

## Enhanced germination but no phenotypic alteration in *cp4 epsps*-transgenic soybean under semi-controlled conditions

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### Abstract

Soybean (*Glycine max* (L.) Merr.) is renowned for its nutritional value and health benefits. Efficient control of undesirable plants is crucial for large-scale soybean production. Combining non-selective herbicides like glyphosate (Roundup®) with *cp4 epsps* transgene-resistant plants has proven effective. Substantial equivalence studies conducted by the Centre for Genetic Engineering and Biotechnology (Cuba) have led to field approval. However, further investigation is needed regarding potential alterations in “minor” agricultural traits, botany, physiology, and biochemistry of this *cp4 epsps*-transgenic variety. This report outlines early germination stages in Petri dishes and plant growth under semi-controlled conditions. The *cp4 epsps* transgenesis enhanced seed vigour, achieving 100% germination at 42 h compared to 54 h for the control group. No visual phenotypic differences were observed at 21 or 60 days after sowing, nor statistically significant differences at 90 days in most biochemical indicators. However, significant differences were noted in the levels of chlorophyll b and soluble phenolics in leaves, and cell wall-linked phenolics in seeds. Despite minor variations observed, they are not significant enough to reject transgenesis as a valuable tool for soybean genetic improvement.

**Keywords:** *Glycine max* (L.) Merr.; plant genetic transformation; public perception of genetically modified organisms; unexpected side effects of transgenesis

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## Introduction

Soybean is an annual herbaceous plant that thrives in spring and summer. Its vegetative cycle spans three to seven months, with a height ranging from 40 to 100 cm (Allen Jr *et al.*, 1987; Singh *et al.*, 2022). The plant's leaves, stems, and pods are covered with hairs, ranging in colour from blonde to greyish-brown (Jahonkulovna *et al.*, 2024). Originating from Asia, soy is one of the five sacred seeds in Chinese culture, renowned for its high nutritional value and various health benefits, earning it the nickname “food of the future” (Valencia Ramírez, 1999; Hymowitz, 2008; Wang and Guo, 2024).

With its significant potential for human nutrition, this grain stands as the premier nutritional alternative due to its high-quality protein content. It offers a promising solution to address the severe malnutrition problems prevalent in third-world countries (Hamza *et al.*, 2024; Li *et al.*, 2024; Messina *et al.*, 2024). Soybean provides various health benefits, including improved blood circulation, diabetes control, enhanced concentration, and high nutritional value (Dwivedi *et al.*, 2024; Li *et al.*, 2024; Wale, 2024). Incorporating soybeans into the diet can also lower cholesterol levels (Rotundo *et al.*, 2024). Numerous scientific studies support these benefits (Mainini *et al.*, 2024). The US Food and Drug Administration (FDA) endorses that consuming 25 g/day of soy protein can reduce the risk of heart disease (Khairnar *et al.*, 2024). Global soybean production for the 2023/24 season is projected to increase by 9.6% compared to the previous cycle, rising from 369.7 to 405.3 million tonnes (e Silva *et al.*, 2024; Hamza *et al.*, 2024; Masih *et al.*, 2024).

Efficient alternatives for controlling undesirable plants are crucial for large-scale soybean production (de la Fuente *et al.*, 2006; Gray *et al.*, 2008; Saldanha *et al.*, 2024). Utilising non-selective herbicides like glyphosate (Roundup®) in combination with resistant plants that carry the *cp4 epsps* transgene has proven to be an effective strategy for addressing this issue in soybean cultivation (Delgado *et al.*, 2015; Soto *et al.*, 2017). However, substantial equivalence studies are essential before cultivating a new transgenic variety in the field (Schauzu, 2000; Kuiper *et al.*, 2002; Paull, 2008; Abrams *et al.*, 2024; Ryan *et al.*, 2024). Such studies, focusing on grain characteristics, have been conducted by the Centre for Genetic Engineering and Biotechnology (Cuba) (Delgado *et al.*, 2015; Soto *et al.*, 2017), leading to the approval of their use in the fields by the Cuban Ministry of Agriculture.

Nevertheless, there remain aspects to be investigated in this *cp4 epsps*-transgenic variety, such as potential alterations in “minor” agricultural traits and the crop's botany, physiology, and biochemistry. This report outlines the early stages of germination in Petri dishes and plant growth under semi-controlled conditions of the transgenic plants, compared to the non-transformed controls.

## Materials and Methods

### *Plant material*

Seeds of *Glycine max* (L.) Merr, var. CIGB L-7 (*cp4 epsps*-transgenic variety) and var. INCA 36 (non-transformed control) were collected in Havana, Cuba. Both groups were cultivated simultaneously in the same environment. The seeds were managed according to the guidelines outlined in the germplasm bank seed management manual (Rao *et al.*, 2007). All seeds were stored in hermetically sealed glass containers at 5 °C until use (Baskin and Baskin, 2014).

### *Experimental design*

Fifteen true-to-type seeds from each variety were placed in Petri dishes ( $\varnothing = 9$  cm) lined with filter paper and supplemented with 10 mL of distilled water (5 Petri dishes with 3 seeds each as replicates). The dishes were incubated in the dark for 96 hours. Germination and seedling growth were recorded every 6 hours.

A second group of seeds was planted in pots (5 L capacity; 20 cm top diameter; 17 cm bottom diameter; 24 cm height) containing 5,720 g of red ferralitic soil. These plants were grown under conditions of  $30 \pm 3$  °C, with a 12/12 h photoperiod and a photosynthetic photon flux density of  $2,000 \pm 195$  (SE)  $\mu\text{mol m}^{-2} \text{s}^{-1}$  at noon for up to 90 d. Four replicates of three seeds each were analysed. The pots were irrigated daily with 500 mL of water. Agricultural traits and levels of chlorophylls (a and b), aldehydes (malondialdehyde and other aldehydes), phenolics (soluble and cell wall-linked), total crude proteins, and nitrogen were recorded in leaves and seeds at harvest (90 d of growth).

#### *Biochemical assays*

Leaf tissues were sampled from three independent replicates of 100 mg each. Chlorophylls were extracted using 5.0 mL of 80% acetone (v/v). The samples were centrifuged at  $14,086.8 \times g$ , 4 °C for 15 min, and the supernatants were collected. Absorbance readings were taken at 646.6 and 663.6 nm using a RAYLEIGH VIS-723G spectrophotometer (Beijing, China) (Porra, 2002). Malondialdehyde and other aldehydes were quantified using a colourimetric method based on their reaction with thiobarbituric acid (Heath and Packer, 1968). Phenolic compounds were extracted with 0.5 mL of methanol and quantified using a spectrophotometer through a colourimetric method involving the Folin-Ciocalteu reagent, expressed as mg of gallic acid equivalents per gram of fresh weight (Gurr *et al.*, 1992).

The total nitrogen content was determined using the AOAC method (2000). Ground plant material (0.5 g) was placed in a Kjeldahl flask with 20 mL of  $\text{H}_2\text{SO}_4$  (98%; w/w), 2 g of  $\text{K}_2\text{SO}_4$ , and 0.2 g of  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ . The flask was first subjected to low heat for 30 min, followed by higher heat for 90 min until the solution turned transparent or light green. After cooling, 200 mL of distilled water was gradually added. The distillation process was commenced by adding 70 mL of 40% NaOH (w/v). The distillation outlet was submerged in 25 mL of 4%  $\text{H}_3\text{BO}_4$  (w/v) with a mixed indicator of 0.1% methyl red (w/v) and 0.1% bromocresol green (w/v) (1:1, v/v, in ethanol). The distillation continued until approximately 200 mL of distillate was collected. The resulting ammonium borate was titrated with a standardised HCl solution ( $0.1 \text{ mol L}^{-1}$ ). Results were expressed as the percentage of nitrogen and protein on a fresh mass basis, using a protein conversion factor of 6.25.

#### *Statistical analysis*

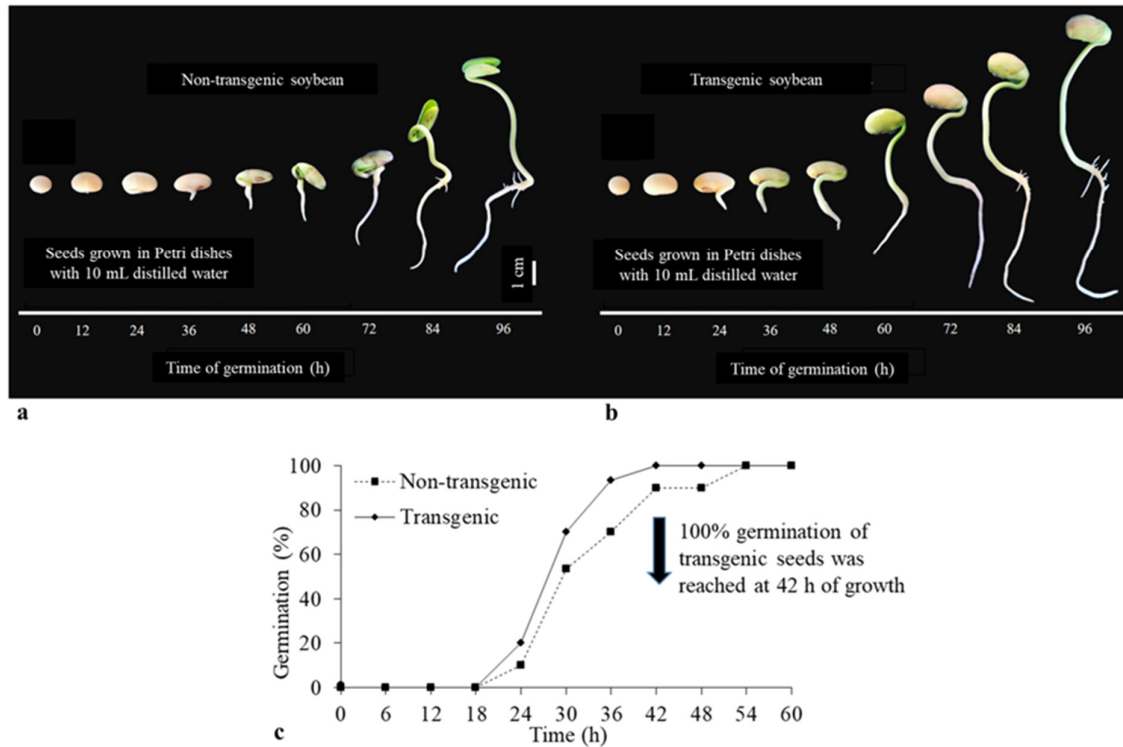
The statistical software SPSS (version 20.0, IBM, Armonk, NY, USA) was used to conduct t-tests (completely randomised experiment;  $p \leq 0.05$ ). The normal distribution and homogeneity of variances were confirmed using the Kolmogorov-Smirnov test ( $\alpha = 0.05$ ) and Levene's test ( $\alpha = 0.05$ ), respectively. Additionally, the overall coefficient of variation (OCV) was calculated as (standard deviation/average) \* 100. The interpretation of results was based on the magnitude of the difference; a larger difference between the two treatments corresponded to a higher OCV (Lorenzo *et al.*, 2015). OCV values were classified into three categories: Low (16.09 to 42.15%), Medium (42.15 to 68.22%), and High (68.22 to 94.28%). These categories were determined by dividing the range of OCVs into three equal parts:  $(\text{Max OCV} - \text{Min OCV})/3 = (94.28\% - 42.15\%)/3 =$  the breadth of each category.

## **Results and Discussion**

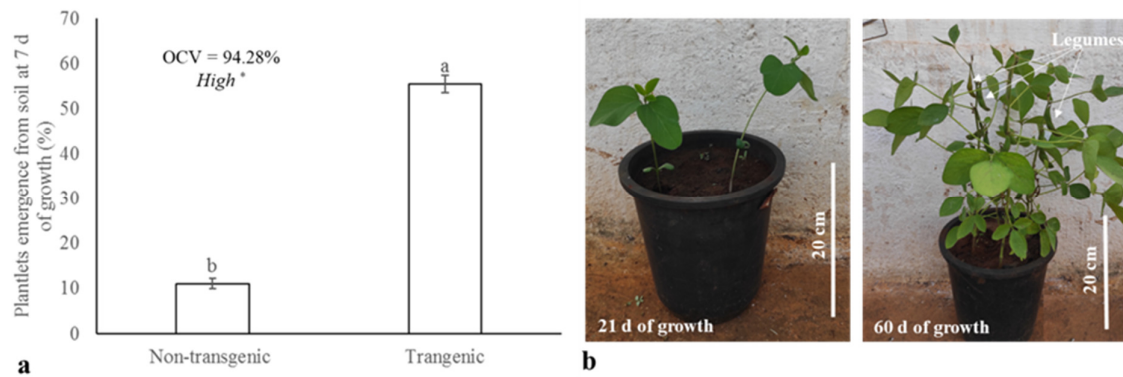
The *cp4 epsps* transgenesis in soybean enhanced seed vigour during germination (Figure 1a, b, c; Figure 2a). Transgenic seeds achieved 100% germination at 42 h of growth in Petri dishes, compared to 54 h for the control group (Figure 1c). However, no visual phenotypic differences between the two plant groups were observed at 21 or 60 d of growth (Figure 2b).

At 90 d of growth, no statistically significant differences were recorded in the following plant phenotypes: combined weight of stems, leaves, flowers, and fruits of adult plants; total leaf weight; total legume

weight per plant; and total number of legumes per plant, although average values were slightly higher in the transgenics than in the control plants, except for the latter parameter (Table 1).



**Figure 1.** Seed germination in Petri dishes: non-transgenic soybean (a), transgenic soybean (b), and dynamics of germination (c)



**Figure 2.** Plantlet emergence from the soil at 7 d of growth (a), averages  $\pm$  SE are shown; results with the same letter are not statistically different ( $t$ -test,  $p > 0.05$ ). Visual phenotypic differences between the two groups of plants could not be recorded at 21 or 60 d of growth. Typical plants are shown (b). \*Overall coefficient of variation (OCV) = (Standard deviation/Average)\*100. Average values were considered to calculate this coefficient. The higher the difference between the two treatments, the higher the overall coefficient of variation. OCVs were only calculated for those indicators with statistically significant differences according to  $t$ -tests. OCVs from 16.09 to 42.15% were classified as Low, from 42.15 to 68.22% as Medium and from 68.22 to 94.28% as High

Additionally, most biochemical indicators showed no significant differences: chlorophyll a, malondialdehyde, and cell wall-linked phenolics in leaves; soluble phenolics in seeds; and other aldehydes, total crude protein, and nitrogen in both leaves and seeds. Certain compounds were not detected in seeds: chlorophylls a and b, and malondialdehyde (Table 1).

Conversely, statistically significant differences were noted in a few traits: levels of chlorophyll b (Medium OCV) and soluble phenolics (Low OCV) in leaves, as well as the content of cell wall-linked phenolics in seeds (Low OCV) (Table 1).

**Table 1.** Plant phenotypes at 90 d of growth (harvest; average  $\pm$  SE)

Parameter		Non-transgenic soybean plants	Transgenic soybean plants	OCV (%)*	Classification of OCV**
Combined weight of stems, leaves, flowers and fruits of adult plants (g)		25.37 $\pm$ 2.33 a	26.27 $\pm$ 2.55 a	---	---
Total leaf weight (g)		6.07 $\pm$ 0.54 a	6.80 $\pm$ 0.48 a	---	---
Total legume weight per plant (g)		10.90 $\pm$ 1.07 a	11.02 $\pm$ 1.12 a	---	---
Total number of legumes per plant.		7.00 $\pm$ 0.56 a	6.88 $\pm$ 0.61 a	---	---
Chlorophyll a ( $\mu\text{g}\cdot\text{g}^{-1}$ FW)	Leaves	231.76 $\pm$ 8.58 a	228.51 $\pm$ 1.67 a	---	---
	Seeds	Not detected	Not detected	---	---
Chlorophyll b ( $\mu\text{g}\cdot\text{g}^{-1}$ FW)	Leaves	27.94 $\pm$ 2.06 a	9.77 $\pm$ 1.13 b	68.13	<i>Medium</i>
	Seeds	Not detected	Not detected	---	---
Malondialdehyde	Leaves	16.50 $\pm$ 2.33 a	18.11 $\pm$ 0.70 a	---	---
	Seeds	Not detected	Not detected	---	---
Other aldehydes	Leaves	247.07 $\pm$ 7.94 a	236.03 $\pm$ 3.30 a	---	---
	Seeds	42.91 $\pm$ 1.83 a	44.30 $\pm$ 0.63 a	---	---
Soluble phenolics ( $\text{mg}\cdot\text{g}^{-1}$ FW)	Leaves	4.31 $\pm$ 0.07 b	5.41 $\pm$ 0.36 a	16.09	<i>Low</i>
	Seeds	1.17 $\pm$ 0.17 a	1.09 $\pm$ 0.01 a	---	---
Cell wall-linked phenolics ( $\text{mg}\cdot\text{g}^{-1}$ FW)	Leaves	3.21 $\pm$ 0.21 a	3.63 $\pm$ 0.26 a	---	---
	Seeds	2.38 $\pm$ 0.13 b	3.37 $\pm$ 0.17 a	24.50	<i>Low</i>
Total crude protein (%)	Leaves	16.58 $\pm$ 0.07 a	16.64 $\pm$ 0.11 a	---	---
	Seeds	29.89 $\pm$ 0.47 a	31.09 $\pm$ 0.24 a	---	---
Nitrogen (%)	Leaves	2.65 $\pm$ 0.01 a	2.66 $\pm$ 0.02 a	---	---
	Seeds	4.78 $\pm$ 0.08 a	4.95 $\pm$ 0.03 a	---	---

Results with the same *letter* are not statistically different (t-test,  $p \geq 0.05$ ).

\*Overall coefficient of variation (OCV) = (Standard deviation/Average)\*100. To calculate this coefficient, average values were considered. The higher the difference between the two treatments, the higher the overall coefficient of variation

\*\*OCVs were only calculated for those indicators with statistically significant differences according to t-tests. OCVs from 16.09 to 42.15% were classified as *Low*, from 42.15 to 68.22% as *Medium* and from 68.22 to 94.28% as *High*

Recent studies have documented the genetic transformation of soybeans by several research groups (de Campos *et al.*, 2020; Fan *et al.*, 2020; Luan *et al.*, 2020; Nguyen *et al.*, 2020; Pareddy *et al.*, 2020; Soto *et al.*, 2020; Adachi *et al.*, 2021; Xu *et al.*, 2022; Enriquez-Obregón *et al.*, 2023). For instance, de Campos *et al.* (2020) evaluated different generations of transgenic (*cp4 epsps* gene) and non-transgenic soybean plants using proteomics and metabolomics. Their analysis revealed that all generations and varieties of soybean can be differentiated through discriminant analysis. According to their findings, transgenic plants appear to be more stable than their non-transgenic counterparts.

Plant transgenesis can cause unintended consequences, including undesirable traits, allergen introduction, genetic contamination, and reduced diversity (Kumar *et al.*, 2022). Risks include gene transfer, hybridisation, and resistance (Breckling *et al.*, 2011). Some genetically modified crops may contain toxins, such as heavy metals, and differ nutritionally from unmodified crops (Bakshi, 2003). Transgenic pineapples show altered mineral composition (Yabor *et al.*, 2017, 2020). Cross-pollination can create herbicide-resistant superweeds (Samuels, 2013). Bt toxin affects insect populations (Kumar *et al.*, 2020), impacting ecosystems. Glyphosate-resistant crops contribute to monarch butterfly declines (Brower *et al.*, 2012). Some modifications yield beneficial traits without negative effects (Karim *et al.*, 2007).

Although our study identified some unexpected biochemical variations in the *cp4 epsps* -transgenic soybean variety, particularly in chlorophyll b and phenolic compound levels, these differences remained within the expected variability observed in conventional soybean cultivars. Furthermore, no statistically significant differences were recorded in major plant phenotype indicators, such as overall biomass, legume production, and leaf weight, underscoring the stability of this transgenic modification in terms of plant development.

Importantly, one of the most notable benefits of transgenesis observed in this study was the improved germination rate in transgenic soybean seeds, which achieved 100% germination significantly earlier than non-transgenic counterparts. This enhanced seed vigour has valuable implications for agricultural efficiency, ensuring faster and more uniform crop establishment.

#### **Authors' Contributions**

Conceptualization: DPD, YA, LP, CC, GL, MLTF, OV, GEO, LPB and JCL; Data curation: DPD, LP, CC and GL; Formal analysis: YA, GL, MLTF, OV, LPB and JCL; Funding acquisition: OV, GEO and JCL; Investigation: DPD, YA, LP, CC, GL and LPB; Methodology: DPD, YA, LP, GL, MLTF, OV, GEO, LPB and JCL; Project administration: JCL; Resources: YA, LP, CC, GL, OV, GEO, LPB and JCL; Software: JCL; Supervision: LPB and JCL; Validation: YA, GL, OV, LPB and JCL; Visualization: YA, MLTF, OV, LPB and JCL; Writing - original draft: OV and JCL; and Writing - review and editing: OV and JCL. All authors read and approved the final manuscript

#### **Ethical approval** (for researches involving animals or humans)

Not applicable.

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#### **Conflict of Interests**

The authors declare that there are no conflicts of interest related to this article.

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