weeks, no GUS activity
EHA 105 (pCAMBIA 1201)	Whole seed embryo	4	20	Minimal growth. All embryos died within 6 weeks
	Half seed embryo	2	10	Minimal growth. All embryos died within 6 weeks
EHA 105 (pCAMBIA 1301)	Whole seed embryo	2	10	Slow growth. All explants died within 3-6 weeks
	Half seed embryo	2	10	Slow growth. All explants died within 3-6 weeks
EHA 105	Whole seed embryo	2	10	Single shoot regeneration. No GUS activity
	Half seed embryo	2	10	Single shoot regeneration. No GUS activity

TABLE 2. Transformation of mature seed embryos of *Phaseolus vulgaris* L var. Rose coco (Kenyan) co-cultivated on MSB5 basal medium

Agrobacterium strain	Explant	Replicates	Number of explants	Remarks
LBA 4404 (PBI 121)	Whole seed embryo	4	20	Slow growth, all explants died within 2-3 weeks
	Half seed embryo	2	10	Slow growth, all explants died within 2-3 weeks
LBA 4404	Whole seed embryo	2	10	Multiple bud regeneration after 3 weeks, poor root growth, no GUS activity
	Half seed embryo	2	10	Multiple bud regeneration after 2 weeks, no GUS activity
EHA 105 (pCAMBIA 1201)	Whole seed embryo	4	20	Shoot and root growth, all embryos died within 2-3 weeks
	Half seed embryo	2	10	All explants died within 2-3 weeks
EHA 105	Whole embryo	2	10	Multiple bud regeneration after 3 weeks, no GUS activity
	Half seed embryo	2	10	Multiple buds within 3-4 weeks, no GUS activity

regeneration was observed in 3 out of 20 explants within the same period. The half seed embryos infected with the same strain and were cultured on the same medium failed to show any significant growth, and died within 3 weeks. The control whole and half seed embryos infected with *A. tumefaciens* strains LBA 4404 and EHA 105 regenerated multiple buds within 2-4 weeks (Table 2).

In variety Mwitemania 33 mature whole seed embryos infected by stab inoculation with *A. tumefaciens* LBA 4404 (pBI 121) and co-cultivated on MSB5 medium supplemented with 10  $\mu$ M BAP for three days turned green after nine days of culture on medium with 10  $\mu$ M BAP, 50 mg L<sup>-1</sup> kanamycin and 250 mg L<sup>-1</sup> cefotaxime (Table 3). The explants exhibited single shoot meristem growth in addition to root regeneration. One out of three explants selected at random after 12 days of culture on shoot regeneration and selection medium exhibited GUS activity evidenced by the blue staining at the embryonic axis (Fig. 1 A). Fourteen out of the 30 remaining embryo explants died within 20 weeks. Eleven out of the 16 surviving embryo explants exhibited shoot growth, three showed both shoot and root growth while two failed to show any significant growth of either the shoot or root region despite regular subculture to shoot regeneration and selection medium of the same composition every two to three weeks.

The number of surviving embryo explants from those initially infected (Table 3) continued to decline in subsequent subcultures and only nine regenerated to form plantlets on shoot induction and selection medium consisting of 10  $\mu$ M BAP, 50 mg L<sup>-1</sup> kanamycin and 250 mg L<sup>-1</sup> cefotaxime after 22 weeks. Two out of the nine plantlets removed at random were both positive for GUS activity when tested after 22 weeks.

However, the transformation was chimaeric as both the shoot and the root regions showed partial expression of the GUS gene (Fig. 1 B).

Five out of the seven 30 weeks' old plantlets subcultured onto a fresh shoot induction and selection medium exhibited normal shoot growth but root growth was poor. There was also presence of GUS activity in both the shoot and root segments of two plantlets selected at random when tested after 32 and 40 weeks (Fig. 1 C and 1 D). Two out of 28 (7.1%) embryo explants from the lot initially infected with *A. tumefaciens* LBA 4404 (pBI 121) excluding those used in the GUS assay exhibited growth on shoot regeneration and selection medium after 40 weeks. However, the two plantlets eventually died when transferred to vermiculite for hardening; therefore, mature plants were not raised. The negative control explants infected with *A. tumefaciens* strain LBA 4404 and were cultured on shoot regeneration medium consisting of 10  $\mu$ M BAP and 250 mg L<sup>-1</sup> cefotaxime after co-cultivation on medium with 10  $\mu$ M BAP exhibited shoot and root meristem growth and developed multiple buds after 12 days. There was no GUS activity when the explants were tested after nine days, as well as after 21 and 32 weeks.

All the 28 whole mature seed embryos explants of var. Mwitemania, infected with *A. tumefaciens* strain EHA 105 (pCAMBIA 1301) and were cultured on MSB5 medium with 10  $\mu$ M BAP, 50 mg L<sup>-1</sup> hygromycin and 250 mg L<sup>-1</sup> cefotaxime after co-cultivation on medium with 10 mM BAP, died within 4 weeks (Table 3). However, the 24 negative control explants infected with *A. tumefaciens* EHA 105 developed multiple buds within 12 days. There was no GUS activity in the shoots regenerated from negative control explants when tested after two and six weeks.

TABLE 3. Transformation of whole mature bean seed embryos of var. Mwitemania (Kenyan)

<i>Agrobacterium</i> strain	Replicates	Number of explants	Remarks
LBA 4404 (PBI 121)	6	33	GUS activity present in 12 days explants to 40 weeks old plantlets
LBA 4404 (negative control)	2	10	Multiple bud regeneration after 12 days. No GUS activity
EHA 105 (pCAMBIA 1301)	6	28	No GUS activity. Explants died within 9-28 days
EHA 105 (negative control)	5	24	Multiple buds after 12 days. No GUS activity

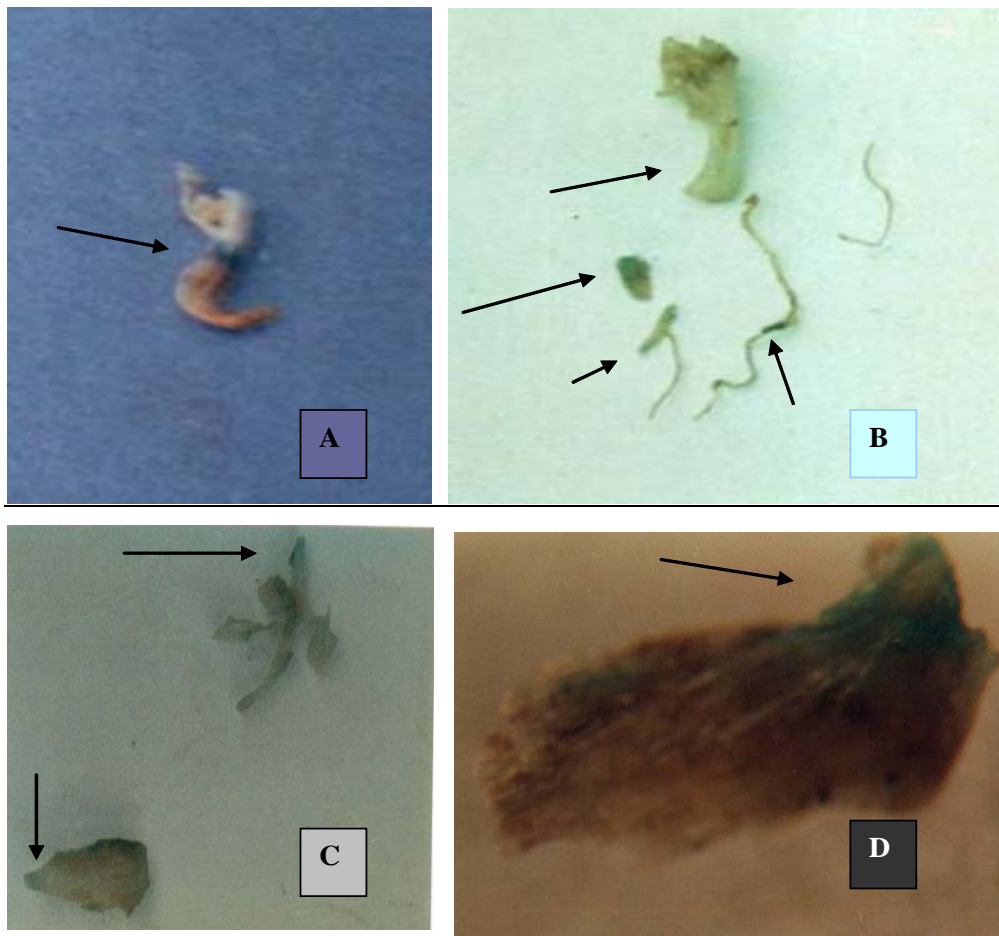


Figure 1. Transformed common bean explants infected with *A. tumefaciens* LBA 4404 (pBI 121) expressing the GUS gene (see arrows) (A) A seed embryo showing expression at the embryonic axis 12 days after infection (B) Shoot and root sections from a 22 weeks old plantlet (C) Expression in shoot and root regions 32 weeks after infection (D) A root section 40 weeks after infection.

## DISCUSSION

Mature seed embryos of variety Mwitmania (Table 1) and Rose coco (Table 2) that were stab inoculated with *A. tumefaciens* LBA 4404 (pBI 121), but were co-cultivated on MSB5 basal medium could not be transformed. The absence of growth hormones during co-cultivation of explants and poor regeneration in *P. vulgaris* resulted in very limited growth, which may have been insufficient for transformation to occur. A suitable regeneration system is necessary for recovery of transformed plants as *Agrobacterium* usually damages plant tissues during infection (Zhang *et al.*, 1997). Zygotic bean embryo explants infected with either *A. tumefaciens* EHA

105 (pCAMBIA 1201) or *A. tumefaciens* EHA 105 (pCAMBIA 1301) exhibited limited growth and died within six weeks. This was probably because the 50 mg L<sup>-1</sup> hygromycin used for selection may have been toxic to the tissues subsequently making regeneration of multiple buds difficult. Also strain EHA 105 though previously used in rice transformation (Hiei *et al.*, 1994) may be unsuitable for common bean transformation.

Evidence of transformation of bean varieties was shown by the fact that infected seed embryo explants co-cultivated on medium with 10 µM BAP could be grown on shoot regeneration and selection medium consisting of 10 µM BAP, 50 mg L<sup>-1</sup> kanamycin and 250 mg L<sup>-1</sup> cefotaxime for 40 weeks (Table 3). The transformed seed

embryos developed directly into a single shoot and root system and exhibited GUS activity.

The expression of GUS activity in the shoot region of germinating *P. vulgaris* var. Mwitmania seed embryo explants infected with *A. tumefaciens* LBA 4404 (pBI 121) was an indication that transgenic plants can be recovered. The non-continuous position of the blue staining zones in the bean shoot indicated that the transformation was chimaeric as none of the events was in continuing cell lineages of the meristem. The wound site created by stabbing the zygotic bean explants was necessary for *Agrobacterium* infection. It also exposed the interior layers of the meristem to *Agrobacteria* infection. Transformation of these layers would allow a subsequently arising germline to pass on the acquired transgene through the seed (Lewis and Bliss, 1994). Wounded plant tissues also produce phenolic compounds necessary for *Agrobacterium* infection (Kado, 1991).

Presence of the growth regulator BAP in both co-cultivation and shoot regeneration media greatly enhanced transformation and the survival rate of the transformed bean explants. This result is in agreement with previous studies in which presence of growth regulators in co-cultivation medium has been shown to enhance transformation in the common bean (Zhang *et al.*, 1997), and in pea, *Pisum sativum* (Schroeder *et al.*, 1993). In previous studies, stab inoculation of intact meristems led to recovery of transgenic soyabean (Chee *et al.*, 1989), but not common bean. Zhang *et al.* (1997) demonstrated that in *P. vulgaris* the explant tissue, the *Agrobacterium* strain and the presence of growth regulators in the co-cultivation medium were found to be important in transformation.

Transformation of common bean with the particle gun though has yielded transformed plants (Aragao *et al.*, 1996; 2002; Rech *et al.*, 2008), may produce lower levels of stable inheritance and is more expensive when compared to the *Agrobacterium* method. Despite the fact that transgenic bean plants were not recovered, the stab inoculation method described is a promising one and requires further study as there are limited reports on successful transformation of *P. vulgaris* using *Agrobacterium*. Also, reducing the period of

bean explant exposure to selection medium containing antibiotics to three to four weeks could lead to recovery of transformed plants as in the case of biolistic transformation (Aragao *et al.*, 2002; Rech *et al.*, 2008).

## CONCLUSION

Mature seed embryos of *Phaseolus vulgaris* var. Mwitmania can be transformed by *Agrobacterium tumefaciens*. Of the vectors tested, *A. tumefaciens* LBA 4404 (pBI 121) transforms the bean embryos. Histochemical staining for GUS activity has confirmed expression in the germinating seed embryos of variety Mwitmania infected with *A. tumefaciens* LBA 4404 (pBI 121). In order for transformation to occur, cytokinin is necessary in both the media used for co-cultivation and selection of transformed plants.

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## REFERENCES

- Aragao, F.J.L., Vianna, G.R., Albino, M.M.C. and Rech, E.L. 2002. Transgenic dry bean tolerant to the herbicide glufosinate ammonium. *Crop Science* 42:1298-1302.
- Aragao, F.J.L. and Rech, E.L. 2001. Transgenic common bean. In: Bajaj, Y.P.S. (Ed.). *Biotechnology in Agriculture and Forestry*, vol. 47. *Transgenic crops II*. Springer\_Verlag, Berlin Heidelberg.
- Aragao, F.J.L., Ribeiro, S.G., Barros, L.M.G., Brasileiro, A.C.M., Maxwell, D.P., Rech, E.L. and Faria, J.C. 1998. Transgenic beans (*Phaseolus vulgaris*) engineered to express



- viral antisense RNAs show delayed and attenuated symptoms to bean golden mosaic geminivirus. *Molecular Breeding* 4:491-499.
- Aragao, F.J.L., Barros, L.M.G., Brasileiro, A.C.M., Ribeiro, S.G., Smith, J.C., Sanford, J.C., Faria, J.C. and Rech, E.L. 1996. Inheritance of foreign genes in transgenic bean (*Phaseolus vulgaris* L.) co-transformed via particle bombardment. *Theoretical and Applied Genetics* 93:142-150.
- Aragao, F.J.L., Sá M.F.G, Davey, M.R., Brasileiro, A.C.M., Faria, J.C. and Rech, E.L. 1993. Factors influencing transient gene expression in bean (*Phaseolus vulgaris*) using an electric acceleration device. *Plant Cell Reports* 12: 483-490.
- Aragao, F.J.L., Sá, M.F.G, Almeida, E.R., Gander, E.S. and Rech, E.L. 1992. Particle bombardment – mediated transient expresión of a Brazil nut methionine- rich albúmin in bean (*Phaseolus vulgaris* L.). *Plant Molecular Biology* 20:357- 359.
- Arellano, J., Fuentes, S.I., Castillo-Espana and Hernández, G. 2009. Regeneration of different cultivars of common bean (*Phaseolus vulgaris* L.) via indirect organogenesis. *Plant Cell Tissue and Organ Culture* 96:11-18.
- Atkins, C.A. and Smith, M.C.P. 1997. Genetic transformation and regeneration of legumes. In: Legock, A., Bothe, H. and Puhler, A. (Eds.). *Biological Fixation of Nitrogen for Ecology and Sustainable Agriculture*. Springer - Verlag, Berlin Heidelberg 39:283 - 304.
- Brasileiro, A.C.M., Aragao, F. J. L., Rossi, S., Dusi, D.M. A., Barros, L. M. G. and Rech, E. L. 1996. Susceptibility of common and tepari bean to *Agrobacterium* spp. strains and improvement of *Agrobacterium*-mediated transformation using microprojectile bombardment. *Journal of American Society for Horticultural Science* 121: 810-815.
- Chee, P.P., Fober, K. A. and Slighton, J.L. 1989. Transformation of soybean (*Glycine max*) by infecting germinating seeds with *Agrobacterium tumefaciens*. *Plant Physiology* 91:1212- 1218.
- Colpaert, N., Tilleman, S., van Montagu, M., Gheysen, G. and Terry, N. 2008. Composite *Phaseolus vulgaris* plants with transgenic roots as research tool. *African Journal of Biotechnology* 7 (4):404 - 408.
- Dang, W. and Wei, Z. 2007. An optimised *Agrobacterium*-mediated transformation for soybean for expression of binary insect resistance genes. *Plant Science* 173:381–389
- Dillen, W., Engler, G., van Montagu, M. and Angenon, G. 1995. Electroporation-mediated DNA delivery to seedling tissues of *Phaseolus vulgaris* L. (common bean). *Plant Cell Reports* 15(1-2):119-124.
- Dillen, W., De Clercq, J., Goosens, A., Van Montagu, M., Angenon, G. 1997. *Agrobacterium* mediated transformation of *Phaseolus* beans. *Theoretical and Applied Genetics* 94:151-158.
- Eapen, S. and George, L. 1994. *Agrobacterium tumefaciens* mediated gene transfer in peanut (*Arachis hypogaea* L.). *Plant Cell Reports* 13:582-586.
- Franklin, C.I., Trieu, T.N., Cassidy, B.G., Dixon, R.A. and Nelson, R.S. 1993. Genetic transformation of green bean callus via *Agrobacterium* mediated DNA transfer. *Plant Cell Reports* 12:74 - 79.
- Gamborg, O.L., Miller, R. A. and Ojima, K. 1968. Nutrient requirements for suspension cultures of soybean root cells. *Experimental Cell Research* 50:151-158.
- Gatica Arias, M.A., Valverde, J.M., Fosencá, P.R. and Melara, M.V. 2010. *In vitro* plant regeneration system for common bean (*Phaseolus vulgaris* L.): Effect of N6-benzylaminopurine and adenine sulphate. *Electronic Journal of Biotechnology* 13 (1):1-8
- Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. 1994. Efficient transformation of rice, *Oryza sativa* mediated by *Agrobacterium* and sequence analysis of the boundaries of T-DNA. *Plant Journal* 6:271-282.
- Hoekema, A., Hirsch, P.R., Hooykaas, P.J.J. and Schilperoort, R.A. 1983. A binary plant vector strategy based on separation of vir and T-region of the *Agrobacterium tumefaciens* Ti-plasmid. *Nature* 303:179 - 181.
- Hood, E., Gelvin, S.B., Melchers, L. and Hoekema, A. 1993. New *Agrobacterium* helper plasmids for gene transfer to plants. *Transgenic Research* 2:208 - 218.

- Jefferson, R.A. 1987. Assaying chimeric genes in plants: the GUS gene fusion system. *Plant Molecular Biology Reporter* 5:387-405.
- Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. 1987. GUS fusions:  $\beta$ -Glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO Journal* 387-405.
- Kado, C. I. 1991. Molecular mechanisms of crown gall tumorigenesis. *Critical Rev. Plant Science* 10:1-32.
- Kim, J.W. and Minamikawa, T. 1997. Stable delivery of a canavalin promoter- $\beta$ -glucuronidase gene fusion into French bean by particle bombardment. *Plant Cell Physiology* 38(1):70-75.
- Leon, P., Planckaert, F. and Walbot, V. 1991. Transient gene expression in protoplasts of *Phaseolus vulgaris* isolated from a cell suspension culture. *Plant Physiology* 95:968-972.
- Lewis, E.M. and Bliss, F.A. 1994. Tumour formation and  $\beta$ -glucuronidase expression in *Phaseolus vulgaris* inoculated with *Agrobacterium tumefaciens*. *Journal of American Society for Horticultural Science* 119:361-366.
- Liu, H.K., Yang, C. and Wei, Z.M. 2004. Efficient *Agrobacterium tumefaciens* mediated transformation of soybeans using an embryonic tip regeneration system. *Planta* 219:1042-1049.
- Miller, J.H. 1972. Experiments in Molecular Genetics. Cold Spring Harbour. New York, USA.
- McClellan, P., Chee, P., Held, B., Simental, J., Drong, R.F. and Slightom, J. 1991. Susceptibility of dry bean (*Phaseolus vulgaris* L.) to *Agrobacterium* infection: Transformation of cotyledonary and hypocotyl tissues. *Plant Cell Tissue and Organ Culture* 24:131-138.
- Murashige, T. and Skoog, F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiology Plantarum* 15:473-497
- Paz, M.M., Martinez, J.C., Kalvig, A.B., Fonger, T.M. and Wang, K. 2005. Improved cotyledonary node method using an alternative explant derived from mature seed for efficient *Agrobacterium*-mediated soybean transformation. *Plant Cell Reports* 25:206-213.
- Popelka, J.C., Gollasch, S., Higgins, T.J.V. 2006. Genetic transformation of cowpea (*Vigna unguiculata* L.) and stable transgene transmission to sexual progeny. *Plant Cell Reports* 25:304-309.
- Puonti-Kaelas, J., Eriksson, T. and Engstrom, P. 1990. Production of transgenic pea (*Pisum sativum* L.) plants by *Agrobacterium tumefaciens*-mediated gene transfer. *Theoretical and Applied Genetics* 80:246-252.
- Rech, E.L., Vianna, R.G. and Aragao, F.J.L. 2008. High efficiency transformation by biolistics of soybean, common bean and cotton transgenic plants. *Nature Protocols* 3 (3):410-418.
- Russell, D.R., Wallace, K.M., Bathe, J.H., Martinell, B. J. and McCabe, D.E. 1993. Stable transformation of *Phaseolus vulgaris* via electric-discharge mediated acceleration. *Plant Cell Reports* 165-169.
- Schenk, R.V. and Hildebrandt, A.C. 1972. Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. *Canadian Journal of Botany* 50:199-204.
- Schroeder, H.E., Schotz, A.H., Wardley-Richardson, T., Spencer, D. and Higgins, T.J.V. 1993. Transformation and regeneration of two cultivars of pea (*Pisum sativum* L.). *Plant Physiology* 101:751-757.
- Svetleval, D., Velcheva, M. and Bhowmik, G. 2003. Biotechnology as a useful tool in common bean (*Phaseolus vulgaris* L.) improvement. *Euphytica* 131:189-200.
- Velcheva, M., Svetleval, D., Petkova, S. and Perl, A. 2005. *In vitro* regeneration and genetic transformation of common bean (*Phaseolus vulgaris* L.)-problems and progress. *Science Horticulture* 107:2-10.
- Zhang, Z., Coyne, D.P. and Mitra, A. 1997. Factors affecting *Agrobacterium*-mediated transformation of common bean. *Journal of American Society for Horticultural Science* 122 (3): 300-305.
- Zambre, M.A., Clerq, J. and Vranova, E. 1998. Plant regeneration from embryo derived callus

- in *Phaseolus vulgaris* L. (common bean) and *P. acutifolius* A. Grey (tepary bean). *Plant Cell Reports* 17:626-630.
- Zambre, M., Goossens, A., Cardona, C., Van Montagu, M., Terryn, N. and Angenon, G. 2005. An efficient and reproducible *Agrobacterium*- mediated transformation systems for cultivated *Phaseolus acutifolius* (tepary bean). *Theoretical and Applied Genetics* 110: 914-924.