Factors Affecting *Agrobacterium*-mediated Transformation of Common Bean

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ABSTRACT. Factors influencing *Agrobacterium tumefaciens*-mediated transformation of common beans (*Phaseolus vulgaris* L.) were examined using an intron-containing β -glucuronidase (GUS) gene as a reporter system to develop a repeatable transformation protocol. Tissue culture procedures used were based on direct shoot organogenesis. Two *A. tumefaciens* strains—A2760 and EHA105—were used, with emphasis on the former due to its overall higher infection rate. Eleven common-bean genotypes were compared for susceptibility to strain A2760 or EHA105. The pinto bean 'Othello' was used extensively in testing different transformation conditions. Factors significantly affecting transformation rate were *Agrobacterium* × host interactions, explant maturity, preculture and cocultivation conditions, and selection schemes, based on transient GUS gene expression. The best transformation conditions were the use of susceptible genotypes and explants derived from mature seeds, preconditioning of explants in a medium containing 20 μ mol of benzyladenine (BA) in darkness or on a filter paper, dipping explants in high concentrations of *Agrobacterium* cell suspension ($OD_{650} = 0.8-1.0$) followed by a long-term (6-day) cocultivation period on a semisolid agar medium in the presence of cytokinin or 3-day cocultivation on a moistened filter paper, and the use of lethal levels of selective agents. About 4% of explants, or 14% of regenerated shoots or buds, were putatively transgenic, as indicated by GUS blue staining throughout the entire shoot or bud, after explants were transformed with *Agrobacterium* strain A2760 using an optimized protocol.

Agrobacterium tumefaciens-mediated DNA transfer has been successfully explored for transformation of many dicots, including some leguminous crops such as soybean (*Glycine max* Mer.) (Chee et al., 1989; Falco et al., 1995; Hinchey et al., 1988), peanut (*Arachis hypogaea* L.) (Eapen and George, 1994), and pea (*Pisum sativum* L.) (Grant et al., 1995; Puonti-Kaerlas et al., 1990; Schroeder et al., 1993). This DNA delivery system has been used to introduce insect tolerance into pea (Shade et al., 1994).

Common bean (*Phaseolus vulgaris* L.) is an important food legume worldwide. The development of an efficient gene transfer system would greatly benefit breeding programs and enhance research investigating molecular processes. Although considerable efforts have been made (Franklin et al., 1993; Lewis and Bliss, 1994; Mariotti et al., 1989; McClean et al., 1991), an *Agrobacterium*-mediated transformation system has not yet been developed.

The slow progress in *P. vulgaris* transformation using *A. tumefaciens* delivery is attributed to lack of knowledge about factors affecting transformation. Only host × *Agrobacterium* interactions, effects of acetosyringone, and types of explant tissues on common-bean transformation have been reported previously (Lewis and Bliss, 1994; McClean et al., 1991). More research is needed on factors affecting *A. tumefaciens*-mediated transformation in common beans to develop a successful transformation scheme.

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The use of an intron-containing β -glucuronidase (GUS) reporter gene (Vancanneyt et al., 1990) has greatly facilitated analysis of transformation events in many other plant species. This assay system has at least two major advantages. It allows in situ analysis and quantification of transformation events by directly observing GUS blue sectors in explant tissues. False readings caused by *A. tumefaciens* contamination are eliminated due to the presence of an intron in the GUS coding region.

Therefore, our research objective was to investigate factors influencing *A. tumefaciens*-mediated DNA transformation of *P. vulgaris* using the GUS reporter gene system to develop a repeatable transformation protocol.

Materials and Methods

BINARY VECTOR AND AGROBACTERIUM. The original P35S GUS INT plasmid (Vancanneyt et al., 1990) containing the second intron (IV2) of the *ST-LS1* gene was provided by Eugene W. Nester (Dept. of Microbiology, Univ. of Washington). The GUS INT region is flanked by the 35S 5' promoter and the 35S 3' terminator (about 3 kb). This GUS INT cassette has been subcloned into the *Hind* III site of the binary vector B-28 (A. Mitra, unpublished data). The B-28 binary vector carries a *npt* II cassette in the T-DNA borders (Fig. 1). The B-28 vector without the P35S GUS INT fragment was used as a negative control. Thus, B-28/35S GUS INT and B-28 vectors were tested for GUS positive and negative activities in plant cells, respectively, either by electroporating them into tobacco protoplasts or by bombarding them into bean tissues using a biolistic PDS/1000 He device (BioRad). These two vectors were subsequently mobilized into *A. tumefaciens* strains A2760 (Hoekema et al., 1983) and EHA105 (Hood et al., 1993), respectively, by direct DNA transformation (An et al., 1988). The absence of GUS expression of B-28/P35S GUS INT vector in the *A. tumefaciens* cells was confirmed by testing for the absence of GUS activity in transformed *A. tumefaciens* cells compared with a GUS-positive reaction in transformed tobacco protoplasts under the same testing conditions.

PLANT MATERIAL AND REGENERATION PROCEDURES. Common-

Fig. 1. A schematic diagram depicting the T-DNA region of binary vector pAM1051 containing P35S GUS INT gene. RB and LB = T-DNA right border and left border, respectively; *npt II* = neomycin phosphotransferase II gene; P35S = cauliflower mosaic virus 35S promoter; GUS/intron/GUS = intron-containing β -glucuronidase (GUS) reporter gene; Tnos = nos terminator.

bean genotypes of Andean (A) and Meso-American (MA) origins were provided by James Steadman (Dept. of Plant Pathology, Univ. of Nebraska, Lincoln). Seeds of pinto 'Othello', great northern (GN) 'Harris', Alubia 33-1, Instituto Colombiano Agropecuario (ICA) 'Pijao', and Cranberry were planted in the greenhouse to obtain seeds for transformation experiments.

Plant regeneration was based on a direct shoot organogenesis protocol previously described by Mohamed et al. (1991, 1992) with some modifications. Explants were prepared using three different procedures. In procedure 1, mature healthy seeds of uniform sizes from different bean genotypes were disinfested for 20 min in 20% (v/v) commercial bleach (5.25% NaOCl) plus a few drops of Tween-20 and rinsed five times with sterile water. Seeds were germinated on Gamborg's B₅ (Gamborg et al., 1968) medium, amended with 10 μ M of BA (abbreviated as B₅BA10) (Table 1), solidified with 8 g·L⁻¹ Difco Bacto agar, and adjusted to pH 5.8 before autoclaving for 20 min. Each disinfested seed was placed in a 2 × 15-cm Pyrex tube (Scientific Products Division, Baxter Diagnostics Inc., McGaw Park, Ill.) containing 5 mL of B₅BA10 medium. Cultures were incubated in a 16-h photoperiod (40 μ mol·m⁻²·s⁻¹ from cool-white fluorescent tubes) at 25 °C for 2 to 3 weeks or until axillary shoots or buds emerged. Seedling node explants were prepared by removing epicotyl and hypocotyl sections and two-thirds of each cotyledon. Nodal regions were then lightly scratched or macerated (5 to 10 parallel slices 0.5 mm deep vertical to the sliced region) with a no. 10 sterile scalpel blade after the emerging axillary shoots or buds were removed. In procedure 2, disinfested seeds were germinated on a sterile moistened filter paper in 1.5 × 10-cm petri dishes at 25 °C for 1 week under a 16-

h photoperiod until green hypocotyls appeared. Explants were then prepared with a no. 10 scalpel blade by following sequential steps: a) only 3 to 5 mm of the hypocotyl region adjacent to the nodal region was retained, b) then each seed was split in half by cutting through the hypocotyl region, and c) the whole epicotyl region and all vascular tissues of the nodal region were removed. The explant was then wounded as in procedure 1. In procedure 3, immature bean seeds were isolated from green pods, about 21 d postflowering, and disinfested as described above. Seedcoats were removed and explants were prepared as described in procedure 2 except that no hypocotyl region was retained.

The explants prepared using these three different procedures were cultured in 1.5 × 10-cm petri dishes (six explants per petri dish), abaxial side in contact with the shoot induction medium B₅BA10 (Table 1). Cultures were grown at 25 °C under a 16-h photoperiod (40 μ mol·m⁻²·s⁻¹ from cool-white fluorescent tubes) for 2 to 3 weeks or until shoots emerged. Shoots longer than 1 cm were excised and transferred to Gamborg's B₅ basal medium (Gamborg et al., 1968) without BA for rooting. Explants, after their shoots were removed, were transferred to a fresh shoot induction medium containing Murashige and Skoog (MS) basal salts (Murashige and Skoog, 1962) and B₅ vitamins amended with 10 μ M BA (abbreviated as MSB₅ + BA10). All tissue culture media and reagents were purchased from Sigma Co., St. Louis, unless otherwise stated.

TRANSFORMATION PROCEDURES. A single colony of *A. tumefaciens* strain A2760 or EHA105, containing B-28/GUS INT or B-28, was inoculated into 5 mL of yeast extract and peptone (YEP) (Difco Laboratories, Detroit) liquid medium supplemented with

Table 1. Tissue culture media, hormonal concentrations (μ Mol), and antibiotics (mg·L⁻¹) used for seed germination, cocultivation, shoot induction, and postcocultivation selection of common bean².

Media-procedure	Media-procedure-selection
Seed germination	Shoot induction-selection
B ₅ + BA10	B ₅ or MSB ₅ + BA10
B ₅ + BA20	B ₅ + BA10 + Kan25 + Carb200 + Cef200
B ₅ + BA30	B ₅ + BA10 + Kan50 + Carb200 + Cef200
Filter paper	B ₅ + BA10 + Kan75 + Carb200 + Cef200
Cocultivation	B ₅ + BA10 + Kan100 + Carb200 + Cef200
MS or B ₅ + BA10	B ₅ + BA10 + Kan125 + Carb200 + Cef200
MS + 2,4-D, 20	B ₅ + BA10 + Tim60
MS(-), liquid	Rooting-selection
B ₅ (-) + BA20, liquid	B ₅ + Kan 50
Filter paper	

²Kan = kanamycin; Carb = carbenicillin; Cef = cefotaxime; Tim = timentin; BA = benzyladenine; MS = Murashige and Skoog (1962) basal medium; B₅ = Gamborg et al. (1968) B₅ basal medium; MSB₅ = MS (Murashige and Skoog, 1962) salts, and B₅ (Gamborg et al., 1968) vitamins.

10 mg·L⁻¹ of kanamycin and 5 mg·L⁻¹ of tetracycline (abbreviated as YEPKT). Cultures were placed on a platform shaker at 150 revolutions/min for 48 h at 28 °C. This suspension was then subcultured once in 1-L flasks containing 250 mL of YEPKT liquid medium at 28 °C overnight until it reached a late logarithmic phase (OD₆₄₀ = 0.8–1.0). Explants, ranging from 20 to 40 per replication, were soaked in strain suspensions for 5 to 15 min, blotted dry on a sterile paper towel, and then placed abaxial side down on the B₅ semisolid agar (6 g·L⁻¹) cocultivation medium with 10 μmol BA (Table 1). All petri dishes were wrapped with parafilm. Explants were cultured in darkness for 3 d at 28 °C. Following cocultivation, explants were washed thoroughly with B₅(-) (Gamborg's B₅ basal salts with minimal organic compounds, pH 5.8) liquid medium and stained with X-gluc solution (see the following section for GUS staining procedures). However, some parameters for transformation procedures were varied.

To study the effect of dark preconditioning and cytokinin (BA) on *A. tumefaciens* infection rate and GUS gene expression, seeds were germinated either in darkness or 16 h photoperiod (40 μmol·m⁻²·s⁻¹ from cool-white fluorescent tubes) at 25 °C on the B₅ medium supplemented with 10, 20, or 30 μmol BA (Table 1) until shoot buds emerged (14 to 21 d). Explants were prepared following procedure 1 and then cocultivated with strain A2760.

To test for susceptibility of bean genotypes to *A. tumefaciens* strains, seeds of 'Othello' and 'PC50' were germinated and explants were prepared according to procedure 1. Explants were then cocultivated with A2760 and EHA105, respectively. Additional equal numbers of explants of these two genotypes were cocultivated with A2760 and EHA105 that contained only B-28 without the GUS intron as a negative control under the same experimental conditions. After cocultivation, explants were transferred to shoot induction–selection medium (B₅ + BA10 + Kan25 + Carb200 + Cef 200) (Table 1). Explants were stained with X-gluc 21 d following culturing selection. The same procedures were used to screen nine additional bean genotypes except that only A2760 was used.

To examine the effect of explant maturity on transformation efficiency, bean genotypes Alubia 33-1, ICA 'Pijao', and pinto 'Othello' were planted and grown as described above, and immature cotyledonary explants were prepared according to procedure 3. Mature explants were prepared according to procedure 2. Freshly prepared explants were then cocultured with A2760.

To examine the effects of plant growth regulators present in the cocultivation media, mature seeds of pinto 'Othello' and GN 'Harris' were germinated on moistened filter papers in 1.5 × 10-cm petri dishes at 25 °C for 8 d at a 16-h photoperiod (40 μmol·m⁻²·s⁻¹ from cool-white fluorescent tubes). Explants from seeds were prepared as in procedure 2, immersed in the A2760 cell suspension for 5 to 15 min, and cocultivated on filter paper or on MS medium in the presence of either cytokinin (10 μmol BA) or auxin (20 μmol 2,4-D) (Table 1).

To study the effect of types of cocultivation media, explant age, and postcocultivation selection on transformation, seeds of 'Othello' were germinated and explants were prepared as in procedure 2. Explants were cocultivated with A2760 and either stained immediately or transferred to shoot induction–selection medium amended with 60 mg·L⁻¹ timentin and 75 mg·L⁻¹ kanamycin (Table 1) under a 16-h photoperiod for 21 d, followed by staining for GUS activity. For liquid-medium cocultivation, all procedures were the same as for the semisolid (agar) medium cocultivation except that 250 μL of late-logarithmic phase *A. tumefaciens* culture was added to the 25 mL of MS(-) basal salts with minimal organics, pH 5.8 (Murashige and Skoog, 1962), liquid medium in 1.5 × 10-cm petri dishes.

Lethal and sublethal concentrations of kanamycin were determined based on a dosage-response experiment (data not shown) using B₅ + BA10 medium with various concentrations of kanamycin (Table 1.).

GUS STAINING. The GUS staining solution used throughout all experiments included 0.5 mg·mL⁻¹ X-gluc (cyclohexylammonium salt) (Gold Biotechnology, Inc., St. Louis), 0.5% (v/v) Triton-100, 80% of 50 mmol NaPO₄ (pH 7.0)(v/v), and 20% (v/v) methanol anhydrous (stored at -20 °C) (Thomas et al., 1992). Before adding the GUS staining solution, explants were washed once with 0.4 mol mannitol and then with 100 mmol NaPO₄. After the staining solution was added, explants were incubated at 37 °C in darkness until GUS blue sectors were visible (about 5 to 12 h) and destained with 70% ethanol. Several changes of 70% ethanol were necessary to eliminate chlorophyll. Explants were subsequently examined for GUS expression with a dissecting microscope.

INFECTION RATE. GUS activity was determined by the histochemical method as described above and expressed as GUS blue sectors. An explant was considered infected if there was at least one GUS blue sector. In a few studies, infection rates were estimated by observing the GUS blue sector in only certain tissues. Levels of GUS expression, when compared, were evaluated based on the number of GUS blue sectors per explant. Therefore, the infection rates were calculated using the number of infected explants divided by the total number of explants for each replicate. Factorial or split-plot designs, either in a completely random design or in randomized complete-block arrangements, were used

Fig. 2. Effect of dark preconditioning and cytokinin [benzyladenine BA]) concentrations on infection rate of the common-bean genotype 'Othello'. Data are least square means from two replications. Each bar represents standard error of a least squares (ls) mean. No error bar was presented for each column in the light treatment due to the zero infection. Treatment ls means with the same letters are not significantly different as determined by Duncan's multiple range test (DMRT) at $P \leq 0.05$.

with at least two replications in each experiment. Means were presented as least square means (lsmeans) due to some missing data. Duncan's multiple range tests (DMRTs) or protected LSDs were used for mean comparisons only when F test showed statistical significance ($P \leq 0.05$).

Results and Discussion

DARK PRECONDITIONING AND PRECULTURE OF EXPLANT SEEDLINGS WITH CYTOKININ. Culturing seedling node explants in darkness before cocultivation increased *A. tumefaciens* infection rates ($P \leq 0.001$) compared to those grown under light treatment (Fig. 2). In addition, various levels of BA affected infection rate, with 20 μmol being optimal. Pretreating explants with different growth regulators also influenced gene transfer in other crops (Akama et al., 1992; Bondt et al., 1994; Jacq et al., 1993; Janssen and Gardner, 1993; Li et al., 1992; Sangwan et al., 1991). Initially, it was presumed that seed germination and preculturing seedlings in darkness might increase explant susceptibility to *Agrobacterium*, because etiolated seedlings had thinner cell walls and cuticles. This treatment could also affect chemical composition of the seedlings and was also reported to promote shoot organogenesis (Mohamed et al., 1992). The dark treatment was then used in all subsequent experiments unless otherwise specified.

SUSCEPTIBILITY OF BEAN CULTIVARS TO AGROBACTERIUM STRAINS. Preliminary screening for *Agrobacterium*-host compatibility using A2760 and EHA105 included two bean genotypes—'PC50'

(A) and 'Othello' (MA). The strain EHA105 induced fewer GUS blue sectors than A2760 on both bean genotypes ($P \leq 0.05$) (Table 2). 'PC50' was more susceptible than 'Othello' to both strains. Infection rates, however, were not different either between the two bean genotypes or between the two *Agrobacterium* strains, based on percentage of chimeric shoots with GUS blue sectors (data not shown). This was due to higher infection of cotyledons with strain A2760 than with strain EHA105. No significant interactions were detected between strains and genotypes ($P > 0.05$) for infection rate on cotyledonary tissues. No difference in infection was observed between strains for meristematic nodal regions, which were involved in direct shoot regeneration in our protocols. However, because of its greater infection ability, strain A2760 was chosen for use in the remaining experiments.

Using the same transformation procedure as above, nine additional bean genotypes were screened as follows: navy bean 'Seafarer', black bean ICA 'Pijao', red bean Zamorano, black bean 'Compuesto Nigra Chimaltenango', pink bean Red Mexican 235, red bean Garbancillo Zarco G15821, black bean Bolita (all MA), white bean Alubia 33-1, and cranberry bean (both A) origin. They represented centers of subdomestication of MA and A origins, respectively (Schoonhoven and Voysest, 1991; Skroch et al., 1992). Susceptibility (as determined by infection rate) of these genotypes to strain A2760 was significantly different ($P \leq 0.05$) as determined by protected LSD. All MA bean genotypes were not infected by this strain (no infection) except for ICA 'Pijao' and G15821, which showed 3.9% and 6.3% infection rates, respec-

Table 2. Host $\times$ *Agrobacterium tumefaciens* interaction between two common bean genotypes and *Agrobacterium* strains.

<i>Agrobacterium</i> strains	PC 50		Pinto Othello	
	Infection rate (%) ²	No. GUS sectors per explant ²	Infection rate (%)	No. GUS sectors per explant
A2760	84.4 a (13.6)	9.8 (1.1)	51.9 b (17.5)	4.6 (1.2)
EHA105	48.1 b (13.6)	4.2 (1.4)	21.8 c (13.6)	2.8 (1.3)

²Data are least square (ls) means and from three replications. Numbers with the same letters are not significantly different ($P > 0.05$) as determined by protected LSD. The numbers within parenthesis indicate the standard error of a lsmean. GUS = β -glucuronidase.

Table 3. Effect of explants, derived from either mature or immature seeds, of three common bean genotypes on infection rate and β -glucuronidase (GUS) gene expression.

Bean genotypes	Immature explant		Mature explant	
	Infection rate (%) ²	No. GUS sectors per explant ²	Infection rate (%)	No. GUS sectors per explant
Alubia	51.1 b (0.63)	19.9 (12.9)	85.3 a (0.96)	148.6 (12.9)
Pinto Othello	6.3 d (0.63)	2.5 (0.7)	42.8 c (0.96)	21.5 (21.2)
ICA Pijao	0.0 f (0.00)	0.0 (0.0)	4.0 e (0.96)	0.7 (2.7)

²Least square (ls) means of a duplicate. Numbers followed by different letters indicate a significant difference ($P \leq 0.05$) as determined by DMRT. The numbers within parenthesis indicate the standard error of a lsmean.

Table 4. Effect of cytokinin (BA), auxin (2,4-D), and filter paper on β -glucuronidase (GUS) expression during cocultivation of two common bean genotypes with *Agrobacterium tumefaciens* strain A2760.

Treatments	Great northern Harris		Pinto Othello	
	Infection rate (%) ²	No. GUS sectors per explant ²	Infection rate (%)	No. GUS sectors per explant
BA, 10 mM	19.4 b	1.4 (2.4)	24.0 b	2.6 (2.0)
2,4-D, 20 mM	0.0 a	0.0 (0.0)	14.0 a	2.0 (2.6)
Filter paper	40.0 c	3.3 (1.6)	50.0 c	23.6 (2.1)

²Least square (ls) means of three replications. Numbers with the same letters within a column are not significantly different ($P > 0.05$) as determined by DMRT. The numbers within parenthesis indicate the standard error of a lsmean.

tively. The two A genotypes were more susceptible to the strain than the MA genotypes, with Alubia 33-1 being most susceptible (85.7%) and cranberry bean ranked second (41.4%).

EXPLANT MATURITY. Mature and immature explants were prepared from three susceptible genotypes as determined above following procedures 2 and 3, respectively. A significant interaction ($P \leq 0.001$) between genotypes and explant maturity was also found, suggesting that the susceptibility of different bean genotypes changed with maturity (Table 3). Strain A2760 induced higher infection rates ($P \leq 0.001$) on mature than immature explants of all three genotypes. The GUS activity, as determined by the number of GUS blue sectors, also showed higher levels in mature than in immature explants. This effect of maturity has not been reported previously.

EFFECT OF PLANT GROWTH REGULATORS IN COCULTIVATION MEDIA. Gene transfer could be inhibited by auxin (Li et al., 1992) and enhanced by cytokinin (Bondt et al., 1994) and filter paper (Janssen and Gardner, 1993). Filter papers were superior for inducing GUS activity, whereas auxin (2,4-D) reduced the infection rate and GUS gene expression (Table 4). Explants cocultivated on filter paper also showed more GUS blue sectors than those on other cocultivation media. The reason for the enhanced GUS expression from the control treatment on filter paper is not clear. It is possible that the phosphate starvation of *Agrobacterium* cells (Aoyama et al., 1991; Li et al., 1994), due to the lack of nutrient supply on the filter paper, enhanced DNA transfer.

TYPE OF COCULTIVATION MEDIUM, EXPLANT AGE, AND SELECTION SCHEME. Two media for cocultivation, liquid and semisolid (Table 1), were examined across two different explant ages and selection schemes for their effects on infection rate and GUS gene expression using procedure 2 for explant preparation. In spite of no difference in explant survival rate (data not shown), the percentage of explants expressing GUS activity cocultivated on the semisolid medium was significantly higher over two explant ages (Table 4). In contrast, explants cocultivated in liquid medium did not show any *Agrobacterium* infection when no selection pressure was imposed. These results confirm previous findings in peanut cocultivation where transformation efficiency was greater on a semisolid medium than on a liquid medium (Mansur et al., 1993). Additionally, 8-d-old explants were consistently found to be more susceptible to strain A2760 than 4-d-old explants ($P \leq 0.05$), no matter whether or not a postcocultivation selection was imposed. On the other hand, kanamycin ($75 \text{ mg} \cdot \text{L}^{-1}$) in the medium enhanced the recovery of transformed cells significantly ($P \leq 0.05$). This was also demonstrated by Hinchey et al. (1988) in

soybean transformation, even when liquid coculture medium was used (see also Table 5). We consider this kanamycin concentration lethal, based on our dosage response study in which $25 \text{ mg} \cdot \text{L}^{-1}$ of kanamycin bleached 75% of shoots, whereas $50 \text{ mg} \cdot \text{L}^{-1}$ of kanamycin completely prevented shoot formation of common bean cotyledons (data not shown).

LENGTH OF COCULTIVATION. The length of cocultivation required for achieving maximum gene transfer was genotype dependent, ranging with most plant species from 2 to 5 d. The highest infection rate was observed at 6 d at 25°C and at 8 d at 28°C . When explants were cocultivated for 6 d at 25°C in darkness, 4% of the explants, or 14.3% of the shoots or buds, showed GUS activity throughout the entire bud. Explant survival rate was reduced in darkness (Zhang, 1996), but increased even in dim light during the cocultivation periods (data not shown). Dim light also greatly enhanced shoot and bud induction without reducing transformation rates during this 6-d period (data not shown).

In general, the higher *A. tumefaciens* infection rates also corresponded to the higher levels of GUS activity, as evaluated by the number of GUS blue sectors. Apparently, little infection occurred in meristematic areas compared with cotyledonary tissues. The reason for this difference is not clear. Because only meristematic regions are competent for shoot regeneration of *P. vulgaris* using direct organogenesis, increasing infection in these regions was critical and, therefore, was our main concern throughout this investigation. This was eventually accomplished using optimized procedures that have not been reported previously. Thus, the recommended overall procedures for transforming common bean should include 1) using susceptible genotypes and explant tissues derived from mature seeds, 2) germinating seeds on B₅BA20 agar medium for 2 weeks in darkness or on a moistened filter paper for 1 week in a 16-h photoperiod, 3) thoroughly wounding explant tissues at nodal regions following the explant preparation procedures as described above, 4) dipping explants in logarithmic phase ($\text{OD}_{650} = 0.8\text{--}1.0$) of *A. tumefaciens* cell suspension for 5 to 15 min, blotting dry on a paper towel, followed by a 6-d cocultivation on the B₅ agar medium in the presence of BA, or a 3-d cocultivation on a moistened filter paper (only for explants germinated on filter papers) in a dim light, 5) culturing explants on a postcocultivation selection medium (B₅BA10) containing lethal concentrations of selection reagents ($75 \text{ mg} \cdot \text{L}^{-1}$ kanamycin in our case), and transferring antibiotic resistant shoots (0.5 cm in length) into B₅ rooting medium with reduced concentrations of selective agents (e.g., $50 \text{ mg} \cdot \text{L}^{-1}$ kanamycin or lower).

Table 5. Effect of types of cocultivation media, explant age, and postcocultivation selection on infection rate and β -glucuronidase (GUS) gene expression of common bean genotype 'Othello'.

Medium type	Explant age	GUS staining 0 d postcocultivation, K_0^z		GUS staining 21 d postcocultivation, K_{75}^y	
		Infection rate (%) ^x	No. GUS sectors per explant ^x	Infection rate (%)	No. GUS sectors per explant
B5, semi solid	4-d seedlings	69.3 c (5.4)	15.9 (3.4)	83.1 b (5.4)	31.1 (3.1)
	8-d seedlings	71.1 c (8.2)	19.7 (3.7)	100.0 a (9.3)	28.1 (4.9)
MS(-), liquid	4-d seedlings	0 e (0.0)	0.0 (0.0)	9.8 d (9.3)	2.1 (7.4)
	8-d seedlings	0 e (0.0)	0.0 (0.0)	69.8 c (9.3)	4.0 (9.3)

^z K_0 = no kanamycin present in medium.

^y K_{75} = $75 \text{ mg} \cdot \text{L}^{-1}$ kanamycin present in medium.

^xLeast square (ls) means of two replications. Numbers with the same letters are not significantly different ($P > 0.05$) as determined by protected LSD. The numbers within parenthesis indicate standard error of a ls mean.

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