Bean tissue culture and genetic transformation with *Agrobacterium*

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INTERNATIONAL JOURNAL OF HORTICULTURAL SCIENCE

AGROINFORM
Publishing House, Hungary

Key words: Phaseolus vulgaris, bean callus, Agrobacterium, lindan resistance, GUS transformation.

Summary: In this paper we report the establishment methods of a rapidly growing callus culture of *Phaseolus vulgaris* bean as well as the conditions required for a high level of transient gene expression using *Agrobacterium*-mediated transformation. A vector is containing both the lindan-resistance gene as a selectable marker, and GUS gene as a screenable marker. By using hypocotyl explant and vertical culture on B5 medium supplemented with 1 mg/l kinetin- and 2,4-D 2 mg/l and subcultured every 3-4 weeks, we can recommend to get a good and much callus from bean. This will help in introducing foreign DNA into callus cells. One strain of *Agrobacterium* carrying plasmid as vector for introducing foreign DNA into plant cells was used. At different concentrations of lindan; 3, 4 and 4.5 mg/l, the transformed Maxidor callus survived and grew over a period of 6 month and subcultured every 3-4 weeks, but the control callus died. Callus were assayed for GUS activity to confirm the expression of the GUS gene using the histochemical assay test. The GUS gene was also correctly expressed in callus cultures grown on 4mg/l lindan-selected medium, the typical blue colour in the histochemical assay using the X-gluc as substrate. But the control, non-transformed callus was not able to grow in the presence of lindan, neither showed a positive reaction in the in vitro assays.

Introduction

Difficulties in obtaining plants from somatic cells or protoplasts in *Phaseolus* species have till now hampered the production of transgenic plants by the application of the most common methods available for the introduction of foreign DNA into cells (*Genga* et al. 1990). Gene transfer technology, in fact, requires reproducible protocols for in vitro culture and efficient plant regeneration from protoplasts, cells or organs (*Mariotti* et al. 1989). Protoplast transformation and particle bombardement are the two proven methods for gene transfer to legumes (*Giovinazzo* et al. 1993). In the case of bean no stable transformation and regeneration method is routinely available (*Giovinazzo* et al. 1993) and the level of transient gene expression reported by *Dron* et al. (1988) is very low.

The new expression cassette containing the well-characterised genes for the enzyme hygromycin B phosphotransferase (HPH) (*Timmermans* et al. 1990) and ß-glucuronidase (GUS) (Jefferson et al. 1987) was tested.

Callus were induced from immature cotyledons (genotype G02771) sliced in three pieces and placed on SH medium supplemented with 2 mg/l pCA, 0.4 mg/l 2,4-D, 1mg/l 6BAP, and 0.6% agar (*Dixon* & *Fuller* 1976). In this paper we report the establishment methods of a rapidly growing callus culture of *Phaseolus vulgaris* bean as well as the conditions required for a high level of transient gene expression using *Agrobacterium*-mediated transformation carrying the A281 pRGG bar H1 plasmid (*Hood* et al. 1987 and 1993), a vector containing both the lindan-resistance gene as a selectable marker, and the ß-glucuronidase (GUS) gene as a screenable marker, and in this report we describe the successful transformation of bean callus.

Several researchers used *Agrobacterium*-mediated transformation of bean, carrying herbicide and antibiotic resistance gene, antibiotic resistance gene and gene GUS (*Genga* et al. 1990, *Mariotti* et al. 1989, *McClean* et al. 1991, *Becker* et al. 1994, *McClean* et al. 1988, and *Lewis* & *Bliss* 1994). Another researchers used *Agrobacterium*-mediated transformation of soybean, carrying the same genes (Facciotti et al. 1985) and pea (*Puonti-Kaerlas* et al. 1989, *De Kathen* & *Jacobsen* 1990).

Materials and methods

Plant material: Seeds of *Phaseolus vulgaris* cv. *Fönix* and *Maxidor* were used as source of plant material.

Preparation of the explants and callus induction: Two cultivars, *Fönix* and *Maxidor* were used in this study. Dry bean seeds were sterilised and germinated by the methods of (*Eissa Ahmed* et al. 1999). After 7–10 days, two types of explant was prepared: shoot apex and cut the hypocotyl two parts. After explant preparation cultured on B5 medium according to *Gamborg* et al. (1968) by two methods, vertical or horizontal in glass bottle 55 x 90 mm. This medium contains macro and microelements, and supplemented with 1mg/l kinetin and 2,4- dichlorophenoxyacetic acid (2,4-D) 2 mg/l (pH 5.7) adjusted before autoclaving; and 20 g/l sucrose and 8g/l agar-agar; within one week, produced the callus. After 3–4 weeks, the explants were removed and the callus was transferred to fresh B5 medium and subcultured evrey 3–4 weeks.

Sometimes the explants were cultured on half MS medium according to *Murashige & Skoog* (1962) major salts+minor elements supplemented with B5 vitamins+0.5 or 0.75 mg/l benzyladenine (BA) and 0.1 mg/l naphthaleneacetic acid (NNA)+20 g/l sucrose+7 g/l agar-agar (pH 5.7).

Effect of Lindan on the growth of callus (Lindan curve procedure): Callus were treated by different concentrations of Lindan 0.5, 1, 2, 3, 4 and 5 mg/l Finale (150 g/l) on the same media by using 12 replicates per each treatment, and the callus were weighted after 56 days from the first day of treatment. Statistical analysis was made to define which dose sufficient to kill the control callus.

Culture media and growth of bacteria: *Agrobacterium tumefaciens* strain A281 pRGG bar H1 was grown on LB-medium according to Maniatis et al. (1982) at 28 °C with appropriate antibiotics kanamycin 50 mg/l.

Co-cultivation with* Agrobacterium *and transformation: For callus transformation, bacteria were grown overnight in LB-medium, and suspended in a modified LB-medium and co-cultivated 3 days with the callus and cultured on B5 medium (pH 5) for 3 days and transferred to B5 medium (pH 7) for 3 days.Transformed callus were selected on 2-4 mg/l lindan. Bacterial growth was controlled by addition of 375-500 mg/l Augmentin or 200 mg/l Claforan (Cefotaximum) (Hoechst) to the medium.

Callus proliferation and genetic selection: Callus inoculated with *Agrobacterium* were maintained for 3 months. Secondary callus (E1 generation) were removed from the original inoculated callus (E0) at 3 months after inoculation.

To screen for transformants, callus (E2-7 generation) of each subclone were transferred to the same medium supplemented with 2–4.5 mg/l lindan over 6 months period and 3–4 week transfer schedule.

Transformation tests: Callus derived from 3mg/l lindan-selected medium were cultured on solidified medium supplemented with 4 mg/l lindan to test their ability to form an actively growing callus in the presence of herbicide.

Callus were histochemically evaluated for the presence of GUS activity according to the method of (*Hinchee* et al. 1988). GUS (ß-glucuronidase) was measured histologically (using X-Gluc[5-bromo-4-chloro-3-indolyle-ß-D-glucuronidecyclo-hexylammonium salt]). Callus were incubated overnight in X-Gluc solution and immediately examined under microscope.

Results and discussion

Callus induction: About 600 bean seedling from *Fönix* and *Maxidor* were used in this study including shoot apex and hypocotyl. Callus were induced from hypocotyl and shoot apex explants in B5 medium supplemented with 1 mg/l kinetin and 2,4-D 2 mg/l within one week, but *Giovinazzo* et al. (1993) induced callus from immature cotyledons (genotype G02771) sliced in three pieces and placed on SH medium supplemented with 2 mg/l pCH, 0.4 mg/l 2,4-D, 1 mg/l 6BAP and 0.6% agar. *Giovinazzo* et al. 1993 found that in approximately 80% of the cultured cotyledons, callus formation was observed within 1 week and subcultured every 20 days. *Mohamed* et al. 1993 prepared cotyledonary leaf explants from 2 week-old seedlings, the explants were cultured for callus induction on medium containing 4% sucrose and 0.2 g/l^{-1} casein hydrolysate, and supplemented with combinations of 2,4-D (4, 8 or 16 mM) and kinetin (0, 1, 2 or 4 µM).

Our results indicated that by using hypocotyl explant culture, from the two cultivars we got a good and much callus than shoot apex explant culture, and vertical explant culture in both hypocotly and shoot apex gave a good and much callus than horizontal culture in the two cultivar *Fönix* and *Maxidor*. B5 medium supplemented with 1mg/l kinetin and 2,4-D 2 mg/l produced a good and much callus than half MS medium supplemented with 0.5 or 0.75 mg/l BA and 0.1 mg/l NAA in the two cultivars. Finally in order to get a good and very much bean callus from *Phaseolus vulgaris* cv. *Fönix* and *Maxidor* we can recommend by using hypocotyl explant and vertical culture on B5 medium supplemented with 1mg/l kinetin and 2, 4-D 2 mg/l and subcultured every 3–4 weeks, and this will help in introducing of foreign DNA into cells.

Effectivness of lindan on the growth of control callus: Our initial experiments focused on the demonstration of which concentration of lindan added to the callus culture of the two cultivars *Fönix* and *Maxidor* was affected to the callus after 40–50 days from the initial culture, and we observed that 0.5 mg/l lindan (Finale 150 g/l) was sufficient to kill the callus of *Fönix* and *Maxidor*, and phenotypically brown and died. Statistical analysis showed that 0.5 mg/l lindan was sufficient to kill the control callus of *Fönix* and *Maxidor* also *Fig. 1.*

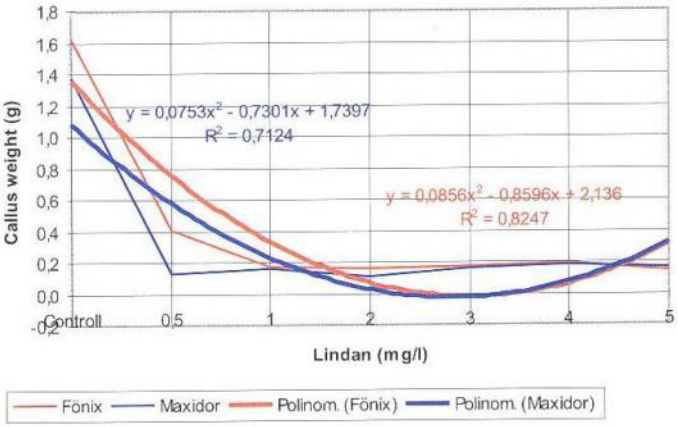

Figure 1 Effect of varying concentrations of lindan 0, 0.5, 1, 2, 3, 4 and 5 mg/l media on the growth of Fönix and Maxidor callus cultures grown into B5 medium.

Transformation of Maxidor callus: *Agrobacterium tumefaciens* and *A. rhizogenes* are known to infect dicotyledon species. The virulence is mediated by A281 pRGG bar plasmid which is able to transfer into the plant DNA the bar and gus-region of their genome. pRGG plasmid are of special interest in genetic engineering as vectors for intro-

ducing foregin DNA into plant cells. In the present work 1 strain of *Agrobacterium* was used.

Effect of the selection medium on callus growth: The herbicide lindan (AgrEvo GmbH Berlin) was added to the media at various concentrations 2, 3, 4 and 4.5 mg/l respectively, and their effects on growth and color over a period of 6 months and 3-4 weeks transfer schedule to screen for transformants callus. Lindan; 2, 3, 4 and 4.5 mg/l allowed growth for about eight weeks. And we found that at all these concentrations of lindan, the transformed *Maxidor* callus survived and grew but control died and co-cultivated *Fönix* callus, and by this method behaved in asssay of transient gene expression *Fig. 2.*

Figure 2 Maxidor callus, non-transgenic callus were not able to grow in the presesnce of 5 mg/l lindan (around center) while transgenic callus resistance (at the center).

Resistance of the selected callus was varified by their ability to repeated by grow on the same selective medium. Our initial experiments focused on the demonstration and optimization of transient expression of lindan resistance and gus genes (bar and gus) 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 callus was conducted. As shown in *Fig. 2* control callus (without bar gene transferred) did not grow when plated on media containing lindan at any of the concentrations tested. Callus transformed with the bar gene yielded much and big quantity any time when plated on media containing lindan 2 mg/l and gave much higher plating efficiencies and a corresponding many-fold increase in the number of lindan resistan callus. In addition, large numbers of resistant colonies were recovered at that time on media containing lindan at 2 and 3 mg/l. Large numbers of transformed callus were also obtained when selection (lindan at 4 and 4.5 mg/l was applied later). These results indicate that both plating efficiency and transformation frequency are dependent on the mode and timing of selection. Increased recovery of transformants occurs when selection pressure is applied later. *Christou* et al. (1987) obtained the same results by using soybean callus and chimeric genes coding for resistance to the aminoglycoside antibiotics kanamycin and G418. And soybean (Glycine max cv. „Forrest") has been successfully transformed with *Agrobacterium* containing a bacterial kanamycin resistance gene linked to the 5' portion of a soybean small subunit carboxylase gene by *Facciotti* et al. (1985) and they found that transformed callus demonstrated light induction of the chimeric gene as measured by increased levels of kanamycin resistance. *Puonti-Kaerlas* et al. (1989) obtained transformed callus of pea (*Pisum sativum* L.) by *Agrobacterium tumefaciens* and transformants could be selected on a medium containing kanamycin. Similar findings were made by *Genga* et al. (1990) and they found that after selection on km-containing media, several kmr-bean callus had been obtained and *McClean* et al. (1991) obtained the same results by using disarmed strain *Agrobacterium tumefaciens* strain C58Z707 to infect cotyledonary nodes and hypocotyls of *Phaseolus vulgaris* and they obtained callus capable of growing in the presence of kanamycin from these infected tissues. Cotyledonary node explants of dry bean were prepared and the nodal region was punctured numerous times and 10 microliters of an overnight culture of C58Z707 (disarmed *A. tumefaciens* strain C58 containing pGA482) then was dripped into the wound surface and the tissue was cocultivated with the bacteria for 3 days at 28 °C in the dark, the explants were then transferred to MS media containing 20 mg/l kanamycin and the kanamycin concentration was increased incrementally to 50 mg/l over a 2 month period and all these steps were made by *McClean* et al. (1988) and they found that callus production of transformed explants continued on this media and control explant did not produce callus at 20 mg/l kanamycin. *Billings* et al. (1997) studied the effect of growth regulators and antibiotics on eggplant transformation by *A. tumefaciens* and they observed that the callus grew on the selection medium supplemented with kanamycin at 50 mg/l. Tobacco callus formation was observed by *Öktem* (1998) in the presence of 10 mg/l broad spectrum herbicides based on gluphosinate, the ammonium salt of phosphinothricin (PPT), 3weeks after co-cultivation in leaf disks infected with *A. tumefaciens* strain LBA4404 carrying the pDHB321.1 binary vector, although callus initiation was observed in some of the control plants in the presence of PPT, development stopped after 40 days of culture, followed by death at the end of 60 days of culture.

Confirmation of transformation (GUS assay): Callus were assayed for GUS activity to confirm the expression of the gus gene using the histochemical assay described by *Jefferson* et al. (1987). The GUS gene was also correctly expressed in callus cultures grew on 4 mg/l lindan-selected medium, the typical blue color in the histochemical assay using the X-gluc as substrate *Fig. 3 and 4.*

Control, non-transformed callus were not able to grow in the presence of lindan, neither showed a positive reaction in the in vitro assays. Similar results obtained by *Genga* et al. (1990) in *Phaseolus* species bean, *De Kathen* and *Jacobsen*

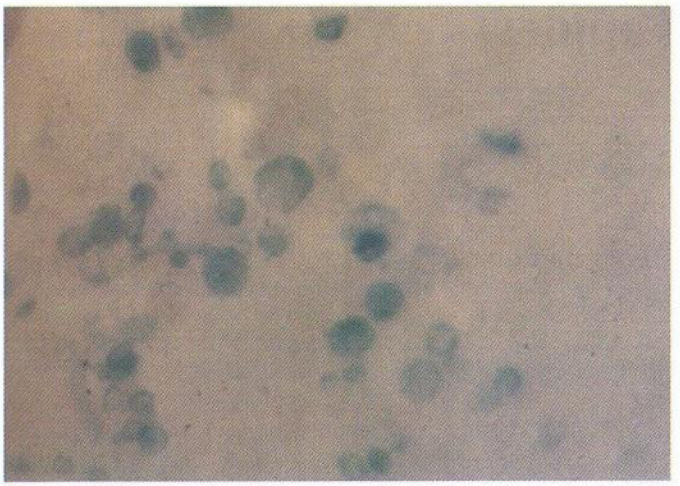

Figure 3 Stable expression of the gus gene in Maxidor callus under the light microscope in all of its cells.

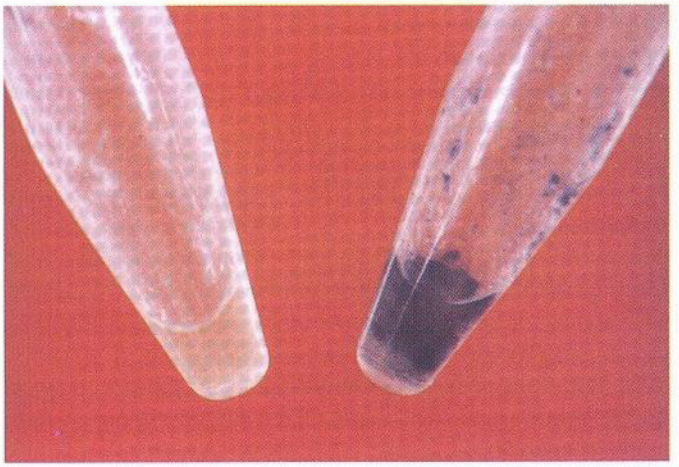

Figure 4 Stable expression of gus gene and qualitative GUS-assay showing ß-Glucuronidase activity in Maxidor bean callus that stained blue, while non transgenic callus not showed a positive reaction.

(1990) in Pisum sativum L., Mariotti et al. (1989) in *Phaseolus vulgaris* L., and *P. coccineus* L., Becker et al. (1994) in *Phaseolus vulgaris*, Lewis and Bliss (1994) in *Phaseolus vulgaris*, and Hinchee et al. (1988) in soybean.

Acknowledgments

The authors wish to thank Pedryc Andrzej, for his help with the statistical analysis of data and his assistance with the manuscript.

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