Efficient transformation of potato stems segments from cultivar Désirée, using phosphinothricin as selection marker

RESEARCH

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ABSTRACT

This work describes the development of a fast and efficient methodology for *Agrobacterium tumefaciens*-mediated genetic transformation of internodal stem segments from potato (*Solanum tuberosum* L.), cultivar *Désirée*, using phosphinothricin (PPT, glufosinate ammonium) as a selection agent. This methodology has a transformation efficiency of 68%, inducing shoots after 4 to 5 weeks in the presence of 2 mg/L of phosphinothricin. The expression of the *bar* gene in leaf segments from resistant, transformed plants was demonstrated by the chlorophenol red assay, as was its presence in the genome of explants by Polymerase Chain Reaction. An additional observation was the presence of high levels of resistance to phosphinothricin (up to 500 mg/L) in the transgenic clones, with an otherwise normal phenotype. These transgenic plants were transplanted to greenhouses and after aspersion with the herbicide Finale® (Bayer CropScience) at 2.5 L/ha remained healthy and green, in contrast with the non-transgenic controls which died after 7 days.

Keywords: potato, genetic transformation, herbicide resistance, Finale®, PPT, phosphinothricin

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RESUMEN

Transformación eficiente de segmentos de tallos de papa de la variedad *Désirée*, utilizando fosfinotricina como marcador de selección. Se desarrolló una metodología rápida y eficiente para la transformación genética de segmentos internodales de tallos de papa (*Solanum tuberosum* L.), de la variedad *Désirée*, mediante *Agrobacterium tumefaciens*, utilizando fosfinotricina (PPT, glufosinato de amonio) como agente de selección. Esta metodología provocó una eficiencia de transformación del 68% y la inducción de brotes después de 4 a 5 semanas en presencia de 2 mg/L de fosfinotricina. Se empleó exitosamente el ensayo del clorofenol rojo en segmentos de hojas de plantas transformadas, lo cual demostró la expresión del gen *bar*. La presencia de este gen en el genoma de los clones resistentes se confirmó mediante reacción en cadena de la polimerasa. Además, se observó la elevada resistencia de clones transgénicos hasta la concentración de 500 mg/L de fosfinotricina, con un desarrollo fenotípico normal. Estas plantas transgénicas se transplantaron a casas verdes, y después de ser asperjadas con el herbicida Finale® (Bayer CropScience), a una concentración de 2.5 L/ha, se mantuvieron vigorosas y de color verde, a diferencia del control no transgénico, que murió después de 7 días.

Palabras clave: papa, transformación genética, resistencia a herbicida, Finale®, PPT, fosfinotricina

*I*ntroduction

The high agricultural productivity and elevated carbohydrate, vitamin and protein contents of potato (*Solanum tuberosum* L.) have turned this plant into one of the most important crops for human nutrition worldwide. Around 13 000 hectares of potato are planted yearly in Cuba, with an average yield ranging from 18 to 25 ton per hectare (translatin g into 300 000 ton per year [1]. However, these yields could be increased up to 30% if more effective means to stop the incidence of weeds were available [2].

Weed growth remains one of the main agricultural problems in the world, with a detrimental effect in the yields and quality of many economically important crops. In many cases, this situation has been the main reason of existence for projects aimed at the development of herbicide-tolerant transgenic plants, since the directed application of herbicides afforded by their use may result in a significant increase in crop yields [3]. Currently there are no commercially available herbicide-tolerant transgenic varieties of potato, and therefore this productive alternative represents a challenge for our country.

Different methodologies based on the use of *Agrobacterium tumefaciens* as a vector have been described for the obtention of transgenic potato. However, since most of these techniques use antibiotic resistance genes as selection markers [4, 5], there is not much available information about the behavior of this culture in the presence of herbicides as selection agents [6-10]. Phosphinothricin (PPT; glufosinate ammonium), the active ingredient of commercial herbicides such as Basta® o Finale®, constitutes a potentially very useful agent that could be used both for selecting transformed clones and for weed control in the resulting transgenic crops. Obtaining phosphinothricin-tolerant crop varieties has obvious commercial benefits, given the wide spectrum of these herbicides, their low toxicity for human, animals and the environment, and their high acceptance among farmers [11].

Phosphinothricin (PPT) is a glutamate analog that inhibits irreversibly the activity of glutamine synthetase, the enzyme responsible for ammonium assimilation and the regulation of nitrogen metabolism in plants. The inhibition of glutamine synthetase leads to the

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death of untransformed tissues, leading to plant death due to the accumulation of ammonia [12]. Resistance to PPT is mediated by phosphinothricin acetyltransferase (PAT), an enzyme coded by the *bar* gene from *Streptomyces hygroscopicus* [13].

The published potato transformation protocols employing phosphinothricin as a selection marker use either leaf [14, 15] or tuber [16] disc explants. However, it is known that explants based on internodal stem segments are easier to manipulate *in vitro* and have a high regenerative potential [17, 18], constituting therefore an attractive target for increasing the efficiency of the transformation process [19]. Hence, we decided to develop a methodology for the genetic transformation of internodal stem segments from the potato cultivar *Désirée*, based on phosphinothricin as selection agent. Through the use of a regeneration protocol modified in our laboratory [20], transgenic plants with high levels of resistance to phosphinothricin were obtained and further characterized in regards to their phosphinothricin acetyltransferase activity.

Materials and methods

Plant material

This work used potato plants, cultivar *Désirée*, propagated *in vitro* in propagation media (PP) by subculturing to fresh medium every 4 weeks. The pH of the medium was adjusted to 5.7 before autoclaving, which was carried out at a temperature of 121 ºC and 1 atmosphere of pressure. The plants were subjected to a photoperiod of 16 h of light, at a temperature of 25 ºC.

Culture media

The culture media used in this work have been previously optimized in our laboratory [20]:

Propagation medium PP: MS salts [21], 0.4 mg/L thiamine, 100 mg/L *myo*-inositol, 2 mg/L calcium pantothenate, 30 g/L sucrose and 6 g/L phytoagar.

Callus induction medium SC: MS salts, 0.4 mg/L thiamine, 100 mg/L *myo*-inositol, 20 g/L sucrose, 3.5 mg/L benzylaminopurine (BAP), 0.01 mg/L naphthaleneacetic acid (ANA) and 6 g/L phytoagar.

Shoot induction medium SB: MS salts, 0.4 mg/L thiamine, 100 mg/L *myo*-inositol, 20 g/L sucrose, 3.5 mg/L gibberellic acid (GA_3) and 6 g/L phytoagar.

Callus induction medium JC: MS salts, 0.4 mg/L thiamine, 100 mg/L *myo*-inositol, 20 g/L sucrose, 4 g/L mannitol, 3.5 mg/L BAP, 0.2 mg/L AIA, 6 g/L phytoagar.

Genetic transformation of internodal stem segments from potato

The genetic transformation experiments employed the At 2260 strain from *Agrobacterium tumefaciens*, bearing the pCAMBIA 3300 plasmid (CAMBIA, Australia). This vector contains the *bar* gene (coding for the phosphinothricin acetyltransferase (PAT) enzyme) under the regulatory signals of the 35S promoter and the 35S terminator of the *Cauliflower mosaic virus* (CaMV) (Figure 1). The bacterium was grown in liquid medium Luria-Bertani [22] supplemented with 50 mg/L rifampicin, 100 mg/L streptomycin and 30 mg/L kanamycin for 16 h at 28 ºC

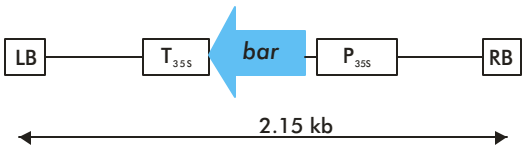

Figure 1. T-DNA fragment from the pCAMBIA 3300 vector bearing the *bar* gene. LB, left border, RB, right border; T_{35s}, 35S terminator from *Cauliflower Mosaic Virus* (CaMV); *bar*, phosphinothricin acetyltransferase gene; P_{35s}, 35S promoter from CaMV.

with an agitation of 100 rpm, until an optical density at 620 nm of 0.7 to 0.9 was reached.

Internodal stem segments from plants 4 to 5 weeks old were used, selecting exclusively the first 6 segments (from 5 to 7 mm) starting from the apex, as described by Enríquez *et al.* (1997) [20]. The minimum lethal concentration of PPT for inhibiting morphogenesis in potato explants was previously found to be 2 mg/L. The internodal stem segments were sliced and infected by submersion for 10 min. into the bacterial suspension, diluted 1:20 in MS salts. Afterwards, the infected explants were placed in SC medium for co-culturing in the dark at a temperature of 22 ºC for 48 hours, after which they were rinsed twice in sterile water and once with water containing 500 mg/L claforan. Excess water was then eliminated by blotting on sterile filter paper, and the explants were cultured under illumination for 10 to 15 days in SC medium containing 500 mg/L claforan and 2 mg/L PPT, followed by transfer to SB medium containing 500 mg/L claforan and 2 mg/L PPT, until the obtention of shoots.

The regenerated shoots, approximately 1 cm high, were separated individually and placed on PP medium supplemented with 500 mg/L claforan and 2 mg/L PPT for rooting. Once rooted, the plants were propagated *in vitro* and left to root again at a higher PPT concentration (5 mg/L).

Chlorophenol red assay

The chlorophenol red (CR) assay [23] was performed on leaf fragments from *in vitro*-grown potato clones resistant to PPT at a concentration of 5 mg/L. The pH indicator was resuspended in ethanol and added to JC medium after its preparation at a final concentration of 50 mg/L, conferring a red-purple color to the medium. The medium + pH indicator was sterilized and then supplemented with 5 mg/L phosphinothricin, without any further color changes. The results of the assay were evaluated after a period of 72 h, during which the leaves were subjected to a photoperiod of 16 h light and 8 h of darkness at a temperature of 25 ºC.

A plate with JC medium containing CR (without explants) was used as a control for the coloration of the medium; and two plates with or without PPT (with untransformed explants) were used as controls for the transgenesis. The assay used 3 plates per transformed clone, and 10 leaf fragments per plate.

Analysis of phosphinothricin-resistant plants by polymerase chain reaction

The genomic DNA from 5 to 6 weeks old potato plants resistant to 2 mg/L PPT and from an untransformed control was purified from 1 g of leaf tissue, following

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the Dellaporta protocol (1983) [24]. Oligonucleotides complementary to the 5′ and 3′ regions of the *bar* gene were used as primers [25], with the sequences 5′-CGAGACAAGCACGGTCAACTTC-3′ (forward primer) and 5′-AACCCACGTCATGCCAGTTC-3′ (reverse primer) and an expected product of amplification by Polymerase Chain Reaction (PCR) of 402 bp. The reactions had a total volume of 30 µL and contained 1X Taq DNA pol buffer (Promega); 1.75 mM $MgCl_2$; 25 pmole of each primer (forward and reverse); 200 µM dNTPs (Promega); 1 U of Taq DNA Polymerase (Promega) and 200 to 600 ng of genomic DNA. The reactions were incubated on a PCR thermocycler (PTC-100TM MJ Research) using a step of 5 min at 95 ºC, followed by 40 cycles of 1 min at 95 ºC, 1 min at 62 ºC and 1 min. at 72 ºC, and a final extension step of 72 ºC for 10 min. The PCR products were analyzed on 0.8% type I (Sigma) agarose gels.

Evaluation of the *in vitro* resistance of the transgenic plants to high PPT concentrations

This assay tested different PPT concentrations (2, 5, 10, 25, 50, 100, 250 and 500 mg/L) on buds from plants propagated *in vitro* at 2 mg/L of PPT, using 2 buds per flask and 4 flasks per concentration. Non transgenic plants from the cultivar *Désirée* were used as controls for each PPT concentration, together with transgenic plants from each clone in PP medium without selection. The assay evaluated the development of leaves and roots, and the height of the plants was measured.

Assay for tolerance to the commercial herbicide Finale® in greenhouse conditions

The assay for tolerance to the Finale® herbicide was performed with 10 transgenic clones (8 plants/clone) and a non transgenic control (20 plants), which were transplanted to pots in greenhouses. The commercial herbicide Finale® (Bayer CropScience, containing 150 g/L of glufosinate ammonium) was aspersed onto the plants, at a dose of 2.5 L/ha (field dose).

Results and discussion

Influence of the efficiency of regeneration on transformation efficiency

The use of phosphinothricin as a selection agent constitutes an alternative to more commonly used antibiotics (e.g. kanamycin) for the transformation of economically important crops such as potato and the selection of transgenic plants [26-29]. The dual role played by this herbicide allows its application for the selection of transformants and also for weed control once the transgenic plants are cultured under field conditions. However, there are only a few publications dealing with the obtention of phosphinothricin-tolerant potato plants [14-16], and none of them uses stem segments as explants. It has been described that the explants obtained from internodal stem segments have a high regenerative potential [17, 18] and, therefore, constitute an attractive candidate for improving the efficiency of the transformation process [1, 14, 19].

This study used an optimized shoot regeneration protocol [20] that is implemented in two stages: callus induction in SC medium, and shoot formation in SB medium. With this methodology, it was possible to obtain yellow and green calluses 10 to 15 days after the infection with *Agrobacterium tumefaciens*, using SC medium containing 500 mg/L of claforan and 2 mg/L PPT (Figure 2A). Normal shoots were obtained 30 days post-infection (Figure 2D and 2E), at a rate of 2.9 to 4.3 shoots per each explant successfully regenerated in the present of PPT. More than 60% of the explants regenerated shoots in PPT selection medium (Table 1), and previous results have shown that the highest levels of regeneration are reached using internodal segments 2 and 3 (counting from the apex down).

Most protocols for potato transformation published so far also use a two-stage system with different culture media for the induction of calluses first and shoots later. This system has worked efficiently for stem [17, 18, 30, 31] and leaf [32-34] segments from different potato varieties, even though there are differences between the hormonal conditions used in these publications. The method, however, was inefficient for the transformation of two *Andean* potato varieties, where higher efficiencies were achieved by regeneration in kanamycin (28 and 34%) using a single culture medium with Zeatin and AIA [35].

A combination of BAP and NAA similar to that used for the SC medium has also been effective for callus induction from leaf segments of an *Andean* potato variety [32], but a combination of NAA, ZR and GA_3 was required for obtaining shoots. However, while the work described here used only GA_3 for shooting, the rate of regeneration in the presence of PPT was higher (Table 1) than that reported for the *Andean* variety in the presence of kanamycin (58%) [32]. The addition of GA_3 to the SB medium promotes the development and elongation of the regenerated shoots (Figure 2D and 2E), and this phenomenon has also been shown to occur in other potato varieties [20, 36]. Nevertheless, Newell *et al*. (1991) [30], who employed a hormonal mixture for stem segments from cultivars *Désirée* y *Kennebec* similar to that used in this work, obtained much lower (13 and 14%) transformation frequencies when selecting under kanamycin than those reached with PPT in this study (68%, calculated as the percentage of explants able to regenerate shoots in the presence of PPT). The regeneration medium plays a major role in obtaining high transformation efficiencies, and we coincide with other authors [15, 37] on the existence of an interaction between the genotype and the optimal regeneration protocol.

The use of SC medium with 2 mg/L of PPT decreased callus formation for most of the infected internodal segments (Figure 2A and C). At the beginning of their development, these calluses were very small and spherical (similar to embryogenic calluses) for some explants (Figure 2B), whereas in other explants they formed a green compact mass (Figure 2C). In spite of these differences, shoots were formed from both types of calluses, and normal plants were obtained in all cases. Similar calluses have also been obtained for the *Russet Burbank* potato variety, and none of the shoots studied in that report turned out to be a chimera [30]. According to some authors, cultured potato cells are highly sensitive to somaclonal variation, and a decreased callus formation can actually be

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beneficial for reducing the influence of this factor [17, 38]. Callus formation is related to the methylation of genes involved in cellular differentiation, ultimately leading to a progressive loss of totipotency and the formation of aberrant shoots [39]. Studies performed on potato stem segments have shown that the transformation process has a significant effect on the formation of calluses, based on the comparison of transgenic and non-transgenic genotypes [40].

Regarding the non-transformed controls, they formed calluses in 100% of the cases (either in one or both tips of the segment) when grown in the absence of selection. After culture for 10 days in regeneration medium (SB), these calluses in turn formed shoots with a regeneration efficiency of 100% (i.e. the percentage of explants that regenerated shoots), at a rate of 7.15 shoots per explant (data not shown). In contrast, there were no calluses or shoots from the explants of these controls when grown in SB medium with 2 mg/L de PPT, acquiring a brownish color and progressing to total necrosis after 30 days of culture.

A total of 452 regenerated shoots were obtained in the presence of the herbicide from 3 separate experiments (Table 1), from which a group of 100 shoots (clones) was selected for rooting in PP medium with claforan and PPT (2 mg/L). After 7 days under selection, 68 clones rooted and developed into normal plants, resulting in a transformation efficiency of 68% (calculated as the percentage of regenerated plants that rooted in the presence of 2 mg/L de PPT). This efficiency is high, as can be seen when comparing this stem transformation protocol with direct selection in PPT to other potato transformation protocols that employ leaf segments as explants [14, 33]. For instance, De Block [14] obtained a transformation efficiency of only 30% with the cultivars *Bintje* and *Désirée* using a three-stage regeneration scheme, in a callus induction medium containing 5 and 10 mg/L PPT (the efficiency was defined as the rate of callus formation in the presence of the active compound); and Barrell *et al*. (2002) [33] did not obtain shoots upon direct selection from leaf explants in 10 mg/L PPT. These authors [14, 33] agree that phosphinothricin is not as efficient as a selection marker for potato when compared e.g. to kanamycin (referring to the regeneration of shoots in the presence of PPT), which differs with our results using the cultivar *Désirée*.

Out of the 68 clones that rooted in 2 mg/L de PPT, 15 clones (22%) were able to root in 5 mg/L PPT. Four of these clones (tolerant to 5 mg/L PPT) exhibited faster growth and rooting rates than the rest, and were therefore propagated *in vitro* in order to test their tolerance to higher PPT concentrations.

Influence of the PPT selection marker on the efficiency of transformation

The use of the visual chlorophenol red assay allowed a fast identification of the transformed tissues expressing the *bar* gene. This assay is based in changes of coloration of the culture medium due to variations in pH caused by the metabolic activity of the cultured explants: freshly prepared JC medium containing CR has a red color after adjusting the pH to 6, as shown in figure 3A; however, when pH decreases, the red tinge of the medium changes to yellow around the leaf segments from metabolically active samples. All the clones evaluated in this assay were positive, with the yellow tinge progressively extending throughout the medium as shown in Figure 3B. The acidification of the medium is an indicator of a physiologically active status for the cells under study [41], and is therefore a surrogate marker for the activity of the PAT enzyme, which detoxifies the PPT from the culture medium. This color change also occurred in the positive control (non transgenic plant in medium without PPT, Figure 3D), although the change was more pronounced around the transgenic explants. Perhaps PPT further stimulates the metabolism of cells bearing the *bar* gene, and although there are no experimental data supporting this interpretation,

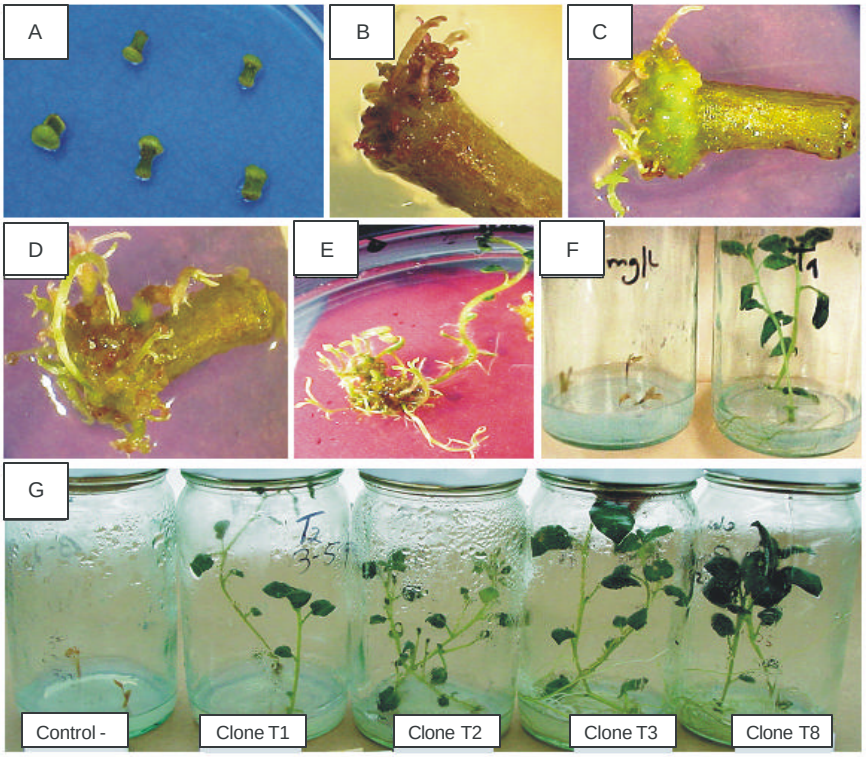

Figure 2. *In vitro* regeneration of transgenic potato plants: growth *in vitro* and selection in PPT.
A. Formation of calluses in internodal stem segments 15 days post infection in SC medium containing claforan and 2 mg/L PPT.
B, C, D and E. Shooting in SB medium with 500 mg/L claforan and 2 mg/L PPT (B-C: 20 days and D-E: 30 and 40 days)
F. Non-transgenic plant rooting in PP medium with 2 mg/L de PPT (left) and transgenic plant rooting in PP medium without selection (right).
G. Transgenic plants rooting in PP medium supplemented with 100 mg/L de PPT; the non-transgenic control is shown to the left.

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Table 1. Shoot regeneration frequency from stem segments grown and selected in 2 mg/L PPT.

Experiments	Number of explants	Number of explants with calluses (%)	Number of explants with shoots (%)	Total number of shoots regenerated in PPT	Number of shoots/explants with shoots
I	60	60 (100)	50 (83.3)	145	2.9
II	60	60 (100)	36 (60)	156	4.3
III	50	40 (80)	39 (78)	151	3.8

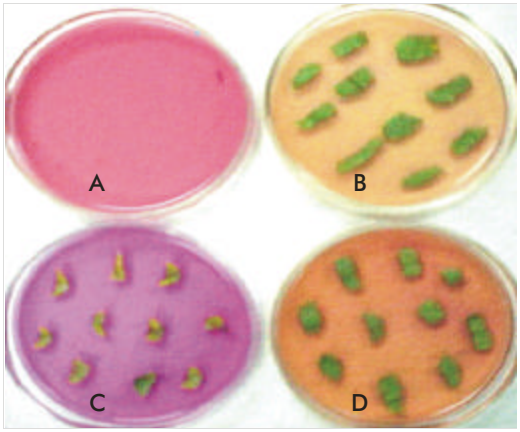

Figure 3. Chlorophenol Red (CR) biological assay.
A. Starting control for the coloration of JC medium with CR.
B. Explants from transgenic plant under selection with PPT (5 mg/L).
C. Explants from non-transgenic plant under selection with PPT (5 mg/L), used as a negative control.
D. Explants from non-transgenic plants used as a positive control.

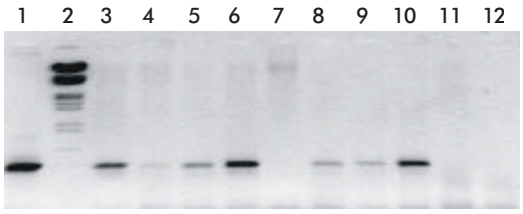

Figure 4. Products of PCR amplification from genomic DNA of potato plants, cultivar *Désirée*, using primers specific for the *bar* gene. The expected product is a 402 bp band. The visualization was performed on 0.8 % agarose gels.
Lane 1. Plasmid pCAMBIA 3300 (Positive control)
Lane 2. Molecular weight marker (λ Eco R I/Hind III)
Lanes 3 to 10. DNA isolated from plants rooting on PP medium with 2 mg/L PPT
Lane 11. DNA isolated from untransformed plant (Negative control)
Lane 12. PCR reaction without template

several authors have reported a stimulatory effect for herbicidal compounds (at sub-lethal concentrations) on the morphogenesis of some plant tissues under different culture conditions [42, 43]. For instance, it has been observed that phosphinothricin at concentrations from 0.5 to 1 mg/L stimulates the production of somatic embryos during the *in vitro* culture of grape [44]. This herbicide also had a cytokinin-like effect on rice tissues [45, 46], and stimulated shoot differentiation and plant regeneration in *Antirrihium majus* L. [47].

Kramer *et al*. (1993) pointed out that the CR assay should not be extended beyond 72 hours in order to avoid the appearance of CR-induced toxicity on the calluses, underlining the non-destructive nature of this test [23, 48]. This assay, for example, has allowed the study, in a short time, of large number of transgenic maize plants bearing the *bar* gen [48].

In the case of the samples that did not tolerate the amount of PPT in the culture media, the pH values increase above 6 due to the accumulation of ammonia, therefore changing the color of the medium from red to red-violet (Figure 3C). The only samples exhibiting this behavior were the leaf explants from non-transformed plants, used as negative controls The absence of the *bar* gene in these cases was further confirmed by PCR, as shown in figure 4.

The 68 plants rooting in 2 mg/L PPT were also tested by PCR, and 98% of these samples yielded the expected amplification product of 402 bp corresponding to the *bar* gene. This constitutes an additional confirmation for the presence of *bar* in the transformed potato plants (Figure 4). Fifteen of the PCR-positive clones also rooted at a PPT concentration of 5 mg/L, and the 4 fastest-growing clones at 5 mg/L PPT were selected for further testing of their tolerance to higher PPT concentrations *in vitro*.

As the result of this experiment, the 32 evaluated transgenic plants (8 plants per clone) rooted at all the tested PPT concentrations, similarly to the non-transgenic clone in the absence of selection (Figure 2F). Rooting, however, was more pronounced in clones T3 and T8 (Figure 2G). After 30 days in PP medium with selection, 3 of the evaluated clones grew normally at PPT concentrations up to 500 mg/L with the exception of clone T1, which did not tolerate the highest tested amount of PPT (Figure 5). The normal growth pattern of these clones was evidenced by the dark green color of their leaves (Figure 2G), whose size and shape were similar to those of the transgenic replicate in nonselective medium (Figure 5); as well as by the height reached by the plants. The results confirm the resistance of the transgenic plants to PPT and the transformation event. Most of the resistant clones reached a higher height than their replicates without selection when grown at PPT concentrations up to 100 mg/L (Figure 5). The high PPT tolerance (up to 500 mg/L) observed during rooting for these clones is higher than that reported by other authors for transgenic clones of cultivar *Mantiqueira* (10 mg/L) [15]

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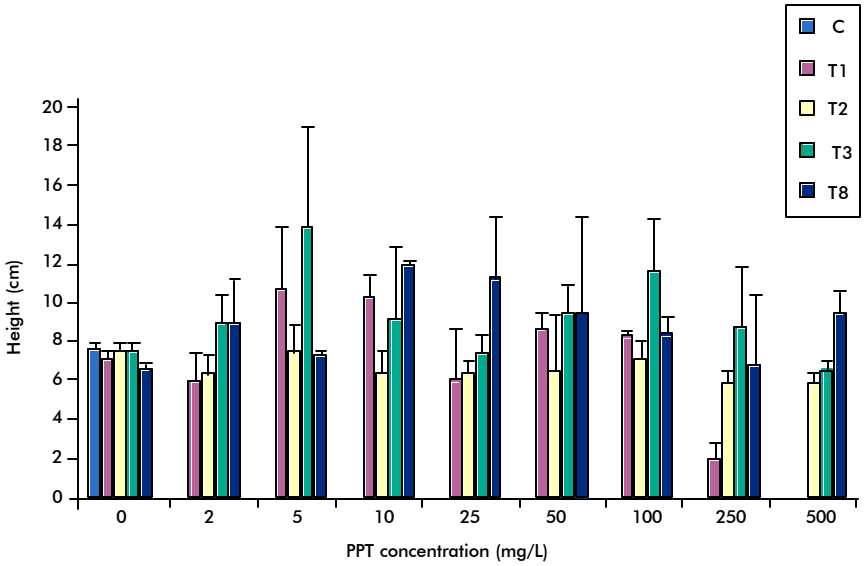

Figure 5. Height of potato plants in propagation medium (PP) at different PPT concentrations. The columns represent the evaluated transgenic clones (T1, T2, T3 and T8) and the non-transgenic control (C). Eight plants were evaluated per clone.

or for transgenic leaves from cultivar Désirée that were able to form calluses at 50 mg/L of PPT [14]. The non-transgenic control was highly susceptible to PPT concentrations of 2 mg/L, with the buds turning brown and dying after 7 days, as shown in figure 2F.

Resistance of the transgenic plants to the commercial herbicide Finale® under greenhouse conditions

In this experiment, 80 transgenic plants (8 plants/clone) expressing the *bar* gene under the 35S promoter and 20 non-transgenic controls were transferred to pots under greenhouse conditions. The plants were aspersed with Finale® at 2.5 L/ha, equivalent to the field dose used for weed control. All the transgenic plants tolerated the herbicide (Figure 6). The non-transgenic controls showed symptoms of chlorosis at days 2 to 3, and died 7 days after the aspersion, in contrast to the transgenic plants which remained green throughout the experiment (Figure 6). Similar results were obtained for transgenic potato plants bearing the *bar* gene obtained from leaf explants of cultivar *Mantiqueira* [15], although in this case the clones showed symptoms of chlorosis during the first days after aspersion which disappeared one week later.

Figure 6. Transgenic (left) and non-transgenic (right) plants in greenhouse conditions, one week after aspersion with 2.5 L/ha of the Finale® herbicide.

Conclusions

The results of this work have allowed the optimization of a simple and reproducible methodology for the genetic transformation of stem segments from the potato cultivar *Désirée*. The shoot regeneration frequency in the presence of 2 mg/L PPT was 83.3%, and the transformation frequency was 68%. This methodology has the advantage of a short regeneration time under PPT selection (around 6 weeks), thereby favoring the development of normal plants. A high correlation was observed between the presence of growth at 5 mg/L PPT, the results of the CR assay, and the results of the analysis by PCR. Additionally, clones with high resistance to phosphinothricin (up to 500 mg/L) were obtained, without any observed abnormalities in their growth and development patterns *in vitro*. To our knowledge, there are no other published studies dealing with *Agrobacterium tumefaciens*-transformed transgenic potato plants bearing the *bar* gene that have reached such a high level of tolerance to phosphinothricin. The tolerance to the herbicide was also observed under green-house conditions, where the transgenic plants remained green and healthy after aspersion with 2.5 L/ha of Finale®. These results illustrate the advantages of the use of stem segments for the obtention of potato plants resistant to high concentrations of this herbicide. Finally, it is important to underscore the fact that there were no phenotypic alterations associated to the transformation process in these herbicide-resistant clones.

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