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Agbiogeneric soybean with glyphosate tolerance: Genetic transformation of new Colombian varieties

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Julian Mora-Oberlaender[1*], Yadira Rodriguez-Abril[1,2], Meike Estrada-Arteaga[1,2], Luisa Galindo-Sotomonte[3], Juan David Romero-Betancourt[1], Jenny Paola Jiménez-Barreto[1,2], Camilo López-Carrascal[4] and Alejandro Chaparro-Giraldo[1]

Abstract: *Soybean (*Glycine max *L.) is a major global crop. Genetic modification of this crop can contribute to enhance yields, reduce pesticide use, and facilitate sustainability, but there is a need for locally adapted genetically modified varieties. Glyphosate tolerant soybean developed under an agbiogeneric model is an attractive prospect as the technology is now mostly in the public domain. This research evaluates the potential for transformation of three soybean varieties (BR1, BR2, and FNS) and an established variety (SK7). The study investigates the interaction between these genotypes and two A. tumefaciens strains, establishes a selection protocol using glyphosate, compares two explant types and evaluates a bacterial removal protocol. Our findings highlight the importance of plant genotype and bacterial strain specificity in the transformation process, with variety BR1 consistently showing the best performance. While challenges remain, this study advances in the development of glyphosate-tolerant soybean varieties as agbiogenerics.*

Keywords: *Herbicide tolerance, public domain,* Agrobacterium tumefaciens*,* Glycine max*, GM crops*

***Corresponding author:**
E-mail: jomorao@unal.edu.co
ORCID: 0000-0003-0304-2380

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[1] Universidad Nacional de Colombia, Grupo de Ingeniería Genética de Plantas, Carrera 30 # 45-03, Bogota, Colombia
[2] Federación Nacional de Cultivadores de Cereales, Leguminosas y Soya, kilómetro 1, vía Cota Siberia, Vereda El Abra Cota, Cundinamarca, Colombia
[3] Instituto de Genética, INTA - Instituto Nacional de Tecnología Agropecuaria, El Ñandú y Gabriel de Aristizábal s/n, Provincia de Buenos Aires, Argentina
[4] Universidad Nacional de Colombia, Facultad de Ciencias, Departamento de Biología, Carrera 30 # 45-03, Bogota, Colombia

INTRODUCTION

Soybean (*Glycine max* L.) is one of the most important crops worldwide. In 2021 almost 130 million hectares were planted, in which over 371 million tonnes were produced (FAOSTAT 2023). Around 100 countries produce soybeans, but over 80% of this production takes place in Brazil, USA and Argentina. Soybean and its derivate products have a wide range of applications as food and feed and for the production of industrial goods. In 2021 Colombia had an annual soybean production of 122 500 tons planted in 48 400 hectares. This production covers only a portion of the demand and results in the need to import more than 1 million tons of whole soybean, oil and soybean meal each year to meet the demand for this agricultural product (Fenalce 2023).

Agrobacterium mediated genetic transformation is an important biotechnological tool that can complement conventional breeding in order to increase crop yields, reduce the amount of pesticides used and increase profits for farmers (Klümper and Qaim 2014). Herbicide tolerance conferred

through genetic modification has been widely implemented in soybean with adoption rates of over 90% in USA, Brazil and Argentina (ISAAA 2019). In particular, glyphosate tolerant soybean allows the use of only one herbicide, a longer period of weed control, less herbicide application events, less use of higher toxicity herbicides, the implementation of no-till agriculture and therefore less greenhouse gas emissions (Brookes and Barfoot 2020).

Introducing conventional or genetically modified (GM) soybean varieties from a subtropical region into a tropical area may involve the adaptation of the crop to the differences in photoperiod and soil conditions, among other factors (Valencia and Ligarreto 2010). Developing locally adapted varieties that also incorporate characteristics provided by modern biotechnology can help supply Colombian and other South American or tropical soybean producers with improved varieties that can increase production and thus partly replace imports.

One of the aspects to consider in the commercial development of a biotechnological crop is the protection of intellectual property (IP). There may exist, associated to a genetically modified crop, a network of patents that protect different aspects of its development, such as genes, regulatory elements, vectors, transformation and regeneration systems, among other aspects (Chaparro-Giraldo and Ávila 2013, Rojas-Arias et al. 2017). These considerations both limits and increases the cost of the commercial release process.

Recently, many patents related to the first commercially released genetically modified crops have expired, as they reached their 20-year protection period in many jurisdictions. This situation allows for the emergence of generic genetically modified crops, or agbiogenics (Jefferson et al. 2015), in which elements and processes in the public domain can be used for the development of new GM varieties.

Rojas-Arias et al. (2017) identified the opportunity for the development of agbiogeneric glyphosate tolerant soybean varieties considering the IP landscape for this characteristic. Jiménez et al. (2020) performed a freedom to operate (FTO) analysis and, using elements from the public domain, designed an expression cassette for the transformation of soybean plants with an optimized version of the *Agrobacterium tumefaciens cp4epsps* gene. Rojas et al. (2018) successfully transformed two Colombian soybean varieties, P29 and Soy SK7 (SK7), using a protocol involving cotyledonary node cocultivation with *A. tumefaciens* strains AGL0 or EHA105.

In 2019 the Colombian National Federation of Cereal, Legumes and Soybean Growers (FENALCE) introduced three new conventional soybean varieties: Brasilera 1 (BR1), Brasilera 2 (BR2) and FNS01 (FNS). These varieties were bred specifically for their high yield and adaptation to the major soybean producing region in Colombia and may prove useful in other tropical areas. These were selected as candidates for the development of new agbiogeneric events due to their agronomic characteristics and their commercial potential.

Soybean is considered a recalcitrant species for genetic transformation mediated by *A. tumefaciens* due to constraining factors that need to be optimized such as the specific interaction between plant genotype and bacterial strain, plant variety dependent regeneration, toxicity of antibiotics, *A. tumefaciens* overgrowth, *in vitro* selection and explant type, among others (Mangena 2019).

In this study we adapted previously developed methodologies (Rojas et al. 2018) in order to evaluate the transformation potential of the three above mentioned soybean varieties (BR1, BR2 and FNS) and one established commercial variety (SK7). Specifically, in the context of the development of an agbiogeneric crop with glyphosate tolerance, we evaluated the interaction between these varieties and two *A. tumefaciens* strains, established an *in vitro* selection protocol using glyphosate as the selection agent, compared two different explant types and established a protocol that minimizes the use of antibiotics during bacterial removal without compromising reactivation and overgrowth.

MATERIAL AND METHODS

Plant Material and Explants

Four different Colombian soybean varieties were used in this study. The seeds for BR1, BR2 and FNS were provided by FENALCE and for SK7 by Semillas Kamerún, a local soybean breeder and seed producer.

Mature soybean seeds with no sign of external damage were selected and surface-sterilized with chlorine gas as

described by Rojas et al. (2018). Selected seeds were placed in a sealed chamber where chlorine gas was produced by the reaction of 4.1 ml 10 N HCl with 100 ml 5% NaClO and left for 16h.

To obtain half-seed explants, sterilized seeds were imbibed in sterile water for 24h and then cut in half longitudinally with a scalpel to separate both cotyledons and the seed coats were removed (Li et al. 2017). To obtain cotyledonary node explants, sterilized seeds were placed on 0.7% solid agar medium and left to germinate for 5 days at 28 °C under a 16/8 (light/dark) photoperiod. After this time, the seed coats were removed, using a scalpel the hypocotyl was cut 5 mm below the cotyledonary junction and the cotyledons were separated. The plumule was removed from the cotyledons and six longitudinal incisions were made at the cotyledonary node (Zhang et al. 1999, Rojas at al. 2018).

Bacterial strains and vectors

Two different *Agrobacterium tumefaciens* strains were used. Strain AGL0 was acquired from an American Type Culture Collection under the denomination ATCC®BAA-100™ and strain EHA105 was kindly provided by Dr. Jershon López from Cenicaña. Two different binary plasmids derived from pCAMBIA 1301 were used (Jiménez-Barreto et al. 2020), both from which the Hygromycin resistance gene was removed and in which the E-IGP expression casette was included. This expression casette inlcudes the soybean polyubiquitin promoter (GmUbi), the Chloroplast Transit Peptide (CTP) from *Petunia hybrida*, a version of the *cp4epsps* gene optimized for expression in soybean and the Nopaline Synthase (NOS) terminator sequence (Jiménez-Barreto et al. 2020). One of the plasmids (pGEIGP) carried the *uidA* (GUS) gene originally included in the binary vector and the other had this gene removed (pEIGP).

Bacterial strains were transformed by electroporation, selected in medium containing kanamycin (50 mg L^{-1}) containing and confirmed by colony PCR using primers 2J (Table 1).

In vitro selection using glyphosate

We tested a previously developed protocol for *in vitro* selection of potentially transformed soybean explants carrying the *cp4epsps* gene using glyphosate in regeneration media (Rojas et al. 2018) on soybean varieties BR1, BR2 and FNS.

In each of six repetitions, 100 cotyledonary node explants from each of the three varieties were obtained as described above and placed in shoot induction medium (SIM) (1X B5 salts, 1X Gamborg vitamins, 3 g L^{-1} sucrose, 1.67 mg L^{-1} BAP, 3 mM MES, 0.7% PTC agar, pH 5.7) for two weeks at the same temperature and photoperiod as described above. After this time, 25 explants of each variety were transferred to fresh SIM with four different glyphosate concentrations (T0=0, T1=50, T2=100, T3=150 and T4=300 µM) after removing the remaining hypocotyl with a scalpel. After two weeks, explants that produced shoots had the remaining cotyledon removed and were transferred to shoot elongation medium (SEM) (1X B5 salts, 1X Gamborg vitamins, 3 g L^{-1} sucrose, 0.7 mg L^{-1} BAP, 0.5 mg L^{-1} GA_3, 0.1 mg L^{-1} IAA, 50 mg L^{-1} glutamine, mg L^{-1} asparagine, 3mM MES, 0.7% PTC agar, pH 5.7) containing 1/4 of the concentration of glyphosate as in SIM (T0=0, T1=12, T2=24, T3=36 and T4=72 µM). SEM was renewed every two weeks and shoots that reached 3 cm or more were individualized and transferred to rooting medium (RM) (0.5X MS salts, 1.5 g L^{-1} sucrose, 0.7% PTC agar, pH 5.7). The number of explants that regenerated and produced elongated shoots was recorded.

Table 1. Primer sequences and PCR conditions

Target	Primers	Sequence	Amplicon length (bp)	Amplification conditions
Soybean Lectin	Le1-F/R (Schmidt and Parrot 2001)	5′ TCCCGAGTGGGTGAGGATAG-3′	66	94 °C x 2 min, 35 x (94 °C x 30 s, 54 °C x 30 s, 72 °C x 30 s), 72 °C x 5 min
		5′ TCATGCGATTCCCCAGGTAT-3′		
cp4epsps (mod.)	2J-F/R (Rojas et al. 2018)	5´CTTTGCTGAAGGAGCTACCG-3′	205	94 °C x 2 min, 35 x (94 °C x 15 s, 54 °C x 45 s, 72 °C x 30 s), 72 °C x 10 min
		5´GTGATCGAGATGCGTAGCAA-3′		
pTiBo542	pTiBo542-F/R (Deeba et al. 2014)	F: 5′-CCCGCTGAGAATGACGCCAA-3′	766	94 °C x 3 min, 35 x (94 °C x 20 s, 61 °C x 20 s, 72 °C x 40 s), 72 °C x 20 min
		R: 5′-CCTGCGACACATCGTTGCTGA-3′		

GUS expression assays

In order to test the interaction between bacterial strain and soybean genotype, the two *A. tumefaciens* strains carrying the pGEIGP vector were used to transform cotyledonary node explants of the four soybean varieties. Twenty explants were used for each strain-variety combination and the assay was repeated once.

Bacterial biomass for cocultivation was obtained by adding 60 µl of bacterial inoculum to 30 ml of LB broth containing 50 mg L^{-1} kanamycon and left in agitation at 28 °C and 200 rpm overnight until optical density at 650 nm (OD_{650}) reached 1.0. This bacterial suspension was centrifuged at 8000 rpm for 4 min and the supernatant was discarded. The pellet was resuspended in 25 ml of cocultivation broth (CCB) (0.1X B5 salts, 1X Gamborg vitamins, 3 g sucrose, 1.67 mg L^{-1} BAP, 0.25 mg L^{-1} GA_3, 20mM MES, 200 µM acetosyringone, pH 5.7) (Rojas et al. 2018). Prepared explants were submerged in resuspended CCM and left for 30 min, then transferred to cocultivation medium (CCM) (CCB with 0.7% PTC agar) with the adaxial side in contact with the medium and left in the dark at 28 °C for three days.

Cocultivated explants were incubated in GUS staining solution (Jefferson et al. 1987, Li et al. 2017) for 48 h in dark at 37 °C, then rinsed in 70% ethanol for one day and observed under a stereoscope.

A similar assay was performed with strain AGL0 and variety BR1 testing three different OD_{650} in the preparation of the inoculum: 0.6, 0.8 and 1. Here, 20 explants were used for each treatment.

A third GUS expression experiment compared cotyledonary node explants and half-seed explants (obtained as described above). Here, 30 explants of each type were cocultivated with the strain AGL0 grown to OD_{650} of 0.6. All other procedures were carried out as already described.

Transformation assays

Cotyledonary node explants of the four soybean varieties were obtained and cocultivated with *A. tumefaciens* AGL0 grown to OD_{650} 0.6 as described above. Half the explants were placed on petri dishes with CCM covered with sterile filter paper and half directly on the medium. After three days at 28°C explants were washed by agitation in sterile water with carbenicillin (400 mg L^{-1}) for 40 minutes. Half the explants from each treatment (CCM + filter paper o just CCM) were washed three times and the others were washed one time. Explants were then transferred to SIM added with 350 mg L^{-1} cefotaxime and 100 mg L^{-1} timentin where bacterial reactivation, if any, was registered.

Subsequent transformation assays used filter paper on CCM and only one wash cycle for bacterial removal. Explants remained in SIM (with antibiotics) for two weeks and then transferred to medium with 100 µM glyphosate, except control explants for which glyphosate-free medium was used. After another two weeks, explants that produced at least one shoot were transferred to SEM (added with 350 mg L^{-1} cefotaxime, 70 mg L^{-1} vancomicin and 24 µM glyphosate). This medium was renewed every two weeks and elongated shoots (3 cm or longer) were collected and individually placed in RM. Containers with plants derived from explants that developed roots were gradually opened to acclimate plants. These were then removed from media and its roots carefully washed to remove all remaining agar. The plants were then potted in a sterile mixture of soil, peat and sand (1:2:2) and kept with a perforated lid for five days after which the lid was removed.

Transgene detection

One leaf was collected from hardened plants for genomic DNA extraction using the CTAB buffer method (Rojas et al. 2018). DNA was quantified by NanoDrop 2000 (Thermo Fisher Scientific). Plant genomic DNA quality was evaluated by electrophoresis and amplification of the soybean lectin gene using the primers Le1-F/R (Schmidt and Parrot 2001) respectively. The presence of remaining *A. tumefaciens* in plant tissues was ruled out by amplification with the primers pTIBO-F/R (Deeba et al. 2014). The presence of the modified version of the *cp4epsps* gene was evaluated with the primers 2J-F/R (Rojas et al. 2018).

Statistical analysis

In vitro tissue culture and transformation assays were evaluated using R software with a significance level of 0.05. Potential differences were assessed through non parametric tests: Kruskal-Wallis and Wilcoxon rank sum test with Bonferroni continuity correction.

RESULTS AND DISCUSSION

Despite an abundance of protocols, approaches and attempts at improving genetic transformation of soybean, it is still considered a recalcitrant species (Xu et al. 2022). Successful transformation of soybean with *A. tumefaciens* depends on factors such as culture medium, plant genotype, type of explant, bacterial strain and density, and their interaction (Mangena 2019). Rojas et al. (2018) reported the transformation of two Colombian soybean varieties with a codon-optimized version of the *A. tumefaciens cp4epsps* gene using only elements in the public domain and showed the importance of the plant genotype and strain specificity.

In order to evaluate this previously established protocol on newly developed soybean varieties of commercial interest, transient expression of GUS in cocultivated cotyledonary explants was used to compare the infection efficiency of two different *A. tumefaciens* strains on these three new varieties (BR1, FNS and BR2) and previously reported SK7 (Figure 1A). At least one explant from each variety was effectively infected by each bacterial strain, but the efficiency of infection of BR1 was significantly higher with both strains compared with the other three varieties, except for SK7 with EHA105 (Figure 1A). Infection efficiency of soybean varieties FNS and BR2 was lower with both strains. *A. tumefaciens* EHA105 performed slightly better with varieties FNS and SK7, while AGL0 was slightly more efficient infecting BR1. This last combination of variety/strain had the overall best performance. In a similar assay, *A. tumefaciens* strain AGL0 at three different growth phases was tested on soybean variety BR1 (Figure 1B). In this assay, 60 explants were cocultivated with this bacterial strain grown to three different optical densities (20 explants for each OD_{650}). The expression of GUS was evaluated visually in the explant tissue as an indicator of effective infection. At OD_{650} 0.6 infection rate reached 100% (20/20 explants), while at OD_{650} 0.8 and 1.0 it was 70% (14/20) and 75% (15/20) respectively.

Using the combination BR1/AGL0 at OD_{650} 0.6, another transformation assay was performed in order to compare two different explant types: cotyledonary node and half-seed. Using transient GUS expression in cocultivated explants as an indicator of infection efficiency, a higher (although not statistically significant) infection of half-seed explants (97% versus 83% for cotyledonary node) was observed. Using four different soybean varieties Paz et al. (2006) reported a transformation efficiency 1.5 times higher for half-seed explants compared to cotyledonary nodes. Our results evaluated infection efficiency rather than final transformation efficiency, but as cotyledonary node explants have been routinely used in the established protocols, further regeneration and transformation assays were carried out with this explant type.

The aim of *in vitro* selection during regeneration of potentially transformed plant tissues is to reduce the possibility of obtaining false positives (regenerated explants that are not effectively transformed) or chimeric plants without

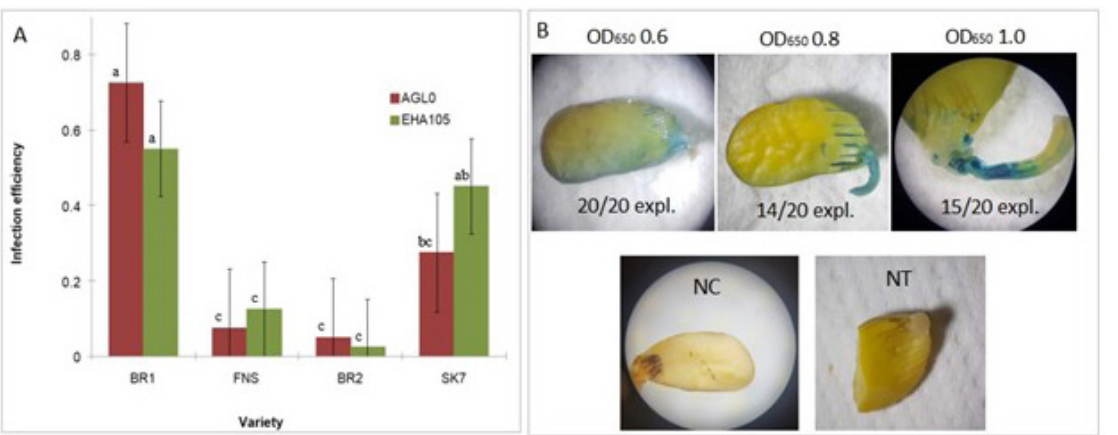

Figure 1. GUS expression in *A. tumefaciens* infected soybean explants. A, GUS positive explants for each soybean variety and bacterial strain used. Different letters denote significant differences between values. B, Soybean BR1 explants assayed for GUS expression after cocultivation with *A. tumefaciens* strain AGL0. Top: Explants where expression was detected after cocultivation with *A. tumefaciens* AGL0 at OD_{650} 0.6, 0.8 and 1.0; NC: Negative control, explant not cocultivated; NT: Cocultivated explant where no GUS expression was detected.

using a selection method that prevents transformed tissues from regenerating. Using glyphosate as the selection agent as has been previously described (Clemente et al. 2000, Rojas et al. 2018) eliminates the need for an additional selection gene in the transformation process, thus reducing potential IP and regulatory burdens. Four selection treatment using glyphosate at different concentrations during weeks 3-4 of shoot induction and during shoot elongation were evaluated. Although the effect of glyphosate in *in vitro* culture can be seen during all phases of regeneration (from shoot induction until hardening) we evaluated its potential as a selection agent for the four soybean varieties up to the shoot elongation stage (Figure 2). An overall decrease of elongation efficiency is evident for the three soybean varieties evaluated as glyphosate concentration in the culture media increases (Figure 2). The effect of the herbicide is evident when comparing selection treatments with the regeneration of explants in glyphoste-free media. Selection treatment T4, in which 300 µM and 72 µM of glyphosate was used in shoot induction and shoot elongation stages respectively was very effective at preventing regeneration, but may be too stringent for use in transformation assays. On the other hand, T1 (50µM/12µM) did not differ significatively from the control (T0)

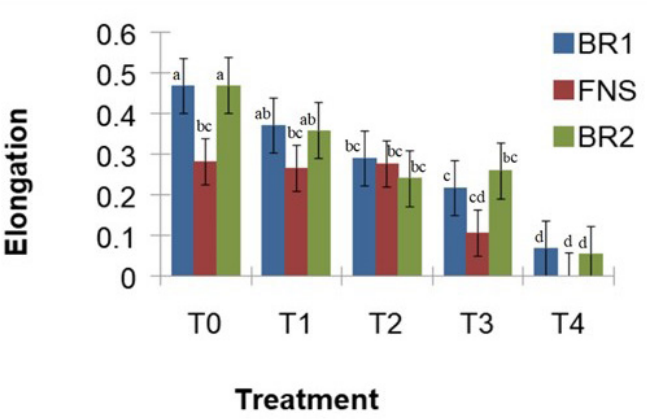

Figure 2. Elongation efficiency of explants from three soybean varieties under four different glyphosate selection schemes. Control: no glyphosate; T1: 50/12; T2: 100/24; T3: 150/36 and T4: 300/72 µM. Different letters denote significant differences between values.

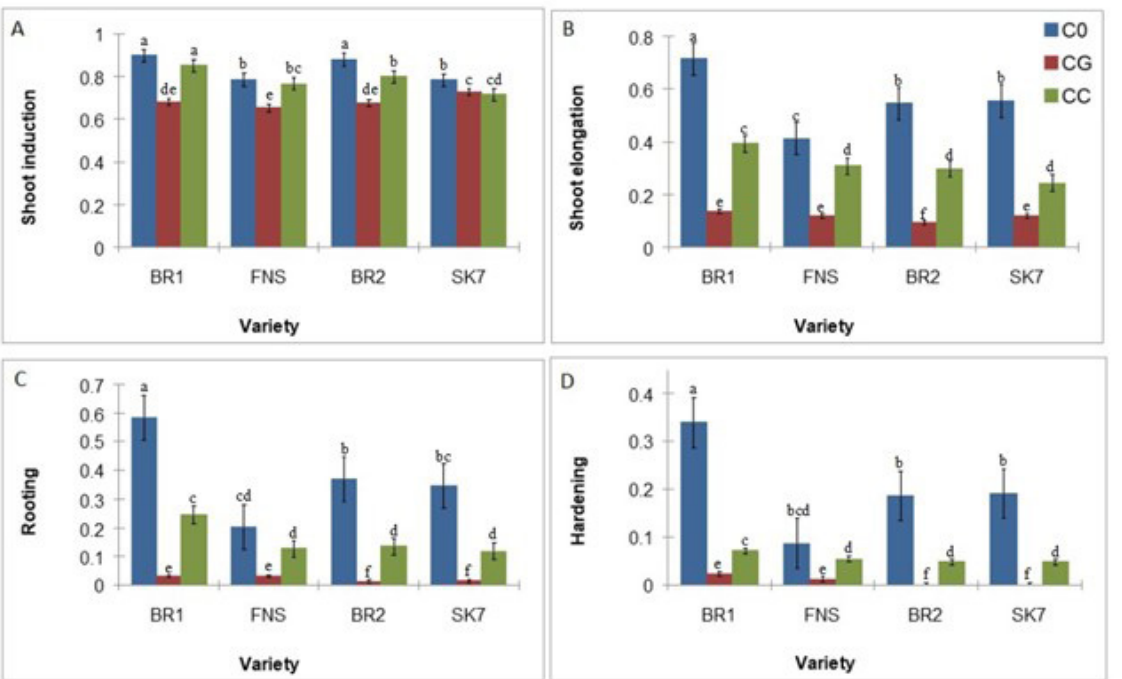

Figure 3. Regeneration efficiency for soybean explants from four varieties at four stages (A, Shoot induction; B, Shoot elongation; C, Plantlet rooting; and D, Plantlet hardening). C0: Explants not subject to cocultivation or selection; CG: explants not subject to cocultivation but under selection scheme 2; CC: explants subject to cocultivation and selection scheme 2. Different letters denote significant differences between values.

and therefore may lead to false positives. T2 and T3 did reduce regeneration significatively and for subsequent transformation assays, T2 (100µM/24µM) was used in order to privilege the obtention of potentially transformed explants.

A. tumefaciens removal from cocultivated explants is a crucial step in order to obtain bacteria-free regenerated plants. Disinfection of explants can be done through mechanical (washing) or chemical (antibiotics) means, or a combination of both. We tested the use of filter paper on solid cocultivation media to reduce the presence of bacteria on the explants and therefore reduce the need for three agitation-rinse cycles. Using only one wash cycle on explants cocultivated without the use of filter paper led to more than 80% reactivation of *A. tumefaciens* during shoot induction. Using filter paper completely eliminated reactivation indistinctively for one or three wash cycles, while some reactivation (~4%) persisted without the use of filter paper despite performing three was cycles. It is thus possible to use only one wash cycle without an increase in bacterial reactivation while reducing antibiotic use.

Eleven different transformation assays were carried out based on the results obtained and above. In these assays, cotyledonary node explants from four soybean varieties were used. *A. tumefaciens* strain AGL0 carrying pEIGP grown up to OD_{650} 0.6 was employed for cocultivation and filter paper was used on cocultivation media. Glyphosate was added to regeneration media during shoot induction and shoot elongation according to selection treatment T2.

As expected, the number of viable explants/plants diminished as regeneration stages progressed, passing from 77% during shoot induction phase to 6% at the end of hardening phase when considering the four varieties under cocultivation (Figure 3). Shoot induction was similar for all varieties, although BR1 and BR2 performed slightly better than the other two with values of 85% and 80% respectively (compared to 76% for FNS and 71% for SK7) (Figure 3A). During this stage, we observed some effect of the use of glyphosate on explants not subject to cocultivation with *A. tumefaciens*, although the full extent of the selection was more evident during later stages of regeneration (Figures 3b-d) in which barely any explants resulted in hardened plants. Consistent with observations presented above, soybean variety BR1 showed a significantly better performance during all stages of *in vitro* regeneration both for explants not subject to cocultivation or selection (controls) and cocultivated explants. For control explants, varieties BR2 and SK7 showed a similar behavior during shoot elongation, rooting and hardening, while FNS produced significantly lower number of successful explants at these stages. However, when looking at cocultivated explants, SK7 produced lower successful elongated shoots, but no significant differences were observed between SK7, BR2 and FNS at rooting or at the final number of hardened plants (Figures 3c, d).

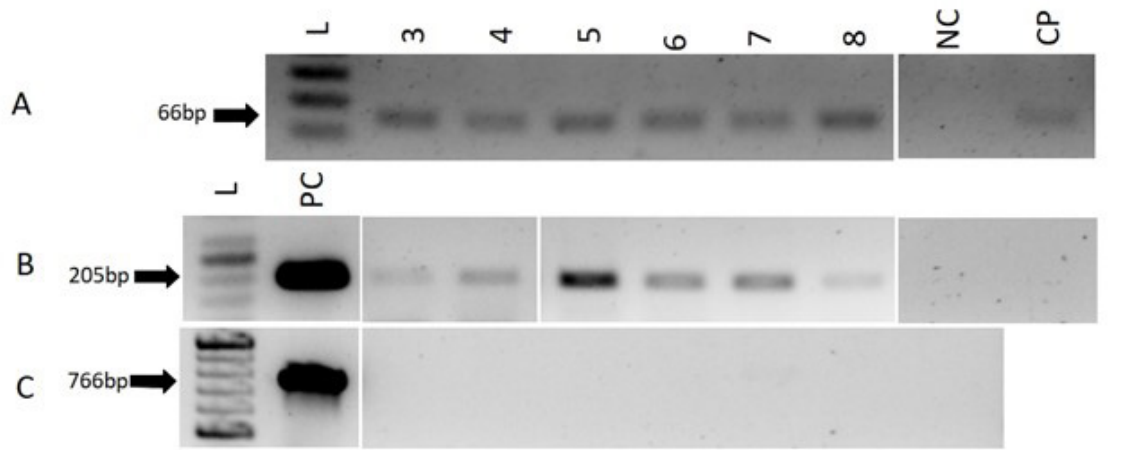

Figure 4. PCR analysis of soybean DNA (see Table 1). A. Soybean endogenous lectin gene, 50bp ladder. B. cp4epsps transgene, 50bp ladder, PC: positive control (pEIGP plasmid). C. *A. tumefaciens* pTiBo542 plasmid, 100bp ladder, PC: positive control (AGL0 DNA). Lanes 3-8 potentially transformed plants. CP: non-transformed plant. NC: negative control.

PCR analysis

Hardened plants that regenerated from cocultivated explants were considered as potential transformants. As shown for six potentially transformed plants (Figure 4) primers Le1-F/R were used to confirm the quality of the soybean DNA extraction, followed by primers 2J-F/R which detect the codon-optimized sequence of the *cp4epsps* gene (Rojas et al. 2018). For these PCR positive plants, the presence of endogenous *A. tumefaciens* carrying the transgene was ruled out by using primers pTiBo542-F/R. This led to the detection of a total of 21 PCR positive plants which included potential transformants of the four different soybean varieties (Figure 4).

Our results show that genetic transformation of new soybean varieties still proves challenging and different aspects of the transformation/regeneration protocol need to be adapted. Still, we were able to evidence differences in the interaction between two *A. tumefaciens* strains and three new Colombian soybean varieties in comparison to previous reports. In particular, of the evaluated genotypes, BR1 seems to present itself as a better alternative for genetic transformation. This represents an advance in the implementation of the agbiogeneric model for the development of glyphosate tolerant soybean as an additional tool for plant breeders.

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