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# Screening of Vietnamese soybean genotypes for Agrobacterium-mediated transgenic transformation

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**ABSTRACT**

Soybean [*Glycine max* (L) Merr.] is one of the most important crops used for human food and animal feed globally. Transgenic soybean covers more than 74% of the global soybean production area, which is an achievement of genetically modified programs. The *Agrobacterium*-mediated method is commonly used for soybean transformation, but the efficiency of this method is affected by various factors including genotypes. Screening of the soybean genotypes suitable for *Agrobacterium*-infection and plant regeneration is the most important step to establish an efficient genetic transformation system. In this study, we screened thirty Vietnamese soybean genotypes including seventeen cultivated soybean genotypes (CSG) and thirteen local soybean genotypes (LCG) for shoot regeneration ability and transient infection via *Agrobacterium tumefaciens* method. Two CSG cultivars, DT22 and VX93, had significantly high efficiencies for shoot regeneration and transient infection compared with the control genotypes Jack and William 82. The shoot regeneration of DT22 and VX93 was 92.32% with 5.75 shoots/explant and 93.35% with 5.92 shoots/explant, respectively, whereas the control genotypes Jack and William 82 had 91.35% with 4.6 shoots/explant and 82.64% with 5.7 shoots/explant. Similarly, the transient infection of DT22 and VX93 was 84% and 86%, respectively, which was comparable with that of Jack (86%) William (82%). The success of transgenic development was confirmed by the β-Glucuronidase staining, PCR, and Basta leaf painting. The results indicated that cultivars DT22 and VX93 could be used for stable *Agrobacterium*-media transformation.

**Keywords:** Soybean, *Agrobacterium*, transformation, transient infection, transgenic.

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## INTRODUCTION

Soybean [*Glycine max* (L) Merr.], one of the most important foods and oil crops, is widely used for human food and animal feed. Significant efforts made from soybean breeding programs have resulted in about a five-fold increment in soybean production during the last decades, although the demand for soybean for food, feed and bio-fuel is increasing (Schmutz et al., 2010). Although the conventional soybean breeding program has made achievements, it has some limitations such as self-pollination inability (Shan et al., 2005). Therefore, there is a need for other approaches such as gene transformation or mutation to soybean improvement. Soybean transformation was first reported in 1988 using an *Agrobacterium tumefaciens* infection with cotyledonary node regeneration (Christou et al., 1988; Hinchee et al. 1988) or particle bombardment of the meristems of immature seeds (McCabe et al., 1988). Of these, *Agrobacterium*-mediated transformation has been

used more widely over the last twenty years. Up to date, 38 events have been approved in 31 countries that helped biotech soybean occupy 74% of soybean area at about 92 million hectares that covered 48% of global biotech crop area (ISAAA, 2019).

An efficient plant regeneration protocol is a prerequisite for the successful application of the genetic transformation approach. Earlier studies have demonstrated that the efficiency of T-DNA transformation depends on many factors such as *agrobacterium* strain, explant types, genotypes, selection system, (Meurer et al., 1998; Liu et al., 2004; Cheng et al., 2004; Paz et al., 2006; Wang and Xu, 2008). To enhance the efficiency of transformation, several significant efforts have been made to establish the regeneration protocol for *Agrobacterium*-mediated transformation. For example, the utilization of different explants such as cotyledonary node (Mante et al. 1989, Sairam et al. 2003), whole cotyledonary node (Ma and Wu, 2008), epicotyl and primary leaves (Wright et al., 1987), primary leaf nodes (Kim et al., 1990), and hypocotyls (Yoshida, 2002); optimal concentration of supplementation such as L-cysteine, sodium thiosulfate, growth hormones, selection agents (Clemente et al., 2000; Olhoft et al., 2003; Cheng et al., 2004; Liu et al., 2008). The genetic factors determine the susceptibility of soybean genotypes to *Agrobacterium* infection and regeneration capacity in the transformation process (Meurer et al., 1998; Donaldson and Simmonds, 2000; Paz et al., 2004). Therefore, screening of suitable soybean genotypes from the germplasm resources for the *Agrobacterium*-mediated genetic transformation has become the focus for optimizing the soybean transformation system and improving the transformation efficiency.

In Vietnam, soybean is one of the important crops used for animal feed and food processing. However, the soybean area is decreasing year by year due to low benefits (100.8 thousand hectares in 2015 to 52.3 thousand hectares in 2019). The major reason for the decreasing soybean production is the low seed yield (about 1.5 tonnes per hectare) that made farmers shift to other crops. Soybean production is meeting only 1/3 of domestic demands that lead to increased import from other countries (average 1.8 million tonnes per year). Vietnamese soybean breeders have been encouraged to improve soybean cultivars with a high value of agronomic traits such as yield, quality, and stress resistance by applying genetic transformation or editing. To determine suitable Vietnamese soybean for high-efficiency *Agrobacterium* transformation, we screened thirty currently growing genotypes using β-glucuronidase (GUS) reporter gene.

## MATERIALS AND METHODS

### Plant materials

Thirty Vietnamese soybean genotypes collected from Vietnam Plant Resource Central were used in this study. Of these, seventeen soybean cultivars were widely planted in Vietnam (cultivated soybean group-CSG), and thirteen cultivars were locally grown (Local soybean group-LSG). Jack and William 82 were used as controls.

### Vector and agrobacterium preparation

The *Agrobacterium tumefaciens* strain EHA105 and plasmid vector pCambia 3301 (Figure 1) were received from Addgene (www.addgene.com). The vector includes a phosphinothricin acetyltransferase (*bar*) gene that confers resistance to herbicide phosphinothricin (PPT), an intron-containing *GUS* gene, and a kanamycin-resistant marker gene for bacterial selection.

Liquid YEP medium (10 $gL^{-1}$ peptone, 5 $gL^{-1}$ NaCl, 5 $gL^{-1}$ yeast extract, pH 7.0) containing 50 $mgL^{-1}$ kanamycin, 25 $mgL^{-1}$ rifamycin, and 50 $mgL^{-1}$ spectinomycin was inoculated with the *A. tumefaciens* EHA105 strain and shaken at 28°C (200 rpm) until the optical density at 600 nm ($OD_{600}$) reached 0.6–0.8. The *A. tumefaciens* culture was centrifuged at 7000 rpm for 15 min at 20°C, and the cell pellet was subsequently resuspended in 15 ml liquid co-cultivation medium (CCM) comprising 0.32 $gL^{-1}$ B5 salts and vitamins (Gamborg et al., 1968), 4.26 $gL^{-1}$ 2-[*N*-morpholino] ethanesulfonic acid (MES, Duchefa, www.duchefa-biochemie.com), 3% sucrose (pH 5.4), filter-sterilized 0.25 $mgL^{-1}$ gibberellic acid (GA3, Duhchefa), 3.3 mM L-cysteine (Sigma, www.sigmadrich.com), 1.67 $mgL^{-1}$ 6-benzylaminopurine (BAP, Duchefa), 1.0 mM dithiothreitol (DTT, Duchefa), and 200 $\mu molL^{-1}$ Acetosyringone (3′,5′-Dimethoxy-4′-hydroxyacetophenone, Sigma).

### Explant preparation and shoot regeneration evaluation

The half-seed explant method used in this study followed the procedure described by Kim et al. (2016). Briefly, mature soybean seeds were surface-sterilized by placing the seeds into a tightly sealed chamber containing chlorine gas, which was produced from a reaction of 95 ml NaCl (12% sodium hypochlorite) and 5 ml 12N HCl, for 16 h. The sterilized seeds were soaked with sterile distilled water at 25°C for about 20 h. The imbibed soybean seeds were cut longitudinally along the hilum to separate the cotyledons and the embryonic axis was excised to obtain half-seed explants (Figure 2A).

To test the shoot regeneration of soybean genotypes, explants were placed in Petri dishes (90 mm ×15 mm) containing solid shoot induction medium (SIM) comprised of 3.2 $gL^{-1}$ Gamborg B5 including vitamins, 0.6 $gL^{-1}$ MES, 30 $gL^{-1}$ Sucrose, 5.3 $gL^{-1}$ agar (Sigma), 1.67 $mgL^{-1}$ BAP, pH 5.6. The number of induced shoots was recorded after 28 days which was defined as: *n*ea/*n*eb×100%, where *n*ea is the number of explants after four weeks of screening and *n*eb is the number of explants before testing.

### Explant preparation and agrobacteria inoculation

Explants were scratched at the embryonic axis by using a No. 11 scalpel blade and dipped in 15 mL of Agrobacterium suspension for 30 min. After inoculation, seven explants were placed upside down on sterile filter paper placed on CCM solidified with 4.8 $gL^{-1}$ agar, and incubated in the controlled growth room under the condition of 18 h/6 h light/dark at 25°C for 5 days.

### Selection and plant regeneration

After 5 days of co-cultivation, explants were briefly washed in liquid shoot induction medium (SIM) containing 3.2 $gL^{-1}$ B5 salt with

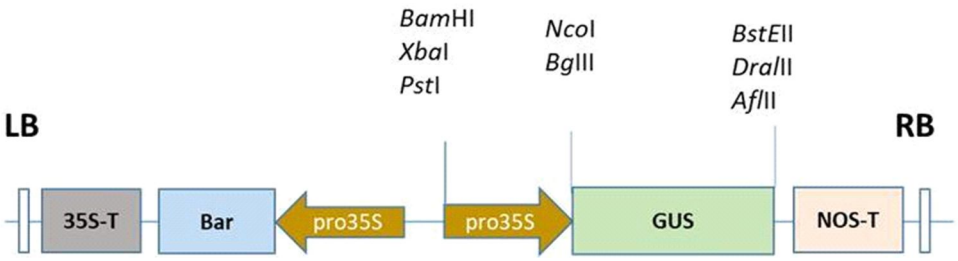


**Figure 1.** T-DNA regions of the binary vector pCAMBIA3301 containing Bar and GUS, driven by the 35S promoter, pro35S; the cauliflower mosaic virus-CaMV35S RNA promoter, 35S-T; CaMV35S polyA; NOS-T, the 3′ terminator region of the nopaline synthase; Bar; phosphinothricin (R). RB—right border; LB, left border.

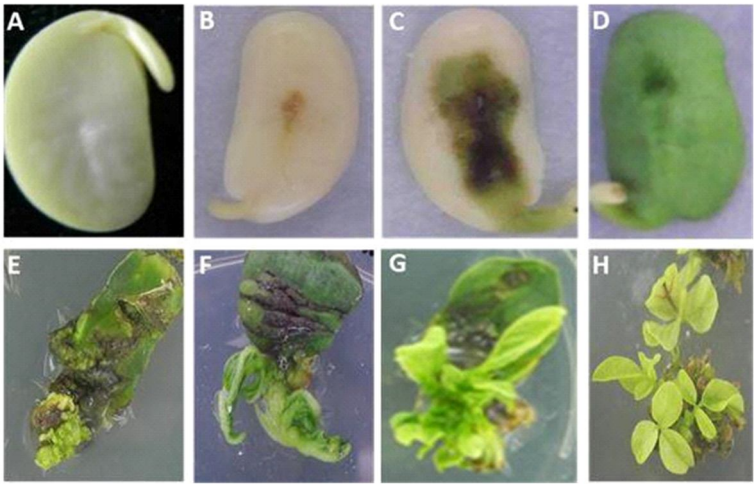


**Figure 2.** The phenotype of explants after 5 days in CCM and 14 days in SIM. A- half seed explant used for transformation; B to D phenotype of explants after 5 days co-cultivated in solid CCM; E to G phenotype of explants after 14 days in the solid shoot induction medium (SIM); H shoot elongation after 14 days in SE medium.

vitamins, 0.6 $gL^{-1}$ MES, 1.67 $mgL^{-1}$ 6-BAP, 250 $mgL^{-1}$ cefotaxime, 50 $mgL^{-1}$ vancomycin, 100 $mgL^{-1}$ ticarcillin, and 3% sucrose, pH 5.6. The explants were then transferred into a solid SIM containing 10 $mgL^{-1}$ PPT and incubated in a growth room at 25°C under an 18 h photoperiod for two weeks. Then, the hypocotyl and shoots were cut-off from the explants and the remaining cotyledons with developing nodules were sub-cultured in a fresh SIM-2 medium containing 5 $mgL^{-1}$ PPT for two more weeks. Then, the half-cotyledon was removed from the explants and transferred into shoot elongation medium (SEM) which was composed of 4.4 $gL^{-1}$ MS salts including B5 vitamins (Murashige and Skoog, 1962), 3% sucrose, 5 $gL^{-1}$ agar, 0.6 $gL^{-1}$ MES, filter-sterilized 50 $mgL^{-1}$ L-asparagine (Duchefa), 100 $mgL^{-1}$ pyroglutamic acid (Duchefa), 0.1 $mgL^{-1}$ indole acetic acid (IAA, Duchefa), 0.5 $mgL^{-1}$ Gibberellic acid (GA3, sigma), 10 $mgL^{-1}$ zeatin (Duchefa), 100 $mgL^{-1}$ ticarcillin (Tic), 250 $mgL^{-1}$ cefotaxime (Cef), 50 $mgL^{-1}$ vancomycin and 5 $mgL^{-1}$ PPT, (pH 5.8). Explants were transferred to fresh SE medium every two weeks until the regenerated shoots were suitable for rooting. Elongated shoots (3–4 cm in length) were excised and placed into rooting medium (RM) containing MS salts and vitamins, 3% sucrose, 5.3 $gL^{-1}$ agar, 0.6 $mgL^{-1}$ (pH 5.8), sterilized 50 $mgL^{-1}$ L-asparagine, 100 $mgL^{-1}$ pyroglutamic acid, 0.1 $mgL^{-1}$ indole acetic acid (IAA), 0.5 $mgL^{-1}$ GA3, 10 $mgL^{-1}$ zeatin, 100 $mgL^{-1}$ ticarcillin, 250 $mgL^{-1}$ cefotaxime, 50 $mgL^{-1}$ vancomycin, and 1 $mgL^{-1}$ indole butyric acid (IBA). After 1–2 weeks, the roots were fully developed to 2–3 cm in length and eventually transplanted in a pot containing the soil in a greenhouse.

**Determination of the transient expression and regeneration rate**

After 28 days of SIM culture, 50 explants were collected for GUS staining. The explants were immediately submerged in GUS staining solution: 0.1 M EDTA (pH 8.0), 50 nM potassium ferrocyanide, 50 nM potassium, ferricyanide, 100 mM X-Gluc, 100 mM phosphate buffer, and placed under a vacuum for 10 min (Jefferson et al., 1987). The samples were incubated overnight in darkness at 37°C and the chlorophyll was removed by submerging the tissue in 70% ethanol. According to the staining results, the explants were divided into four categories: very strong (+++), strong (++), weak (+), and none (-). The transient rate of each category was calculated as: $ne/nt \times 100\%$, where $ne$ is the number of $GUS^{+}$ explants and $nt$ is the number of total stained explants.

**Detection of transgenic soybean plants**

Transgenic soybean plants were verified by leaf painting or spraying, PCR analysis and GUS staining. The plants at the 3rd leaf stage were screened by painting the upper leaf with PPT (100 mgmL$^{-1}$). In order to screen T1 transgenic plants, seeds were sown in the seedling-growing plastic trays in a greenhouse. At the 3rd leaf stage, plants were sprayed with BAYER Basta Glufosinate herbicide. After 3 to 5 days of the herbicide spray, the treated leaves of the non-transgenic died but those of the transgenic plants remained unaffected. For PCR analysis, the genomic DNA of the transgenic soybean was extracted using the CTAB method (Doyle and Doyle, 1987). The 470 bp bar gene coding region was amplified using a primer pair: 5′-GTACCGGCAGGCTGAAGTCC-3′ (forward) and 5′-CGGTCTGCACCATCGTCAAC-3′ (reverse). The amplified products were separated by electrophoresis on a 1% agarose gel for about 20 min and photographed with a Geldoc imaging system (www.bio-rad.com).

## RESULTS

### Shoot induction of soybean genotypes

After 28 days of culture in SIM, soybean genotypes showed different phenotypes of shoot induction. Most SCG produced multiple shoots per explant (Figure 2G, H), but LCG were hard to produce shoot (Figure 2E, F). The shoot induction rate was varied from 48.82 to 93.53% and 1.64 to 5.76 number of shoots per explant (Table 1). Among them, the highest rate of shoot induction and shoots number were obtained at DT22 and VX93 cultivars that showing a mean of 92.32% with 5.75 shoots and 93.35% with 5.92 shoots, respectively. Comparison of regeneration frequency between CSG and LSG showed significant variation (Table 1). The mean shoot regeneration rate of CSG was 74.98% with a range from 45.45 to 93.35%, and that of LSG was 63.21%, which ranged from 40.14 to 79.86%. CSG was not only better at shoot regeneration but also produced higher shoot numbers per explant than LSG. After 28 days of incubation, CSG produced 2.30 to 5.92 shoots (mean 4.19) per explant, which was higher than LSG (1.65 to 2.85 with a mean of 2.15 shoots per explant) (Table 1).

### Transient infection efficiency and shoot regeneration ability of soybean genotypes

Based on the shoot induction ability, we screened the soybean genotypes for the susceptibility to *Agrobacterium* infection using the GUS reporter gene. A half-seed explants were used for transformation as described by Kim et al. (2016). After 14 days of selecting in SIM containing 10 mgL$^{-1}$ PPT, explants produced healthy resistant buds (Figure 3E). Fifty SIM explants for each cultivar were collected for GUS staining. The efficiency of the transient infection was calculated using the variation of GUS expression rate and signals (Table 2). The percentage of GUS$^{+}$ at CSG ranged from 16 to 90% (mean 67.4%) and that of LSG from 22 to 86% (mean 46.7%). Among them, seven cultivars (DT84, DT90, DVN5, DVN6, DT22, VX93 and Cocchum) showed the highest rate (from 82 to 90%) of GUS$^{+}$ compared with the controls, Jack (86%) and William 82 (82%) (Table 2). Interestingly, three CSGs, DT84, DVN5 and DVN6, displayed stronger GUS signal (+++) than other cultivars including controls (Figure 3D), whereas nine CSGs and six LSGs showed as strong GUS expression as controls, and four CSGs and seven LSGs presented weak GUS signals (+) (Figure 3B, Table 2).

**Table 1.** Shoot induction of thirty-two soybean genotypes after 28 days on SIM.

| No. | Genotype | No. of explants | Regeneration rate (%) | No. of shoots per explant |
|---|---|---|---|---|
| **Cultivated soybean group (CSG)** | | | | |
| 1 | A28 | 410 | 53.17$^{fg}$ | 3.63$^{c}$ |
| 2 | DT26 | 356 | 89.89$^{a}$ | 5.76$^{a}$ |
| 3 | DT84 | 343 | 86.88$^{b}$ | 2.93$^{d}$ |
| 4 | DT90 | 423 | 81.56$^{c}$ | 3.21$^{c}$ |
| 5 | DT96 | 534 | 79.21$^{c}$ | 4.85$^{ba}$ |
| 6 | DT2001 | 440 | 70.23$^{de}$ | 4.10$^{bc}$ |
| 7 | DT2003 | 496 | 61.49$^{e}$ | 3.92$^{c}$ |
| 8 | DT2008 | 319 | 45.45 | 3.20$^{c}$ |
| 9 | DVN5 | 487 | 63.86$^{e}$ | 5.54$^{a}$ |
| 10 | DVN6 | 230 | 87.39$^{b}$ | 4.58$^{ba}$ |
| 11 | DVN9 | 570 | 87.37$^{b}$ | 3.87$^{c}$ |
| 12 | DVN10 | 235 | 70.21$^{de}$ | 4.42$^{ba}$ |
| 13 | DVN11 | 453 | 64.02$^{e}$ | 3.67$^{c}$ |
| 14 | DT22 | 456 | 92.32$^{a}$ | 5.75$^{a}$ |
| 15 | D2101 | 356 | 76.97$^{c}$ | 3.57$^{c}$ |
| 16 | D9602 | 342 | 71.35$^{d}$ | 2.30$^{d}$ |

**Table 1.** Continues.

| | | | | |
|---|---|---|---|---|
| 17 | VX93 | 451 | 93.35[a] | 5.92[a] |
| **Mean** | | | **74.98** | **4.19** |
| | | | | |
| **Local soybean cultivars group (LSG)** | | | | |
| 18 | VMK | 427 | 79.86[c] | 2.13[de] |
| 19 | Cọc Chum | 355 | 65.92 | 2.08[de] |
| 20 | VCB | 345 | 71.3[d] | 1.93[e] |
| 21 | Tho Xuan | 213 | 78.4[c] | 2.12[d] |
| 22 | Cuc Luc Ngan | 342 | 61.99[e] | 1.78[e] |
| 23 | Cuc Huu Lung | 256 | 77.34[c] | 2.01[de] |
| 24 | Cuc Ha Bac | 423 | 65.25[e] | 2.34[de] |
| 25 | Doan Ket | 180 | 54.44[fg] | 1.64[e] |
| 26 | Xanh Cao Bang | 310 | 62.9[e] | 2.64[d] |
| 27 | Vang Ha Giang | 386 | 49.74[g] | 1.86[e] |
| 28 | Hoa Tuyen | 279 | 40.14[h] | 1.91[e] |
| 29 | Cuc mat đen | 312 | 65.71[e] | 2.67[d] |
| 30 | Cuc Vo Nhai | 211 | 48.82[g] | 2.85[d] |
| **Mean** | | | **63.21** | **2.15** |
| | | | | |
| 31 | Jack | 351 | 91.35[a] | 4.6[ba] |
| 32 | William 82 | 426 | 82.64[bc] | 5.7[a] |

* Regeneration rate was expressed as a mean; means were compared by common letter are not significant according to Duncan's multiple range test ($P < 0.05$).

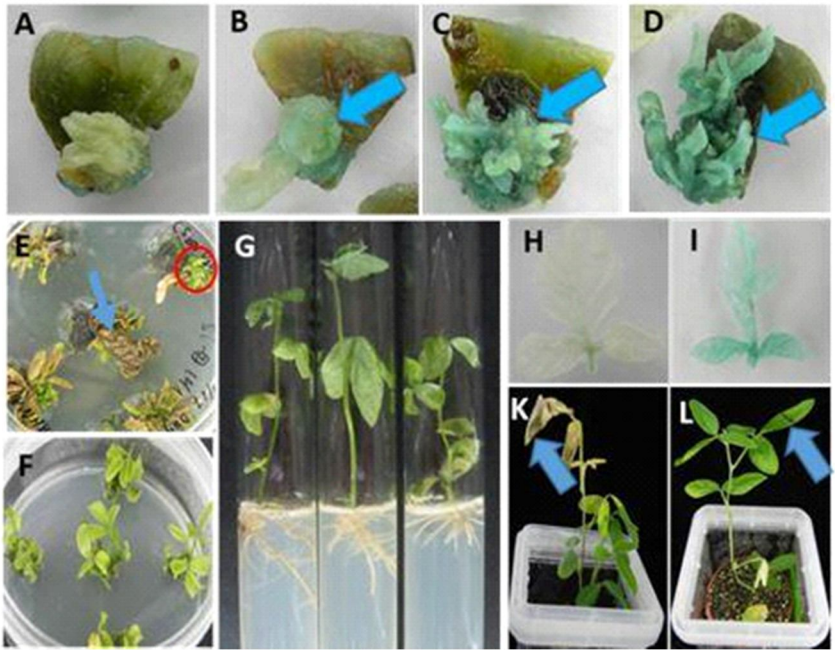


**Figure 3.** GUS expression pattern and regeneration of transgenic plants. A to D: GUS expression patterns after staining with X-glucA were classified: A-non GUS signal (A), B-week (+), C-strong (++), D- very strong (+++), arrows indicate GUS expression; E- shoot induction after 14 days in selection medium, SIM-I applied 10 $mgL^{-1}$ PPT, arrow indicates non-PPT resistant shoots, surround indicates PPT resistant shoots; F- Shoot induction in SE medium after 14 days; G- Root induction after 28 days on rooting medium; H and I leaf GUS staining for non-transgenic (control, K) and transgenic (L) plants, narrows indicate leaf phenotypes after 5 days Basta spray.

**Table 2.** Transient GUS staining of explants infected by *Agrobacterium.*

| No. | Genotype | GUS$^+$/50 tested explants | % GUS$^+$ | GUS signal | No. of explants on SEM | No. of elongated shoots | No. of Basta resistant plants |
|---|---|---|---|---|---|---|---|
| **Cultivated soybean group (CSG)** | | | | | | | |
| 1 | A28 | 8 | 16$^{u}$ | + | 102 | - | |
| 2 | DT26 | 14 | 28$^{r}$ | ++ | 94 | 6 (6.4%)$^{d}$ | 2 |
| 3 | DT84 | 41 | 82$^{cb}$ | +++ | 75 | 2 (2.7%)$^{gf}$ | - |
| 4 | DT90 | 38 | 76$^{d}$ | +++ | 54 | - | - |
| 5 | DT96 | 41 | 82$^{cb}$ | ++ | 89 | - | - |
| 6 | DT2001 | 37 | 74$^{efd}$ | ++ | 112 | 1 (0.9%)$^{i}$ | - |
| 7 | DT2003 | 35 | 70$^{g}$ | ++ | 155 | - | - |
| 8 | DT2008 | 24 | 48$^{on}$ | ++ | 105 | - | - |
| 9 | DVN5 | 45 | 90$^{a}$ | +++ | 115 | 5 (4.3%)$^{e}$ | 2 |
| 10 | DVN6 | 44 | 88$^{a}$ | +++ | 127 | 5 (3.9%)$^{fe}$ | 1 |
| 11 | DVN9 | 36 | 72$^{fg}$ | ++ | 254 | 12 (4.7%)$^{e}$ | 4 |
| 12 | DVN10 | 26 | 52$^{ml}$ | + | 215 | 2 (0.9%)$^{i}$ | - |
| 13 | DVN11 | 31 | 62$^{ki}$ | + | 109 | 5 (4.6%)$^{e}$ | - |
| 14 | DT22 | 42 | 84$^{b}$ | ++ | 256 | 26 (10.22%)$^{c}$ | 16 |
| 15 | D2101 | 33 | 66$^{h}$ | + | 101 | 2 (1.9%)$^{hi}$ | - |
| 16 | D9602 | 35 | 70$^{g}$ | ++ | 98 | - | - |
| 17 | VX93 | 43 | 86$^{ab}$ | ++ | 103 | 16 (15.5%)$^{b}$ | 12 |
| **Mean** | | | **67.4** | | | | |
| **Local soybean cultivars group (LSG)** | | | | | | | |
| 18 | VMK | 28 | 56$^{l}$ | ++ | 201 | - | - |
| 19 | Cọc Chum | 41 | 82$^{cb}$ | ++ | 154 | - | - |
| 20 | VCB | 33 | 66$^{h}$ | ++ | 112 | - | - |
| 21 | Tho Xuan | 37 | 74$^{efd}$ | ++ | 121 | - | - |
| 22 | Cuc Luc Ngan | 13 | 26$^{sr}$ | + | 186 | - | - |
| 23 | Cuc Huu Lung | 23 | 46$^{po}$ | + | 172 | - | - |
| 24 | Cuc Ha Bac | 25 | 50$^{nm}$ | ++ | 165 | - | - |
| 25 | Doan Ket | 14 | 28$^{r}$ | + | 134 | - | - |
| 26 | Xanh Cao Bang | 21 | 42$^{qp}$ | + | 97 | - | - |
| 27 | Vang Ha Giang | 11 | 22$^{ts}$ | ++ | 115 | - | - |
| 28 | Hoa Tuyen | 24 | 48$^{nm}$ | + | 167 | - | - |
| 29 | Cuc mat đen | 32 | 64$^{ih}$ | + | 157 | - | - |
| 30 | Cuc Vo Nhai | 14 | 28$^{r}$ | + | 149 | - | - |
| **Mean** | | | **46.7** | | | | |
| 31 | Jack | 43 | 86$^{ab}$ | ++ | 121 | 28 (23.1%) $^{a}$ | 11 |
| 32 | William 82 | 41 | 82$^{cb}$ | ++ | 135 | 19 (14.1%)$^{b}$ | 9 |

* Regeneration rate was expressed as mean; means were compared by common letter are not significant according to Duncan's multiple range test ($P<0.05$). GUS$^+$: positive stained GUS. GUS signal: + weak, ++ strong, +++ very strong.

To test whether these soybean genotypes can produce transgenic shoots, after 28 days of selection in the SI medium, the explants with healthy buds were transferred to shoot elongation medium (SEM) containing 5 mgL$^{-1}$ PPT. Interestingly, although all of the soybean genotypes produced multiple buds, only 11 out of 17 cultivars of CSG produced healthy shoots and none were observed at LSG after 28 days in the SEM (Table 2). There were two CSGs with a significantly high rate of elongated shoots as VX93 (15.5%) and DT22 (10.2%), whereas the controls resulting in 23.1% (Jack) and 14.1% (William 82). Nine out of seventeen CSGs produced less elongated shoots, ranged from 1 to 16 shoots (0.9% to 6.4%). Six CSGs (A28, DT90, DT96, DT2003, DT2008, and D9602) and all LSGs have no elongated shoots observable (Figure 3E, Table 2). These results indicated

that shoot induction and elongation were strictly governed by soybean genotypes.

## Screening T0 transgenic plants

The elongated shoots with about 3-4 cm length were cut off and transferred to the rooting medium (RM). After 28 days, the plants with healthy roots (Fig. 3G) were transplanted into the soil for transgenic screening. At the 3rd leaf stage, plants were tested by painting PPT (100 $mgL^{-1}$ ) on the upper side of a leaf. After 3 days, all tested plants were identified based on the leaf phenotypes. Due to carrying the bar gene, the transgenic plants can survive after PPT painting. In contrast, the non-transgenic plants will die when exposed to Basta herbicide (Figure 3K, L). The results showed that more than half the number of plants died (57 survival plants out of 129 tested plants) after 3 days of painting (Table 2, Figure 3K, L). In addition, we also verified transgenic plants by leaf GUS staining. All tested plants showed GUS positive, while the negative control plant has no GUS expression (Figure 3H, I). Taken together, the results indicated that 57 T0 plants contained *bar* and GUS genes.

## T1 transgenic plant analysis

The T1 seeds were harvested from individual T0 transgenic plants (Fig. 4A). we collected eight lines derived from DT22 to further analysis in T1 generation. The results of basta screening demonstrated that all tested lines providing positive Basta resistant plants after five days of spraying (Table 3). The Basta segregation ratios of these lines were calculated to determine the inherited pattern. Five out of eight lines were fit for 3:1 ($Basta^{Resistance}$/ $Basta^{Sensitive}$) ratios, the others lines showed distorted Mendelian fashion (Table 3). To confirm the transgenic lines, we conducted PCR analysis for T1 Basta resistant lines. All showed positive with bar gene (Figure 4C).

**Table 3.** Basta segregation ratios in T1 soybean transgenic lines.

| Transgenic lines | Genotypes | No. of T1 plants | Basta segregation | | |
|---|---|---|---|---|---|
| | | | $Basta^{R}$ | $Basta^{S}$ | $Ratio^{R/S}$ |
| 1 | DT22 | 50 | 34 | 16 | 3:1 |
| 2 | DT22 | 38 | 28 | 10 | 3:1 |
| 3 | DT22 | 30 | 5 | 25 | - |
| 4 | DT22 | 50 | 41 | 9 | 3:1 |
| 5 | DT22 | 50 | 4 | 46 | - |
| 6 | DT22 | 35 | 6 | 31 | - |
| 7 | DT22 | 50 | 45 | 5 | 3:1 |
| 8 | DT22 | 25 | 21 | 4 | 3:1 |

*: - : other segregation ration. $Basta^{R}$: Basta resistance. $Basta^{S}$: Basta sensitive.

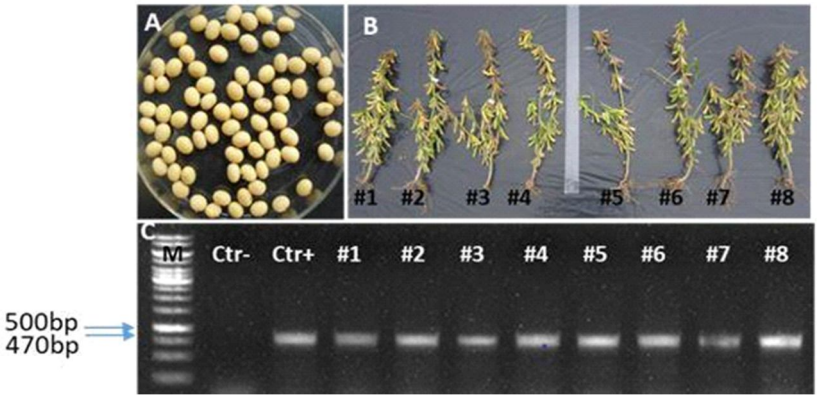


**Figure 4.** PCR confirmation of transgenic plants. A- T1 transgenic seeds; B- T1 transgenic lines at mature phase; C- PCR analysis to confirm bar gene present using bar forward and reverse primers, M- DNA marker 1kb, non-transgenic plant used as negative control (ctr-) and DNA plasmid used as a positive control (ctr+), #1 to #8 transgenic lines.

## DISCUSSION

Efficient plant regeneration is a prerequisite for the successful application of genetic transformation or gene-editing technologies. However, many other factors such as medium composition, explant source, and genotype have also been found to be crucial. The existence of strong genotype specificity in the regeneration capacity of the different cultivars represents a major limiting factor for the advancement of soybean technology (Bailey et al., 1993; Barwale et al., 1986, Raza et al., 2017). Jack and William 82 are well known for model soybean cultivars and commonly used for soybean transformation. However, these cultivars have poor agronomic traits and only suitable for growing in narrow regions. To speed up the soybean transformation program by a country, the selection of suitable genotypes for domestic ecological regions is very necessary. Barwale et al. (1986) evaluated the regeneration of 155 soybean genotypes reporting that the number of shoots formed ranged from 1 to 12. The ability to form multiple shoots appears to be genetically controlled. Reichert et al. (2003) tested adventitious regeneration from hypocotyl explants excised from 18 genotypes showed that all genotypes were capable of producing elongated shoots with healthy roots. Hiraga et al (2007) examined the capacity for plant regeneration through somatic embryogenesis in Japanese soybean cultivars and identified two genotypes Yuuzuru and Yumeyutaka as having the highest regeneration rate. Similarly, Yang et al (2009) screened 98 Chinese soybean cultivars and obtained the greatest average number of plantlets regenerated per explants (1.35) in N25281 variety. Raza et al. (2017) tested nine commercial Australian soybean genotypes for *in vitro* plant regeneration using cotyledonary-node, half split hypocotyl and complete hypocotyl explants. Of which, the Bunya variety showed the best regeneration response using complete hypocotyl with 100% shoot induction explants and 4.1 shoots per explant. However, genotype PNR79 gave 100% shoot regeneration and 10.5 shoots per explant with cotyledonary node.

Our study used cotyledonary node explant to evaluate the shoot regeneration response of thirty Vietnamese soybean genotypes including 17 cultivated soybean genotypes (CSG) that are currently grown in other ecological regions and 13 local soybean genotypes (LSG) that are planted in a specific ecological region. The results showed a variation in CSGs for shoot regeneration rate and number with a mean of 76.98% and 4.19 shoots, respectively. On the other hand, we obtained a lower regeneration rate and shoot number per explant in LSGs with 63.21% and 2.15, respectively. Of which, the highest rate of shoot induction and shoots number were obtained at DT22 and VX93 cultivars. These results are in agreement with previous reports that shoot regeneration response is strictly controlled by soybean genotypes.

*Agrobacterium*-mediated transformation is a high-efficiency method used in transgenic soybean development. To evaluate the genotype's susceptibility to *Agrobacterium*, we employed a transient transformation system using the GUS reporter gene. Based on the GUS expression signals, all of the soybean genotypes showed GUS positive and relatively good in transient infection compared to the control genotypes, Jack and William 82. However, the efficiency of transient infection was significantly different among the tested genotypes. To generate transgenic plants, infected explants can be recovered whole plantlets otherwise transformation program will be failed. In this research, we obtained only 11 out of 17 cultivars of CSGs produced healthy shoots, whereas no elongated shoots were observed in LSGs after 28 days of growth in the SE medium. The results suggested that a high transient infection may not ascertain a successful generation of transgenic plants. Adriana et al. (2018) screened Colombian soybean genotypes for *Agrobacterium*-mediated transformation found that the SK7 variety presented a better regeneration performance from the cotyledonary node and also had the highest transformation frequency.

Although 11 CSGs could produce transgenic plants, two cultivars VX93 and DT22 produced the highest rate of elongated shoots and transformation efficiency compare to Jack and William 82 genotypes. These two CSGs cultivars could be used for transgenic soybean breeding in Vietnam.

### Conclusion

The screening of thirty Vietnamese soybean genotypes for shoot regeneration ability and transient infection via the *Agrobacterium tumefaciens* method showed that CSGs have a higher percentage of shoot regeneration ability and shoot number per explant than that of LSGs. The mean shoots regeneration rate of CSGs was 74.98% with 4.19 shoot per explant, meanwhile, LSG showed 63.21% with 2.15 shoot per explant. All tested soybean genotypes have a significantly high rate of *GUS* transient infection, however, LSG hardly recovered healthy shoots that may be caused by genetics characterization. Among, CSG cultivars, DT22 and VX93 had significantly high efficiencies of shoot regeneration and transient infection compared with the control genotypes Jack and William 82. Shoot regeneration of DT22 and VX93 was 92.32% with 5.75 shoots per explant and 93.35% with 5.92 shoots per explant, respectively; and transient infection of DT22 and VX93 was 84% and 86%. We also confirmed the transgenic plants by GUS staining, PCR, and Basta leaf painting indicating that those cultivars could be used for stable *Agrobacterium*-media transformation.

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## Conflicts of interest

The authors declare no conflict of interest.

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