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EFFECT OF THE SOMATIC EMBRYOS MORPHOLOGY ON PLANT REGENERATION OF SOYBEAN (*Glycine max* L. MERRILL)

Efecto de la morfología de los embriones somáticos en la regeneración de plantas de soya (*Glycine max* L. Merrill)

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ABSTRACT. The established protocols for plant regeneration via somatic embryogenesis in soybean (*Glycine max* L. Merrill) are specific to a small number of cultivar, requiring new studies to provide more efficient and reproducible protocols in a large number of genotypes. This work had as objective to determine the effect of the morphology type of somatic embryos on the germination and plant conversion in soybean cultivar 'Incasoy-27'. Mature somatic embryos were used with different morphologies types that were placed in germination medium. At 30 days the somatic embryos number with complete or partial germination by morphology type was quantified. In the conversion phase it was determined to the 10 days of cultivation the survival percentage of the regenerated plants and it was carried out a morphological characterization of the regenerated plants in comparison with the obtained plants of seeds. It was achieved at 74,38 % of somatic embryos germinated with normal and abnormal morphology. The percentage of survival of regenerated plants reached 70,0 % efficiency. The progeny obtained from the regenerated plants of somatic embryos were morphologically similar to the control plants. The results from this research show that it is possible to allow regeneration of whole plants through somatic embryogenesis in soybean Cuban cultivar 'Incasoy-27'.

Key words: tissue culture, somatic embryogenesis, culture medium

RESUMEN. Los protocolos establecidos para la regeneración de plantas vía embriogénesis somática en soya (*Glycine max* L. Merrill), son específicos para un número reducido de cultivares, lo que requiere de nuevos estudios que permitan disponer de protocolos más eficientes y reproducibles en un mayor número de genotipos. Este trabajo tuvo como objetivo determinar el efecto del tipo de morfología de los embriones somáticos en la germinación y conversión a planta en soya cultivar 'Incasoy-27'. Se emplearon embriones somáticos maduros con diferentes tipos de morfologías, que se colocaron en medio de cultivo de germinación. A los 30 días, se cuantificó el número de embriones somáticos con germinación completa o parcial por tipo de morfología. En la fase de conversión se determinó a los diez días de cultivo, el porcentaje de supervivencia de las plantas regeneradas y se realizó una caracterización morfológica de las plantas regeneradas en comparación con las plantas obtenidas de semilla botánica. Se logró un 74,38 % de embriones somáticos germinados con morfología normal y anormal. El porcentaje de supervivencia de las plantas regeneradas alcanzó el 70,0 % de eficiencia. La descendencia obtenida a partir de las plantas regeneradas de embriones somáticos, fueron morfológicamente similares a las plantas control. Los resultados derivados de esta investigación permitieron demostrar que es posible la regeneración de plantas completas vía embriogénesis somática en soya cultivar cubano 'Incasoy-27'.

Palabras clave: cultivo de tejidos, embriogénesis somática, medio de cultivo

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INTRODUCTION

Somatic embryogenesis is an inductive process that allows non-zygotic plant cells to form somatic embryos with a bipolar structure and without vascular connection with the original tissue (1, 2). In addition, due to non-fusion of the gametes it is possible to achieve a high genetic homogeneity in the regenerated plants (3).

This plant regeneration pathway has been efficiently implemented in different species; however, in soybean has been described in a limited number of genotypes (4), among which the ones of North American origin, Jack, Williams and Fayette as well as Brazilians Bragg and IAS5 (5). For this, the use of growth regulators such as auxins is required, which stimulate cells with embryogenic capacity and induce the formation of somatic embryos (1). However, there is evidence that within the auxins, the leading role corresponds to 2,4-dichlorophenoxyacetic acid (2,4-D). This growth regulator allows induction of repetitive somatic embryogenesis, where the formation of the secondary somatic embryos has a unicellular origin and occurs from primary somatic embryos (6).

Some references associate the formation of an embryogenic cell with 2,4-D and changes occurring in the structure of chromatin by methylation of deoxyribonucleic acid (DNA), leading to genomic reprogramming in somatic cells and expressed. The genes required for embryogenic expression (7). However, the prolonged use of high concentrations of 2,4-D in soybean somatic embryo induction and multiplication media can lead to the appearance of various types of abnormal morphologies during the saturation phase, Efficiency of germination and conversion to plant (8).

Some authors have found aberrations and chromosomal instability in somatic embryos of soybean, attributed to growth regulators during *in vitro* culture (9). Because of this most of the researchers discard the somatic embryos of soy that show abnormal morphologies, reason why the references are few that approach the germination efficiency by morphology type. Others do not use growth regulators in the maturation and germination of somatic embryos; or by manipulation of the growing conditions, as is the case of partial desiccation; it is possible to prevent the early germination (10).

Taking into account that the use of high concentrations of 2,4-D can cause different abnormal morphologies, this work was carried out to determine the effect of somatic embryo type morphology on germination efficiency and plant conversion in soybean cultivar 'Incasoy-27 '.

MATERIALS AND METHODS

The work was carried out at the Institute of Plant Biotechnology in Santa Clara, Cuba. The Cuban soybean cultivar 'Incasoy-27', obtained at the National Institute of Agricultural Sciences (INCA) of Cuba, it was used by natural hybridization, which occurred in the Brazilian genotype 'BR-32'.

Culture conditions

The experiment was developed in a growth chamber with sunlight, with a duration of the luminous period of 12 to 13 hours of light; Temperature of 26 ± 1 °C; intensity of photosynthetic photon flux between 68,0 and 72,0 µmol $m^{-2}s^{-1}$, measured with a luxmeter (Extech 401025, USA).

Somatic embryos were taken with two months in maturation culture medium (10), which contained maltose (6,0 %) as carbon source. For partial desiccation of the somatic embryos, these were placed for four days in sterile Petri dishes (100×10 mm). Each plate contained about 48 somatic embryos which were placed to one end thereof and on the opposite side a fragment of semisolid culture medium (1,0 cm^3), according to Bailey's (11) methodology.

Germination of somatic embryos

11 to 12 mature somatic embryos (replicates) were placed with partial drying per 250 ml glass bottle, distributed in 21 flasks for a total of 242 somatic embryos. Each culture flask contained 30 mL of germination culture medium, MS (12), vitamins B5 (13), sucrose 1,5 %, Gelrite® 0,3 % and pH 5,8. After 30 days of culture, total sperm embryos were quantified and based on this value the number of somatic embryos with complete or partial germination (%) by type of morphology was determined.

Conversion to plant

Subsequently, 100 plants were selected *in vitro* from the germination culture medium. To achieve homogeneity between the plants were taken that were between 5,0-6,0 cm long and subcultured to germination culture medium for five days.

The flasks were covered with aluminum foil and in order to provide greater ventilation, a hole was made in the lid with the aid of a pencil, on the first and third day of culture. At the end the roots were washed with sterile distilled water before transferring the plants to greenhouse conditions. The sterilization of the water was done in autoclave at 121 °C and 1,1 kg cm^{-2} of pressure during 40 minutes.

The acclimatization of the plants under *ex vitro* conditions was carried out in the winter period (March-April). The plants were placed in polycarbamate flasks (500 mL) containing a sterile substrate containing 75 % decomposed organic matter of bovine origin and 25 % of the zeolite mineral homogeneously. The latter is a microporous crystalline natural aluminum-silicate with excellent ion exchange properties, high absorption power and granulation 1,0-3,0 mm (14). Sterilization of the substrate was previously done in an oven at 180 °C for two hours.

The control plants were obtained from botanical seeds in the same culture conditions, with two daily irrigations per microaspersion of three minutes' duration; mean temperature of 27 ± 2 °C and relative humidity 84 ± 5 %. Sunlight was regulated with a black shading mesh, at an intensity of photosynthetic photon flux of 280 to 400 µmol $m^{-2}s^{-1}$.

At ten days, the conversion efficiency was determined from the number of live plants (%), and at 30 days the morphological characters of the regenerated plants from the *in vitro* culture were evaluated. In the same way, the control plants and the offspring of *in vitro* regenerated plants were evaluated at 75 days of culture, which were obtained from mature seeds.

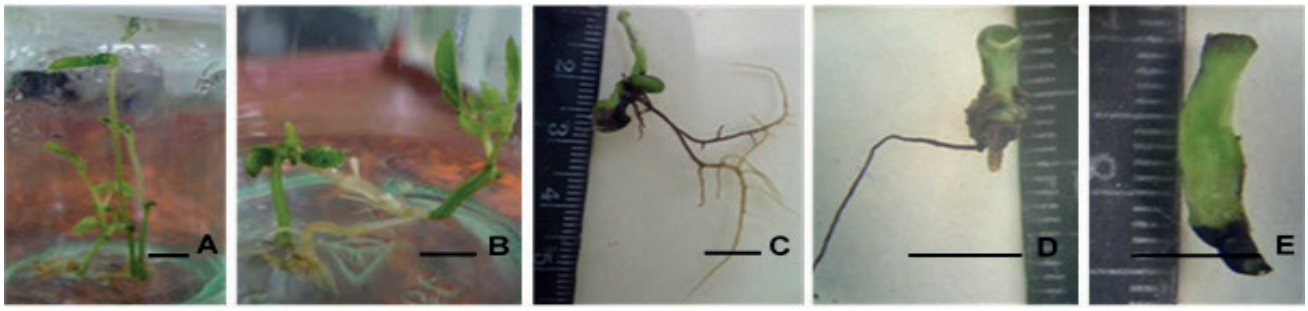
For the morphological evaluation, 50 plants were selected at random from each of the three treatments (plants obtained from botanical seed, plants regenerated from somatic embryos, and offspring from plants from tissue culture) and for comparison the descriptor of this cultivar. The evaluation criteria were: leaf color; number of leaflets in leaves; flower color; mean height of the plant (cm); height to first pod (cm); number of leaves per plant; legumes per plant; grains per legume and color of the seed. For the visual evaluation of these characters, the morphological descriptor of this genotype was taken into account. The height of the plants was determined with a millimeter ruler and measured from the base of the stem to the apical meristem of the plant.

Statistical analysis

A completely randomized design with five replicates was used for the development of the research. Data were analyzed using the Kruskal-Wallis test (15) and the differences between treatments with the Mann Whitney test. The statistical program SPSS (Statistic Package for Social Science) PASW Statistics version 18 was used, with a value of $p < 0.05$ (16).

RESULTS AND DISCUSSION

The evaluation of the morphology of the somatic embryos showed that this influenced their ability to germinate and regenerate soybean plants to cultivate 'Incasoy-27'. Somatic embryos that had a dicotyledonal morphology developed a complete germination where green leaf primordia were visible; with elongation at the roots, hypocotyl and epicotyl (Figure 1A, B). In contrast, somatic embryos with long, fused hypocotyl morphologies had partial germination, which only developed the root (Figure 1C, D). However, somatic embryos were found mainly of fused type, which failed to germinate when they did not develop any meristem (Figure 1E).



(A, B) plants obtained from somatic embryos with complete germination. (C, D) somatic embryos with partial germination, with type morphologies: (C) long hypocotyl; (D). (E) ungerminated somatic embryo with fused cotyledons. Bar = 1,0 cm

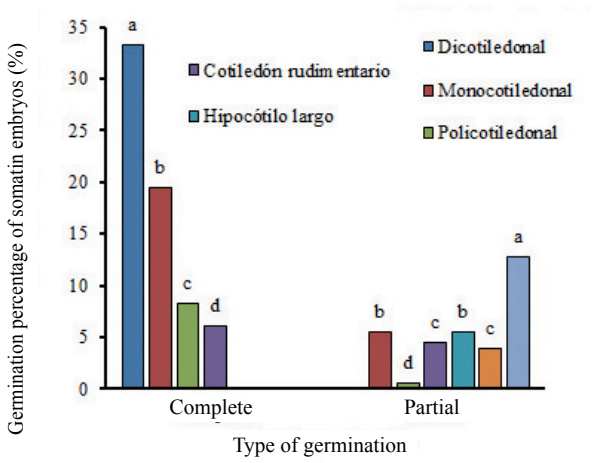
Figure 1. Germination of somatic embryos of soybean (*Glycine max* L.), 'Incasoy-27' in cultivar germination medium with DM salts, vitamins B5 and sucrose 1,5 %, after 30 days

Although germination was achieved in mature somatic embryos, it was found that those with abnormal morphologies represented the largest number, with a value equivalent to 49,59 % distributed as follows: monocotyledonal (18,60 %), polycottonic (6,61 %), rudimentary cotyledon (7,85 %), long hypocotyl (4,13 %), fasciate (2,89 %) and fused (9,51 %). However, the number of somatic embryos with abnormal morphologies doubled the value obtained in normal somatic embryos, with significant differences between them (Table I).

Although it was possible to germinate somatic embryos with abnormal morphologies, there are some types such as trumpet, which have a limited development of caulinar meristem and others of the fasciated type that manage to form the radical meristem, but never caulinar meristem, which affects their ability to germinate (17).

These changes in the bilateral symmetry of somatic embryos may be due to endogenous factors such as auxin polar transport inhibitors during somatic embryo development; as well as by the continuous exposure of tissue to high concentrations of 2,4-D, which interferes with the polar auxin gradient established in somatic embryogenesis (18, 19).

However, from the total number of germinated somatic embryos the dicotyledonal type presented complete germination (Figure 2). Somatic embryos with other types of morphologies classified as abnormal presented complete and partial germination. From the total somatic embryos with complete germination (67,21 %), those with dicotyledonal morphology had the highest percentage (33,33 %), followed by monocotyledonal, polycotyledonal and rudimentary cotyledon, with significant differences between them (Figure 2). In the somatic embryos with partial germination (32,79 %), the morphologies of the fused type had the highest values of germination (12,78 %), followed by monocotyledonal and long hypocotyl (Figure 2).



Bars with unequal letters in the same germination type differ according to the Kruskal-Wallis / Mann-Whitney test, (p <0,05; n = 180)

Figure 2. Effect of morphological type of somatic embryos on the percentage of complete or partial germination in soybean (*Glycine max* L.), 'Incasoy-27' cultivar in germination medium with DM, B5 and sucrose 1,5 % For 30 days

This reduction in the germination capacity of somatic embryos could also be due to an inefficient accumulation of reserve substances during the maturation phase, which limits their ability to recover after the stress caused by the drying process.

There are references that show that the incorporation of abscisic acid (ABA) during the development early stages of somatic embryogenesis favors the synthesis of reserve compounds in somatic embryos (20). This effect of ABA is attributed to it regulates different physiological processes and activates genes linked to stress response (21); while a low endogenous content may reduce the germination capacity and affect its recovery after the drying process.

Table I. Effect of morphological type of somatic embryos of soybean (*Glycine max* L.), 'Incasoy-27' cultivar on germination in culture medium without growth regulators at 30 days of cultivation

Type of morphologies	Somatic embryos		Percentage of germinated somatic embryos (%)	
	Matures	Germinated	Half	Middle range
Normal: Dicotiledonal	69	60	24,79	212,50 b
Abnormal: Monocotyledonal, polycotyled, rudimentary cotyledon, trumpet, long hypocotyl, fasciated, fused	173	120	49,59	272,50 a
Total	242	180	74,38	

Mean ranks with unequal letters in the same column differ according to the Mann-Whitney test (p <0.05, n = 242)

In other studies, there has been a greater accumulation of reserve substances in somatic embryos in the first three weeks in ripening medium, but at the end of this period the content of linoleic and linolenic acid declined, whereas palmitic acids and stearic increased. This may have been related to the germination process of somatic embryos, attributed to a re-mobilization or degradation of some reserve substances during senescence, similar to what happens in other plant tissues (22).

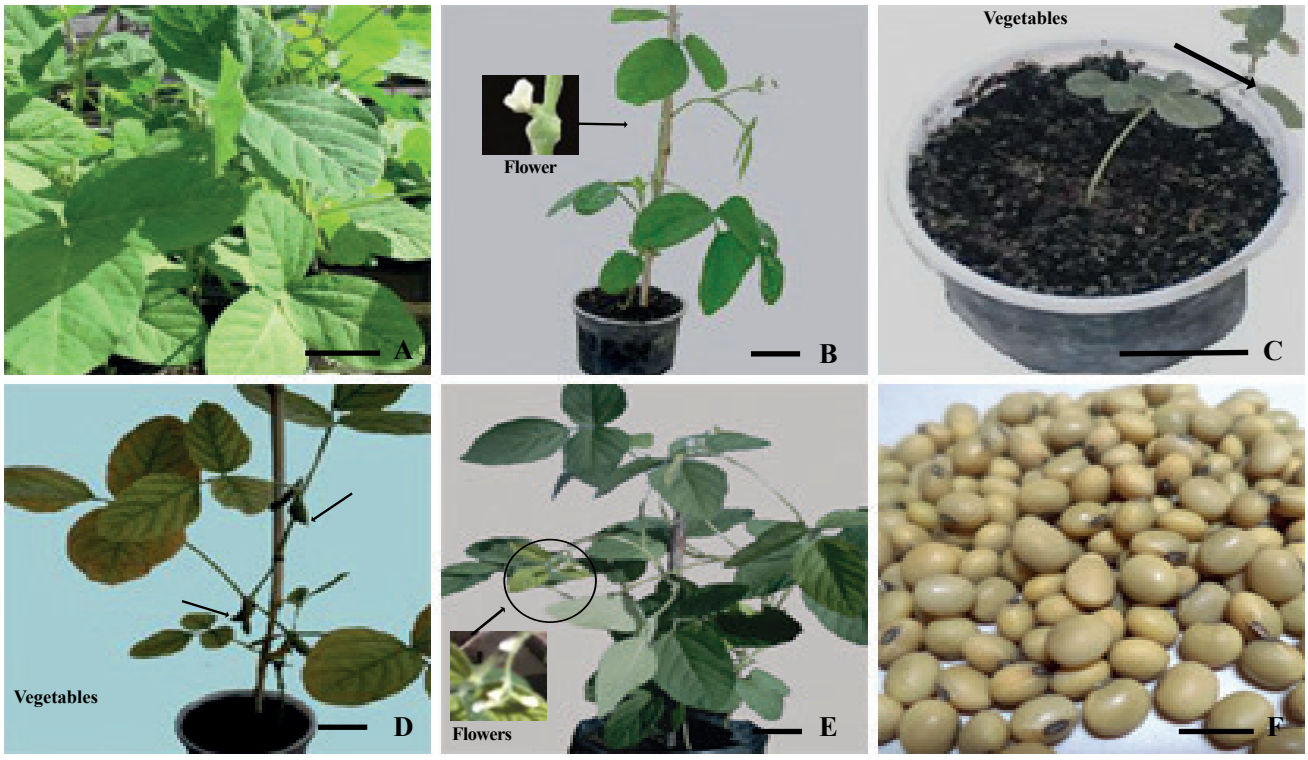
The low germination capacity of somatic embryos has also been described in the scientific literature. In Hindu soybean cultivars after a five-day drying period, 40, 9 % of the somatic embryos were able to germinate (6). The authors noted that the maturation and germination phases of somatic embryos in soybean are not very efficient in many studies due to the high number of somatic embryos with abnormal morphologies that fail to form complete plants.

According to studies (4), after seven days of desiccation the somatic embryos were placed in two concentrations of sucrose (1,5 and 3,0 %) and found the highest percentage of plants with normal growth (53,0 %), In the Hindu genotype 'PS1477' with 1,5 % sucrose. This result is lower than the 67,21 % obtained in the present work with the same concentration of sucrose but with four days of desiccation. It is possible that the use of seven days resulted in greater dehydration in the somatic embryos and affected their germination potential.

From the somatic embryos with complete germination in the cultivar 'Incasoy-27', plants were obtained that were able to adapt to the conditions of *ex vitro* cultivation. The survival rate of regenerated plants was 76,0 % conversion efficiency at 15 days of culture; while 100 % was achieved in control plants obtained from botanical seed.

Control plants (Figure 3A) and those regenerated from somatic embryos had common characteristics such as trifoliate leaves of green color and white flowers (Figure 3B, C). However, plants grown by *in vitro* culture developed a thin stem and, in some cases, limited leaf growth and development, attributed to tissue habituation to *in vitro* culture conditions (Figure 3C).

In vitro regenerated plants began to bloom and fructify prematurely around 15 days (Figure 3D) unlike the control plants, where flowering occurred around 35 to 40 days.



(A) control plants obtained from seeds; (B) plant with flowers obtained from somatic embryo with dicotyledonal morphology; (C) plant with little development and formation of legumes, coming from somatic embryo with dicotyledonal morphology; (D) plant in vitro with legumes at 30 days of culture; (E) offspring with flowers at 45 days in house of cultivation, Bar = 5,0 cm; (F) mature seeds obtained from offspring. The arrows indicate presence of flowers or vegetables, Bar = 1,0 cm.

Figure 3. Plants regenerated from somatic embryos of soybean (*Glycine max* L.), 'Incasoy-27' cultivar

This could be due to the *in vitro* plants coming from a period of 45 days in the germination phase that allowed developing the radical and apical system. In contrast, botanical seed plants took five to seven days to initiate germination. Seeds obtained from plants regenerated *in vitro* gave rise to offspring with normal growth and characteristics of this genotype (Figure 3E, F); in any case, out of type plants were detected.

Plants from somatic embryos had less development than control treatment plants. Likewise no visible differences were found in the color of the seeds, which showed the greenish yellow color of this cultivar. As for the results obtained in the offspring, these plants did not have significant differences with respect to the values obtained in the controls in the same culture conditions (Table II).

The conversion efficiency obtained was higher than the values reached in cultivars 'IAS5' and 'BRSMG68' (5). These authors obtained a 45,0 % conversion in cultivar 'BRSMG68' and 42,0 % in 'IAS5', attributed to an early germination of the somatic embryos without having reached the sufficient maturity, which causes a poor development in the plants *in vitro* and reduces regeneration efficiency. This also indicates that plant conversion efficiency could be influenced by the genotype.

In this regard, in the Brazilian soybean cultivar 'CD 201', 82,0 % of conversion to plant was achieved (23). However, this efficiency decreased in the cultivars 'CD 220' (58,0 %) and 'CD 216' (51,0 %), attributed to the presence of somatic embryos with abnormal morphologies that managed to germinate but did not convert the plant. Other authors concluding the 28 days of germination (4), the *in vitro* plants were transferred to culture flasks containing a mixture of peat-perlite-vermiculite substrates (2: 1: 1), which was hydrated with DM salts 25,0 %) and cultured 28 days at a temperature of 26±1 °C and a photoperiod of 16 hours of light (150 µmol $m^{-2}s^{-1}$). During this period the plants were hardened under controlled conditions before being transferred to the greenhouse, and allowed that after 21 days of *ex vitro* cultivation they achieved an 80,0 % survival in cultivar 'DS 2706 '.

As for the characteristics of the regenerated plants, one of the causes that could have caused that the coming from somatic embryos were smaller than those obtained from seeds, is that the reserves of proteins and lipids in the somatic embryo can decline before occurring the root elongation, due to the lack of the nutritive tissue surrounding the seed (24). This differs from the plants that are obtained from seeds, where the nutritive substances are mobilized from different parts of the plant, towards the seeds that are in development and soon these substances contained in the seeds favor the germination and development of the outbreak.

CONCLUSIONS

- The germination efficiency of somatic embryos and their ability to convert to plant, is related to the type of morphology that they develop.
- Somatic embryos with dicotyledonal or normal morphology presented complete germination.
- Somatic embryos with abnormal morphologies such as monocotyledonal, polycotyledonal and rudimentary cotyledon have complete and partial germination.
- The survival percentage of regenerated plants from somatic embryos is influenced by the type of morphology and efficacy in the maturation of somatic embryos.

Table II. Morphological characteristics of soybean plants (*Glycine max* L.), 'Incasoy-27' cultivar regenerated via somatic embryogenesis and controls obtained from seeds at 75 days in houses of culture

Variables	Botanical seed		Somatic embryogenesis			
			Plants *in vitro*		Offspring	
	Half	Middle range	Half	Middle range	Half	Middle range
Average height of the plant (cm)	59,30	38,42 a	21,40	10,50 b	63,10	42,58 a
Height to the first sheath (cm)	9,05	33,90 a	7,50	19,90 b	9,65	37,70 a
Number of trifoliate leaves	7,35	38,70 a	5,00	15,75 b	7,15	36,98 a
Branches per plant	4,15	33,40 a	3,30	21,60 b	4,35	36,50 a
Legumes per plant	51,30	39,45 a	5,75	10,50 b	54,40	4,55 a
Seeds per vegetable	2,60	33,92	2,15	25,15	2,50	32,42

Mean ranks with unequal letters in the same row differ according to the Kruskal-Wallis / Mann-Whitney test ($p < 0,05$; $n = 50$)

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