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DETERMINATION OF MINIMUM LETHAL CONCENTRATION OF GLUFOSINATE-AMMONIUM FOR SELECTION OF TRANSFORMED SOMATIC EMBRYOS OF SOYBEAN CULTIVAR INCASOY-27

Determinación de la concentración mínima letal de Glufosinato de amonio para seleccionar embriones somáticos transformados de soya cultivar INCASoy-27

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ABSTRACT. The establishment of a genetic transformation program requires a selection *in vitro* system of transformed tissues. In the *Glycine* genus exists few references that employ the Glufosinate-ammoniun that selective agent in transformed somatic embryos. For this reason this work had as objective to determine the minimum lethalconcentration of the herbicide Glufosinate-ammoniun in somatic embryos of Cuban soybean cultivar INCASoy-27, to use as selective agent in genetic transformation programs. The soybean somatic embryos were placed on multiplication medium that contained 2,4-dichlorophenoxyacetic acid 20 mg L^{-1} with different concentrations of Glufosinate-ammonium (1,0; 2,0; 3,0; 4,0; 5,0; 6,0 mg L^{-1}). All concentrations of selective agent caused necrosis in not transformed soybean somatic embryos. The increments of selective agent concentration and culture time increased the necrosis of somatic embryos. Finally was determined the minimum lethal concentration of 6,0 mg L^{-1} Glufosinate-ammoniun in semisolid medium, that selective agent in the genetic transformation of somatic embryos of soybean cultivar INCASoy-27.

Key words: agents, somatic embryogenesis, phosphinothricin, *Glycine max*, genetic transformation

RESUMEN. El establecimiento de un programa de transformación genética de plantas requiere de un sistema de selección *in vitro* de los tejidos transformados. En el género *Glycine* existen pocas referencias que emplean el Glufosinato de amonio como agente selectivo de embriones somáticos transformados. Por ello, este trabajo tuvo como objetivo determinar la concentración mínima letal del herbicida Glufosinato de amonio en embriones somáticos de soya cultivar cubano INCASoy-27, para usarlo como agente selectivo en programas de transformación genética. Los embriones somáticos fueron colocados en medio de cultivo de multiplicación con 20 mg L^{-1} de ácido 2,4-diclorofenoxiacético y diferentes concentraciones de Glufosinato de amonio (1,0; 2,0; 3,0; 4,0; 5,0; 6,0 mg L^{-1}). Todas las concentraciones del agente selectivo causaron necrosis en embriones somáticos no transformados de soya. El incremento de la concentración del agente selectivo y el tiempo de cultivo, aumentó la necrosis de los embriones somáticos. Finalmente se determinó la concentración mínima letal de Glufosinato de amonio 6,0 mg L^{-1}, en medio de cultivo semisólido, requerida para su empleo como agente selectivo en la transformación genética de embriones somáticos de soya cultivar INCASoy-27.

Palabras clave: agentes, embriogénesis somática, fosfinotricina, *Glycine max*, transformación genética

INTRODUCTION

Soybean [*Glycine max* (L.) Merrill], is one of the most important economic crop in world agriculture due to its high oil and protein content in the seeds, so it is useful for food and industrial purposes (1).

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Due to its economic importance, breeding programs have been designed through traditional methods that pose limitations for the low genetic variability potential among cultivars. In this regard, genetic transformation provides the possibility of producing new cultivars breaking off sexual barriers. However, gene transfer is not always stable due to the low efficiency and randomization in the integration of foreign DNA into the genome of the host cell being necessary a selection system of transformed tissues (2).

In soybean, most of the genetic transformation research uses the selective agent the hygromicine antibiotic (3, 4, 5). However, there is a worldwide rejection to the use of antibiotics as selective agents due to the incorporation potential of selection marker genes in human pathogens (6). It should be emphasized that few published articles use the herbicide Ammonium glufosinate in the selection of transformed somatic embryos of this specie (6, 7).

Ammonium glufosinate is a chemically synthesized compound commercially known as Basta® and Finale®. The active ingredient of this compound is a natural tripeptide produced by the bacterium *Streptomyces hygroscopicus* and the herbicide compound of bialaphos. Ammonium glufosinate is a herbicide that inhibits the synthetase glutamine, an enzyme involved in the assimilation of ammonium and in the nitrogen regulation in plants (2).

Based on the above, in order to perform an efficient selection process it is necessary to know the minimum inhibitory concentration of this selective agent that wipes out the maximum number of cells and non-transformed tissues in which the resistant gene incorporated into the plasmid is not expressed. On the contrary, the use of high concentrations can cause the death of those cells where the acquired resistance could be lower than the concentration used.

This research aimed at determining the minimum inhibitory concentration of Ammonium glufosinate on somatic soybean embryos Cuban cultivar INCASoy-27, to use it as selective agent in genetic transformation programs.

MATERIALS AND METHODS

The research was conducted at the Biotechnology Plant Institute of Santa Clara, Cuba. The Cuban soybean cultivar INCASoy-27, produced by the National Institute of Agricultural Sciences (INCA) through natural hybridization from the Brazilian genotype BR-32, was used.

Essays under *in vitro* culture conditions used immature cygotic cotiledons from sheaths of donor plants. Sheaths were disinfected with tap water and detergent. Then they were dipped in ethanol at 70 % (v/v) for 20 seconds and then washed three times with sterile distilled water. At the end, they were dipped into sodium hypochlorite (2,0 %) (v/v) on a laminar flow table for 20 minutes followed by four rinses with sterile distilled water.

Immature cygotic cotyledons were extracted and placed in culture flasks of 250 mL that contained culture medium for the formation of somatic embryos composed of MS salts (8), vitamin B5, 2,4-dichlorophenoxiacetic acid (2,4-D) 40 mg L^{-1}, sucrose 3,0 %, Gelrite® 0,3 % and pH 7, during four weeks of culture. Somatic embryos were transferred to a multiplication culture medium made up of MS salts, vitamin B5, 2,4-D 20 mg L^{-1}, sucrose 3,0 %, Gelrite® 0,3 % and pH 5,8 during three weeks of culture.

Then, somatic embryos were transferred to a multiplication culture medium enriched with different concentrations of Ammonium glufosinate. In so doing, six concentrations of the herbicide were evaluated (1,0; 2,0; 3,0; 4,0; 5,0; 6,0 mg.L^{-1}) and a control treatment for a total of seven treatments.

Ammonium glufosinate was prepared at a concentration of 20 mg.mL^{-1} and it was sterilized by filtration with the help of an sterile membrane filter with a pore size of 0,22 µm. The solutions of the selective agent were added when the culture medium reached an approximate of temperature of 40 °C prior to solidification.

In order to determine the minimum inhibitory concentration of Ammonium glufosinate, five selection cycles of non-transformed somatic embryos were made in the multiplication culture medium that contained the selective agent. Subculture was made every 10 days during seven weeks according to the selection cycles. At the end of the fifth selection cycle, a sample of somatic embryos was taken and transferred to a multiplication culture medium without the selective agent to prove the inhibitory effect on the regenerative capacity of somatic embryos.

Ten Petri dishes of 5,0 cm of diameter were used for each treatment. The plates contained five groups with approximately 20 mg of somatic embryos in their globular stage. The fresh mass (mg) of somatic embryos was determined with an analytical scale (*Sartorius*). For subcultures, somatic embyros were taken with a spatula and dispersed over the culture medium to facilitate a greater contact with the herbicide present in it.

In each subculture, the number of groups of somatic embryos with necrosis was quantified and the mortality percentage was calculated. Moreover, damages caused by the Ammonium glufosinate were described and the area with necrosis in the groups of somatic embryos of each treatment was determined.

Therefore, an optical microscope OPTON (Axioskop) was used with a descriptive scale of the damage degree (9), where: without damage (degree 1), necrosis 25 % (degree 2), necrosis 50 % (degree 3), necrosis 75 % (degree 4) and total necrosis (degree 5).

Petri dishes were sealed with *Parafilm*® and placed on a growth chamber with artificial light, luminous intensity 68-73 µE m^{-2} s^{-1}, photoperiod of 16/8 hurs (light / darkness) at 24±2 °C. Luminous intensity within the growth chamber was measured with a luxometer EXTECH 401025.

Data analysis was made with the *Statistic Packaged for Social Science* (SPSS) version 18. Data related to the damage degree of embryogenic masses were processed by the *Kruskal-Wallis/Mann-Whitney* Test ($p \leq 0,05$).

RESULTS AND DISCUSION

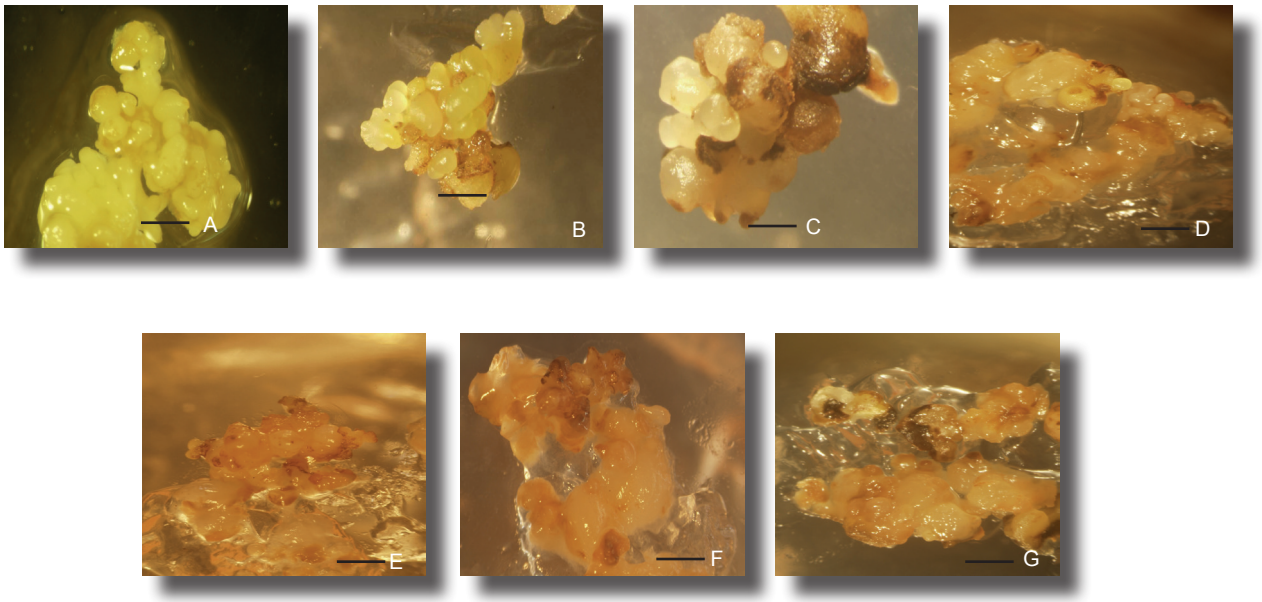
This research could finally define the minimum inhibitory concentration of Ammonium glufosinate used as selective agent of non-transformed somatic embryos in soybean cultivar INCASoy-27, never before described in Cuban soybean genotypes.

All evaluated concentrations of Ammonium glufosinate, caused damages on the non-transformed somatic embryos of soybean cultivar INCASoy-27. After 15 days of culture, somatic embryos acquired a brown color that intensified with the culture time. After 30 days somatic embryos showed a necrotic aspect due to the increased concentration of the selective agent and culture time. However, in the control treatment without the selective agent, somatic embryos retained their yellow color and multiplication capacity (Figure 1).

When analyzing the mortality rate after six weeks of culture with the selective agent, as concentrations of Ammonium glufosinate increased, the damage degree of somatic embryos also increased. It was also observed that concentrations of 0,50 mg L^{-1} and 0,60 mg L^{-1} of the selective agent produced the highest values of damage degrees with significant differences to the rest of the treatments (Table).

The highest mortality values of somatic embryos were reached at the concentrations of 5,0 and 6,0 mg L^{-1} of Ammonium glufosinate with significant differences to the rest of evaluated concentrations. At these concentrations, 80 to 100 % total necrosis of the somatic embryos was recorded (Figure 2).

However, with the concentrations of 3,0 and 4,0 mg L^{-1} of the selective agent, there was necrosis in 50-100 % of the tissue surface and in the total of somatic embryos evaluated (Figure 2).



(A) control, (B) 1,0 mg L^{-1}, (C) 2,0 mg L^{-1}, (D) 3,0 mg L^{-1}, (E) 4,0 mg L^{-1}, (F) 5,0 mg L^{-1}, (G) 6,0 mg L^{-1}. Bar=1,0 mm

Figure 1. Necrosis caused by the concentrations of Ammonium glufosinate in the somatic embryos of soybean cultivar INCASoy-27 after 30 days in the multiplication culture medium

Table. Effect of different concentrations of Ammonium glufosinate on the somatic embryos of soybean cultivar INCASoy-27 after six weeks in the multiplication culture medium

Ammonium glufosinate (mg L^{-1})	Damage degree of somatic embryos	
	Mean	**Mean range**
0	1,00	26,50 e
1,0	2,16	83,72 d
2,0	3,22	144,09 c
3,0	3,98	198,57 b
4,0	4,28	222,12 b
5,0	4,74	267,03 a
6,0	5,00	286,47 a

Different letters in one column are statistically different according to Kruskal-Wallis /Mann Witney Test (p≤0,05)

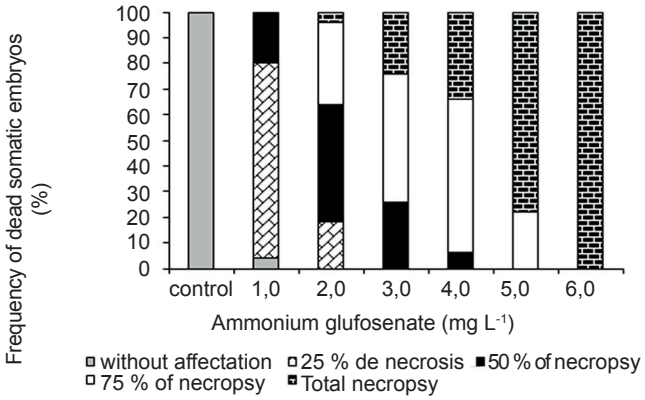


Figure 2. Effect of Ammonium glufosinate concentrations on somatic embryos of soybean cultivar INCASoy-27 after 40 days on a multiplication culture medium

From these results, 4,0 mg L^{-1} was selected as the minimun concentration of Ammonium glufosinate to use as selective agent in transformed somatic embryos of soybean cultivar INCASoy-27 on a semi-solid culture medium.

In this regard, it was taken into account that from this concentration, a 94 % of the somatic embryos evaluated had more than 75 % of the tissue with necrosis (Degree 4), and out of them, 34 % with total necrosis. In addition, these somatic embryos did not recover their regenerative capacity on culture medium without selective agent.

The necrosis observed in somatic embryos is an expression of the phytotoxic effect of Ammonium glufosinate due to the inhibition of the glutamine sinthetase, an essential enzyme for the assimilation of ammonium and the regulation of nitrogen in tissues (10).

The Weed Science Society of America (acronym in English WSSA) defined resistance as the inherited capacity of a plant to survive and reproduce after being exposed to a concentration of a lethal herbicide in the wild species. It can be natural or induced by Genetic Engineering, selection of varieties produced by cell and tissue culture or through mutagenesis (11).

On the other hand, the efficiency of genetic transformation can be improved changing the concentration and duration of the selection regime or by using different markers and selection agents.

The consulted literature (7) refers to the use of hygromicine 30 mg L^{-1} or phosfinotricine 80 mg L^{-1} in the liquid culture medium during the selection of transformed somatic embryos of soybean. As a result, 12 transgenic lines resistant to hygromicine were produced. However, only two lines showed resistance to phosfinotricine, and in one of them, the presence of the bar gen was confirmed. All the plants were sterile, attributed to the age of embryogenic tissues and to the transformation method used.

Ammonium glufosinate has been tested on a wide range of concentrations, according to the use in liquid or semi-solid culture media. In a study, transformed somatic embryos of soybean, cultivar Jack, were placed on a liquid culture medium FN-Lite (from English: *Finer & Nagasawa Lite*) with and without asparagine, and 5,0 a 35 mg L^{-1} of Ammonium glufosinate. As a result, with the use of 2,5 mg L^{-1} of this herbicide, it was possible to inhibit growth in more than 95 % of the somatic embryos after three weeks of culture, with total inhibition after five weeks of culture (6).

In a second study, these authors evaluated 0,16 a 0,5 mg L^{-1} of Ammonium glufosinate on a FN-Lite culture medium 50 % without aparagine. However, the only difference found was that the growth inhibition of somatic embryos started the first week of selection with the use of FN-Lite 50% culture medium, while in the FN-Lite normal culture medium, inhibition was observed from the second week on (6).

The results of this research differ from those previously described (6), which is attributed to the type and composition of the culture medium. The use of the liquid culture medium permits a higher exposure of tissues to contact with the selective agent and reduce the selection time.

On the contrary, on a semi-solid culture medium only the parts of the tissue in contact with the culture medium are exposed to the effect of the selective agent and somatic embryos known as false positive can show up in areas not in contact with the herbicide present on the culture medium.

The consulted literature (12) refers to the use of 5,0 mgL^{-1} of Ammonium glufosinate in the selection of cotyledon nodes of soybean during four weeks. After this period, there was necrosis in the non-transformed tissues while the transformed ones formed shoots in the cultivars Jack (82,8 %) and Williams (67,7 %).

Other authors also observed that 3,0 to 5,0 mg L^{-1} of Ammonium glufosinate are enough to prevent the formation of shoots in the cotyledon nodes of soybean (13). These concentrations are similar to those required to inhibit the multiplication of somatic embryos of soybean according to this study.

The Bialaphos or tripeptid phosfinotricine that is produced by *Streptomyces hygroscopicus,* has also been used as selective agent. Some authors found that no shoot was formed from the cotyledon nodes of soybean by using bialaphos at 5,0 mg L^{-1}, and only 5,0 % were able to regenerate shoots by using 4,0 mg L^{-1}, taking this latter concentration for the selection of transformers (14).

In other legume species as *Phaseolus vulgaris* it was shown that 0,50 mg L^{-1} of Ammonium glufosinate was enough to make that more than 87 % of callus showed total necrosis after eight weeks of culture (9).

Differences found in the concentrations of Ammonium glufosinate required to inhibit the multiplication of somatic embryos could be related to the cell composition of this tissue, that is made up of differentiated cells under active division process that differ from the totally undifferentiated cells forming the callus.

There also studies that have used other types of herbicides and have confirmed the inhibition of the multiplication of somatic embryos of soybean on a liquid culture medium. Such is the case of the herbicide Bispyribac-sodium, an inhibitor of the enzyme acetolactate syntheetase. When 1.0 µM of this herbicide was used, somatic embryos of the cultivar Jack that did not have the transgens *Os-mALS* showed up, and with 2,0 µM did not survive (15).

Taking into account the results expressed herein, they are the first reference of determining the minimum inhibitory concentration of Ammonium glufosinate for its use in the selection of transformed somatic embryos of soybean on a semi-solid culture medium.

CONCLUSIONS

The minimum inhibitory concentration of Ammonium glufosinate (4.0 mg L^{-1}), in semi-solid culture medium required for its use as agent for the selection of transformed somatic embryos of soybean cultivar INCASoy-27 was determined.

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