Efficient Transformation of *Solanum tuberosum* cv. Spunta Mediated by *Agrobacterium tumefaciens* Using Hygromycin as a Selective Agent

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ABSTRACT

Plant genetic transformation is a biotechnological tool with a high impact on plant breeding, since it offers the possibility of introducing new traits in crops to fulfill the demands of production and consumption. The most widely used transformation strategy in potatoes is based on the integration of genes via *A. tumefaciens*. The objective of the present work was to develop a transformation protocol for *Solanum tuberosum* cv. Spunta mediated by *A. tumefaciens* using hygromycin as a selective agent. For this, explants of potato cv. Spunta were transformed using *A. tumefaciens* with a binary vector containing the hygromycin resistance transgene (*hpt*) under the control of the NOS promoter. Molecular and phenotypic determinations were performed to validate both the efficiency of the transformation protocol and the performance of hygromycin as a selection agent. Sixty-two regenerated shoots were obtained from explants inoculated with *A. tumefaciens* (62% regeneration), of which 30 plants were positive for the *hpt* gene. A thirty percent transformation efficiency, calculated as the number of transgenic plants / the number of explants inoculated, was obtained using this protocol. The present work presents a simple and efficient protocol for the genetic transformation of potato cv. Spunta by *A. tumefaciens* using hygromycin as a selective agent.

Keywords: *potato, plant transformation, selection, genetic breeding.*

Introduction

Potato (*Solanum tuberosum* L.) is the third most important food crop in the world, with a total of 22 million hectares and an approximate production of 458 million tonnes. In Argentina, during the 2015/2016 season a production of 2,430,000 t was reported (FAOSTAT, 2018). The cultivar Spunta is the most widely used in Argentina because it has a high yield, adapts to double-cropping regions, and has very good storage capacity (Huarte and Capezio, 2013).

One of the objectives of breeding programs is to increase production in order to meet the demand of the sector. Plant genetic transformation, understood as the controlled introduction of nucleic acids into a recipient genome, is a high-impact biotechnological tool that offers the possibility of obtaining improved crops more rapidly through the introduction of a desired trait, for example: increased production, nutritional quality, and tolerance to both biotic and abiotic stresses (Waterer *et al.*, 2010; Imbo *et al.*, 2016). The transformation methods most widely used at present are based on obtaining transgenic cells by means of *Agrobacterium tumefaciens* or biolistics, followed by the regeneration of complete and fertile plants from *in vitro* culture and selection. During the transformation process, only a few target cells survive and stably incorporate the foreign DNA (Birch, 1997). It is therefore essential to detect transformed cells among the large number of untransformed ones and to establish suitable conditions of tissue culture, gene transfer and selection that allow whole plants to be developed from this strategy. For this, “marker” genes are used that allow transformed explants to grow on selective media, making it possible to distinguish them from untransformed ones.

At present, multiple protocols for potato transformation via *A. tumefaciens* have been reported, using various explants, in different cultivars, and with different selection agents (Millam, 2007; Halterman *et al.*, 2016; Craze *et al.*, 2018; Bruce and Rupp, 2019; Bakhsh *et al.*, 2020). The relevance of this topic is mainly due to the fact that cultivated potato, *Solanum tuberosum* Group Tuberosum L. (2n = 4x = 48), is a highly heterozygous tetraploid crop that is propagated clonally; owing to its lack of sexual reproduction and the concomitant segregation of alleles, genetic engineering is an efficient way of introducing crop-improvement traits more rapidly than conventional breeding (Nadakuduti *et al.*, 2019).

It should be noted that, to date, no potato transformation protocol has been published for the cultivar Spunta.

In addition, various works indicate that *A. tumefaciens*-mediated plant genetic transformation is genotype-dependent (Heeres *et al.*, 2002; Cingel *et al.*, 2010; Barpete *et al.*, 2015; Chakraborty *et al.*, 2020), so it is of interest to develop a genetic transformation protocol for commercial potato cultivars such as Spunta, which will make it possible to apply it in future breeding plans.

Materials and Methods

1. Plant material

Apices of *S. tuberosum* cv. Spunta were cultured *in vitro* on Murashige and Skoog medium (MS; Murashige and Skoog 1X basal medium supplemented with vitamins (Phytotechnology Laboratories, Cat. No. M519); 20 $g \cdot L^{-1}$ sucrose (Biopack, Cat. No. 9789.08); 8 $g \cdot L^{-1}$ agar (Phytotechnology Laboratories, Cat. No. A296)) for 3 to 4 weeks in test tubes for the determination of the sensitivity curve, and in plastic magenta boxes for the transformation assays (Phytotechnology Laboratories, Cat. No. C2100). The MS medium was adjusted to pH 5.8 with 0.1 N KOH or HCl before sterilizing in an autoclave (121 °C, 20 min). For the transformation experiments, five apices were placed per culture vessel, in a laminar flow cabinet, and were cultured in a culture room with a 16/8 h photoperiod (light/dark) (80/90 $\mu E/m^2/s$ cool white light) at 21 °C, until the time of plant transformation.

2. Selection protocol: sensitivity curve to the selective agent

The effects of different concentrations of the selective agent hygromycin were evaluated on untransformed (WT) potato plantlets cv. Spunta over 30 days of *in vitro* culture on BM medium. To construct the sensitivity curve, 5 different concentrations of hygromycin were used (2 $mg \cdot L^{-1}$, 4 $mg \cdot L^{-1}$, 6 $mg \cdot L^{-1}$, 8 $mg \cdot L^{-1}$, or 10 $mg \cdot L^{-1}$). As a control, BM medium without hygromycin was used. For each curve, 10 plantlets were analyzed over four weeks. During this period, the following parameters were recorded three times per week: plantlet height, presence of roots, and number and coloration of leaves.

3. Plant expression vector

For the genetic transformation, the binary vector pHPT (13,277 bp) was used. This plasmid contains the *hpt* gene, which codes for the enzyme hygromycin phosphotransferase, which confers resistance to the antibiotic hygromycin under the transcriptional control of the NOS promoter (nopaline synthase; An *et al.*, 1990) and the NOS terminator between the T-DNA borders. The pHPT vector was introduced into *A. tumefaciens* strain EHA105 by electroporation. Transformed cells were selected for resistance on Luria Bertani (LB) medium supplemented with 25 $mg \cdot L^{-1}$ rifampicin and 50 $mg \cdot L^{-1}$ kanamycin.

3.1 Preparation of the Agrobacterium tumefaciens inoculum for transformation

An initial culture of 5 ml of LB medium was prepared, supplemented with 25 $mg \cdot L^{-1}$ rifampicin and 50 $mg \cdot L^{-1}$ kanamycin, starting from a culture on a fresh plate. The culture was incubated for 16 h at 28 °C on a shaker at 200 rpm. After the 16 h incubation, the 5 ml of culture were transferred to a 250 ml Erlenmeyer flask containing 50 ml of LB medium supplemented with 25 $mg \cdot L^{-1}$ rifampicin and 50 $mg \cdot L^{-1}$ kanamycin. The culture was incubated for 16 h at 28 °C on a shaker at 200 rpm. At the time of transformation, the optical density (OD) was measured at 600 nm.

4. Transformation protocol

Leaves of plantlets grown *in vitro* for 3 or 4 weeks were used as explants. The leaves were cut, removing the petiole. Twenty explants were placed per Petri dish (OneLab Ø90 mm) together with 20 ml of liquid infection medium composed of 20 $g \cdot L^{-1}$ sucrose (Biopack, Cat. No. 9789.08) and 4.3 $g \cdot L^{-1}$ Murashige and Skoog salts with vitamins (PhytoTechnology Laboratories, Cat. No. M519). To each dish, the necessary amount of *A. tumefaciens* inoculum was added to achieve a final OD of 0.2/0.3.

Immediately, the dishes were incubated at 21 °C, in darkness, with shaking at 50 rpm for 45 minutes. The explants were then dried on sterile paper and subcultured on co-culture medium for 48 h at 21 °C with a 16/8 h photoperiod (light/dark; 20 $\mu E/m^2/s$). The co-culture medium is composed of MS medium supplemented with 0.2 $mg \cdot L^{-1}$ naphthaleneacetic acid (NAA; Sigma, Cat. No. NN0640), 0.02 $mg \cdot L^{-1}$ gibberellic acid (GA_3; Sigma, Cat. No. x), 2.5 $mg \cdot L^{-1}$ zeatin riboside (ZR; PhytoTechnology Laboratories, Cat. No. Z899), and 8 $g \cdot L^{-1}$ agar (Phytotechnology Laboratories, Cat. No. A296).

After the co-culture period, a total of 10 explants per plate were subcultured on callus-inducing medium (CIM) for 12 days at 21 °C with a 16/8 h photoperiod (light/dark; 80 to 110 $\mu E/m^2/s$). The CIM medium is composed of MS medium supplemented with 0.2 $mg \cdot L^{-1}$ NAA, 0.02 $mg \cdot L^{-1}$ GA_3, 2.5 $mg \cdot L^{-1}$ ZR, 2.5 $mg \cdot L^{-1}$ cefotaxime (Phytotechnology Laboratories, Cat. No. A296), 100 $mg \cdot L^{-1}$ ampicillin (Sigma, Cat. No. 7177-48-2), and 8 $g \cdot L^{-1}$ agar.

Subsequently, the explants were subcultured every 15 days on shoot-inducing medium (SIM) at 21 °C with a 16/8 h photoperiod (light/dark; 80 to 110 $\mu E/m^2/s$). The SIM medium is composed of MS medium supplemented with 0.2 $mg \cdot L^{-1}$ NAA, 0.2 $mg \cdot L^{-1}$ GA_3, 2.5 $mg \cdot L^{-1}$ ZR, 400 $mg \cdot L^{-1}$ cefotaxime, 100 $mg \cdot L^{-1}$ ampicillin, 8 $mg \cdot L^{-1}$ hygromycin, and 8 $g \cdot L^{-1}$ agar.

As the apices formed (5 to 10 mm in size), they were carefully cut from the calli and transferred to tubes containing MS medium and kept at 21 °C under a 16/8 h photoperiod (light/dark; 80 to 110 $\mu E/m^2/s$).

The explants continued to be transferred at 15-day intervals to SIM medium. The dissection of the new apices was carried out so as to ensure that the new shoots came from independent calli.

5. Detection of the hpt gene by polymerase chain reaction (PCR)

DNA extraction from the plantlets derived from the transformation assay was carried out as described by Haymes and collaborators (1996). The DNA concentration was determined by absorbance at 260 nm with a digital spectrophotometer (SmartSpect™ 3000 BIORAD, USA). DNA quality was determined by means of the A_{260}/A_{280} ratio and by electrophoresis on 1% agarose gels stained with GelRed (Genbiotech, Argentina).

The DNA extracted from the samples was used as a template to amplify the *hpt* gene by PCR. For this purpose, the primers Hig Fw and Hig Rv (5′ gaagatgttggcgacctcgta 3′ and 5′ aagttcgacagcgtctccga 3′, respectively) were used. As a negative control, DNA from potato plants cv. Spunta WT was used, and as a positive control, plasmid DNA from the pHPT vector was used. The amplification conditions were: 94 °C for 3 min; 35 cycles of 94 °C for 30 sec, 52 °C for 15 sec, 72 °C for 1 min; and finally 1 cycle at 72 °C for 5 min. The products obtained were confirmed by electrophoresis on 1% agarose gels stained with GelRed (Genbiotech, Argentina) in the presence of molecular weight markers (Axygen, USA) and revealed under ultraviolet light.

6. Statistical analysis

For the statistical analysis of the phenotypic data from the sensitivity curve, a repeated-measures ANOVA was performed, with a subsequent Bonferroni analysis, using the GraphPad Prism 4.0 program (version 6.00 for Windows; GraphPad Software, La Jolla, California, USA, www.graphpad.com).

For the statistical analysis of the data from the transgenic plants, a Wilcoxon test (Mann-Whitney U) was performed using the InfoStat statistical program (Di Rienzo *et al.*, 2012).

Results

1. Sensitivity curve to the selective agent in potato plants cv. Spunta

Different concentrations of hygromycin were evaluated to determine the sensitivity of untransformed potato plantlets to the selective agent. The effect of the treatment was evaluated for each of the sampling days. During the first 3 days of treatment, all plants subjected to the different doses of hygromycin showed a coloration similar to the control (figure 1). In the case of the 2 mg·L^{-1} dose, the plants showed a coloration similar to the control plants throughout the whole experiment. At the 4 mg·L^{-1} dose, significant differences with respect to the control were found from day 9. At the remaining doses, the difference was evident from day 7. At the end of the experiment, the 10 mg·L^{-1} dose was the only concentration of selective agent that showed significant differences with respect to all the other doses from day 7. The statistical analysis indicated significant differences in the interaction between selective-agent dose and time ($p<0.0001$).

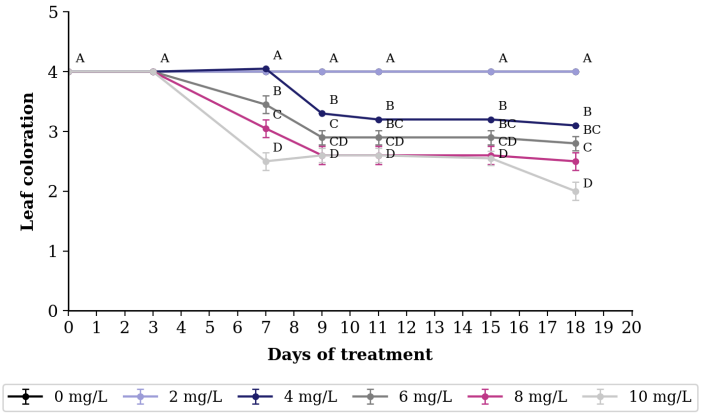

Figure 1. Evolution of leaf coloration in potato leaves subjected to different concentrations of hygromycin. Data are mean ± standard error of 10 plants. For color classification, the following criterion was used: dark green (4), light green (3), yellow-green (2), and yellow (1). Comparison of the different hygromycin concentrations within each of the evaluated time points. Different letters indicate significant differences $p<0.05$.

Regarding the other evaluated parameters, the plantlets that grew under hygromycin concentrations of 2, 4, and 6 mg·L^{-1} formed roots. These treatments did not show significant differences with respect to the control throughout the whole period of the experiment (figure 2). The plantlets that grew on medium supplemented with 8 mg·L^{-1} hygromycin presented roots, but their appearance occurred later than in the control. The differences observed were statistically significant ($p<0.0001$). From day 9, the plantlets that grew on medium with 8 mg·L^{-1} hygromycin presented roots, the same as the control, but it should be noted that their growth was slower (unpublished data). The plantlets that grew on medium with 10 mg·L^{-1} hygromycin differed markedly from the control from day 7; this difference was significant ($p<0.0001$) and was maintained until the end of the treatment.

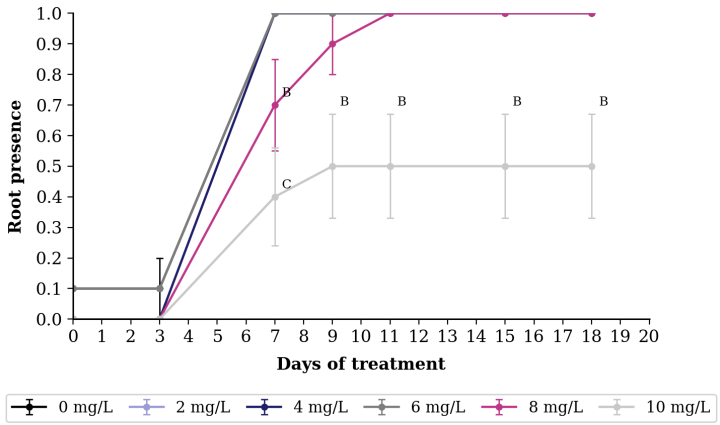

Figure 2. Evolution of root formation in potato plantlets subjected to different concentrations of hygromycin. Data are mean ± standard error of 10 plants. 1 indicates presence of roots, 0 indicates absence of roots. Comparison of the different hygromycin concentrations within each of the evaluated time points. Different letters indicate significant differences ($p<0.05$).

For the number-of-leaves parameter, the effect of the different concentrations of selective agent was evaluated for each of the sampling days. The plantlets that grew on medium with 2 mg·L^{-1} hygromycin did not show significant differences from the control throughout the treatment (figure 3). The plantlets that grew on medium with 4 mg·L^{-1} hygromycin differed from the control from day 11; these differences were statistically significant and were maintained until the end of the treatment. The plantlets on medium with 6, 8, and 10 mg·L^{-1} hygromycin differed from the control from day 7. It should be noted that there were no significant differences among the treatments with 6, 8, and 10 mg·L^{-1} hygromycin, since in none of the three treatments was the appearance of new leaves observed.

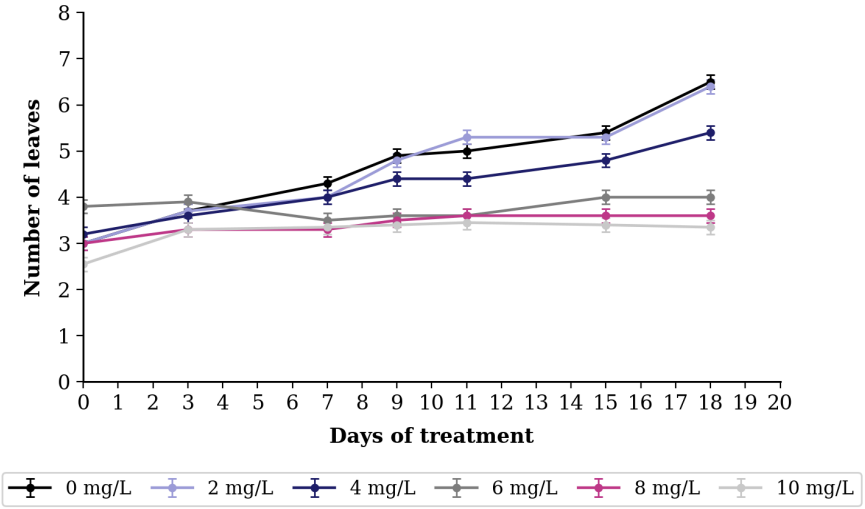

Figure 3. Evolution of the number of leaves in potato plantlets subjected to different concentrations of hygromycin. Data are mean ± standard error of 10 plants. Comparison of the different hygromycin concentrations within each of the evaluated time points. Different letters indicate significant differences $p<0.05$.

For the plant-height parameter, the effect of the different concentrations of selective agent was evaluated for each of the sampling days. From the start of the experiment, the plantlets were found to present different heights, which is observed in the differences found among the treatments on day 0 (figure 4). For this reason, it was decided to evaluate the growth parameter (difference between final and initial height) in the different treatments with hygromycin (figure 4, table 1). The increase in hygromycin concentration negatively affected the growth of the plantlets (figure 4). Significant differences were found in the growth of the plants growing under 2, 4, 6, 8, and 10 mg·L^{-1} hygromycin.

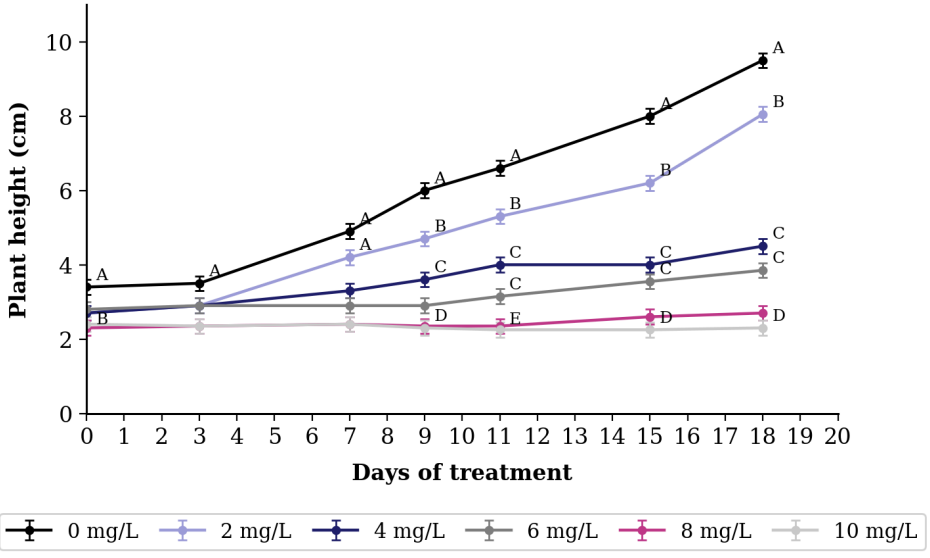

Figure 4. Evolution of height in potato plantlets subjected to different concentrations of hygromycin. Data are mean ± standard error of 10 plants. Comparison of the different hygromycin concentrations within each of the evaluated time points. Different letters indicate significant differences $p<0.05$.

Hygromycin concentration	0 mg·L^{-1}	2 mg·L^{-1}	4 mg·L^{-1}	6 mg·L^{-1}	8 mg·L^{-1}	10 mg·L^{-1}
Growth (cm)	6.06 ± 0.35 ^{A}	5.4 ± 0.23 ^{B}	1.83 ± 0.35 ^{C}	1.06 ± 0.14 ^{C}	0.44 ± 0.10 ^{D}	−0.13 ± 0.12 ^{D}

Table 1. Statistical analysis of the growth data (difference between final and initial height). Comparison of the different hygromycin concentrations. Different letters indicate significant differences $p<0.05$.

2. Production of transgenic potato plants cv. Spunta

A total of 100 explants were inoculated with a culture of *A. tumefaciens* at a final OD_{600} of 0.27 per plate. Sixty-two putatively transgenic apices were obtained from the 100 inoculated explants (62% regeneration efficiency). On the selective SIM medium supplemented with 8 mg·L^{-1} hygromycin (according to what was obtained from the analysis of the hygromycin sensitivity curve), a total of 276 calli were obtained, of which only 56 developed apices (apex-induction efficiency of 20.28%).

Additionally, 30 uninoculated explants were used as regeneration controls, from which 24 apices were obtained (80% regeneration efficiency). On the selective SIM medium without hygromycin, a total of 32 calli were obtained, of which only 9 developed apices (apex-induction efficiency of 28.12%).

Callus formation was observed at the cut edges of the explants after 12 to 15 days of culture on CIM medium; approximately 3 calli formed per explant. The calli were compact, granular, and green (figure 5a). The explants produced more calli during the following subcultures until reaching the total (276) between 8 and 9 weeks post-inoculation. No morphological differences were observed between the calli derived from inoculated explants and those from the control without antibiotic.

Apex formation was visualized between weeks 10 and 11 post-inoculation (figure 5b). These developed on the surface of the calli, and no morphological differences were observed between the apices from inoculated explants and those from the control (figure 5c). All the apices were dissected from the calli upon reaching an approximate height of 1 to 2 cm and were placed individually in tubes with MS medium (figure 5d). The calli produced apices during the following subcultures until reaching the total (62 inoculated and 24 control) between 17 and 18 weeks post-inoculation; after this time, no formation of new apices was observed, so the subcultures of the calli on SIM medium were ended.

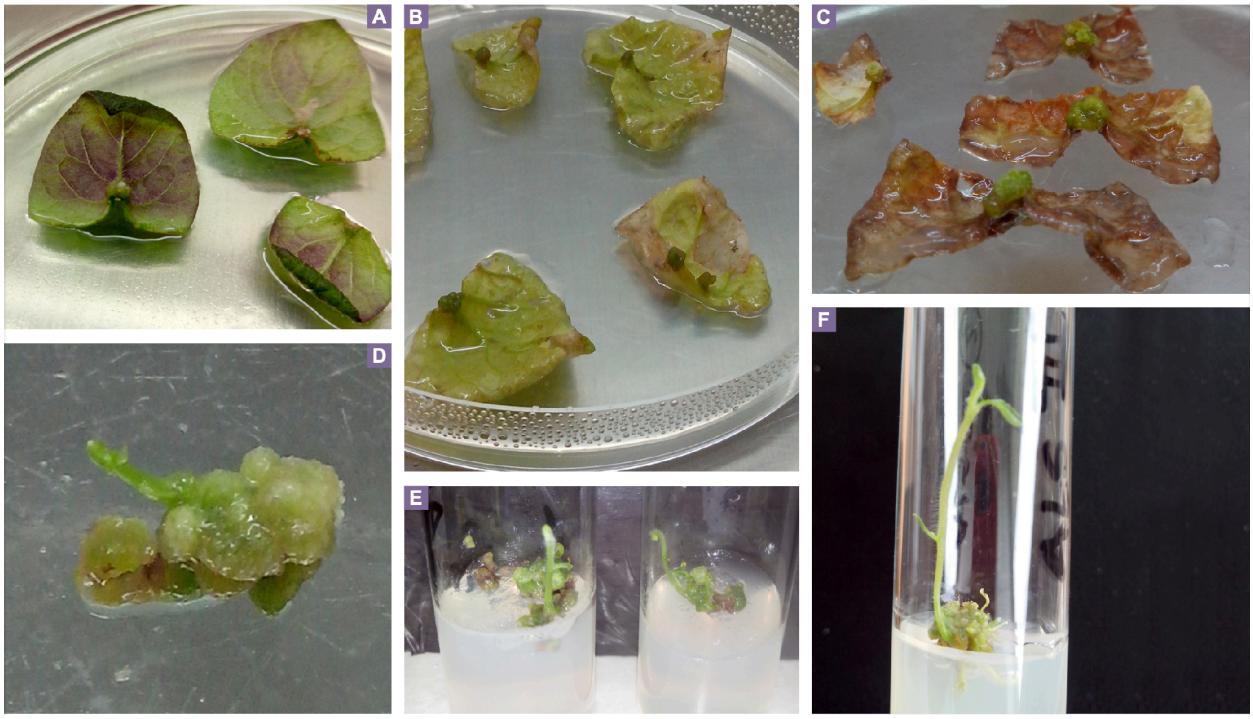

Figure 5. Genetic transformation of potato cv. Spunta mediated by *Agrobacterium tumefaciens*. A, B and C: calli obtained on CIM culture medium with selection; D: apices from explants inoculated on SIM medium; E and F: apices and plantlets from explants inoculated on BM medium (in test tube), cut from the callus.

2.1 Evaluation of transgenic plants cultured on selective medium

In order to corroborate the results of the sensitivity curve, the concentration of 8 $mg \cdot L^{-1}$ hygromycin was selected to evaluate its efficacy as a selection agent for transgenic plants of cv. Spunta. The concentration of 8 $mg \cdot L^{-1}$ hygromycin was selected because it was the lowest concentration at which significant differences were observed in all the parameters studied, compared with the controls.

The 62 apices of potato cv. Spunta derived from the *A. tumefaciens*-mediated genetic transformation assays were taken and subcultured in tubes containing MS medium supplemented with 8 $mg \cdot L^{-1}$ hygromycin, and were evaluated phenotypically and molecularly in order to confirm the presence of the *hpt* transgene.

2.2 Molecular evaluation

After three weeks, genomic DNA was extracted from the 62 plantlets subcultured on MS medium. The extracted DNA was of good quality, since an intact high-molecular-weight band was observed and the A_{260}/A_{280} ratio was in the range of 1.6 to 2.3. A PCR was then performed in order to determine the presence of the *hpt* gene. Of the 62 plants analyzed, 30 were positive for the presence of the *hpt* gene (*hpt*+, 648 bp) and were classified as transgenic plants. The remaining 32 plants did not present the *hpt* gene, so they were classified as untransformed plants and were considered escapes. These results indicate that the transformation efficiency obtained was 30%, calculated as the number of transgenic plants obtained / the number of explants inoculated.

2.3 Phenotypic evaluation of hpt+ plants and untransformed plants

The plants were separated into two groups, those positive for *hpt* (*hpt*+) and those negative for *hpt* (*hpt*−) (escapes); they were placed in tubes containing BM supplemented with 8 $mg \cdot L^{-1}$ hygromycin, and the parameters measured previously during the sensitivity curve were evaluated. In *hpt*+ plants, the appearance of new leaves was observed, an effect not observed in *hpt*− plants ($p=0.0001$; 1.25±0.14 and 0±0). The coloration of all *hpt*+ plants was intense green, whereas in *hpt*− plants it varied from yellow to yellowish green ($p<0.0001$; 4±0 and 1.38±0.16). In the case of growth, it was observed to be greater in *hpt*+ plants, whereas in *hpt*− plants growth was almost null ($p=0.0002$; 3.34±0.28 cm and 0.09±0.05 cm). Finally, the presence of roots was evaluated. Although not all the plants developed roots, their appearance was greater in *hpt*+ plants than in *hpt*− plants ($p=0.0201$; 0.67±0.15 and 0.13±0.11).

Discussion

The development of a reliable regeneration protocol is a crucial step for genetic transformation in potato (Ahmed *et al.*, 2018), because genotype is an important factor that affects both regeneration efficiency and genetic transformation efficiency (Borna *et al.*, 2010; Ahloowalia *et al.*, 1982; Romano *et al.*, 2003). The selection of healthy explants from the wild-type potato plant is critical to generate a high frequency of calli, shoots, and root regeneration (Khatun *et al.*, 2012).

In the present work, a reproducible protocol for the genetic transformation of potato cv. Spunta was adjusted for the first time, using *Agrobacterium tumefaciens* and the antibiotic hygromycin as the selective agent. Using this protocol, a transformation efficiency of 30% was achieved. In various works, higher transformation efficiencies are reported, in a range between 60% and 90% (Beaujean *et al.*, 1998; Douches *et al.*, 1998; Khatun *et al.*, 2012; Borna *et al.*, 2010; Ahmed *et al.*, 2018; Bakhsh *et al.*, 2020) for cultivars such as Désirée, Lemhi Russet, Kaptah Vandel, Cardinal, Heera, Diamant, Granola, Lady Olympia, Agria, and Innovator. In none of these works is cv. Spunta included, so it is not possible to make an adequate comparison between the transformation efficiencies reported in the mentioned works and ours.

The lower transformation efficiency obtained in our assay may be explained by factors such as genotype, the selection agent, the construct used for the transformation, or the Agrobacterium strain used (Roman *et al.*, 2015). On the one hand, 50% of the regenerating apices obtained on SIM medium turned out to be escapes (*hpt*−). This fact was also reported in the transformation of cv. Desiree (Roman *et al.*, 2015). The high number of escapes may be due to the great *in vitro* regeneration capacity that characterizes certain

potato cultivars (Cañedo and Cisneros, 2004), allowing the proliferation and development of certain untransformed regenerants in the presence of the selective agent. As a result, a plant with natural resistance or tolerance is obtained (López and Chaparro, 2007). It is also possible that several of these regenerants were not in permanent contact with the medium containing the selective agent, allowing their development (Monserrat *et al.*, 2001). This may be due to the globular shape presented by the calli, where not all of the callus is in contact with the medium containing the selective agent. It is for this reason that the apices obtained on SIM medium should be cultured individually in tubes containing BM medium supplemented with 8 $mg \cdot L^{-1}$ hygromycin, to ensure the contact of the apex with the selection agent and thus reduce the number of plants in the molecular analyses.

On the other hand, the antibiotic hygromycin was used for the first time as a selective agent in the genetic transformation of *Nicotiana tabacum* by the group of Van den Elzen (1985), and in 1989 it was used for the first time in potato (*Solanum tuberosum* x *S. phureja* and *Solanum tuberosum* x *S. chacoense*) by Masson (1989). Subsequently, it was used in numerous works as a selective agent in the transformation of various potato and sweet potato cultivars (Surov *et al.*, 1998; Okada *et al.*, 2001; Cavatorta *et al.*, 2011; Orbegozo *et al.*, 2016), with its action not being described in cv. Spunta. Through the hygromycin antibiotic sensitivity assays, the lethal dose for untransformed explants was determined to be 8 $mg \cdot L^{-1}$ hygromycin. In accordance with the results obtained, and after comparing the different antibiotic sensitivity curves, it was concluded that the increase in the concentration of the selective agent in the culture medium caused a decrease in growth, discoloration of the tissue, a decrease in the number of leaves, and impaired root formation. The plants that presented the best morphophysiological parameters on selective medium carried the *hpt* gene. The selection method proposed for cv. Spunta (8 $mg \cdot L^{-1}$ hygromycin) presented an identification percentage of 48.38% among the transgenic plants of the untransformed plantlets.

In relation to the use of hygromycin during the process of obtaining transformants, it is concluded that the efficiency was low (48.38%) in the present work; this was determined by comparing the results obtained with previously reported assays that describe the use of the same antibiotic, but in different varieties (M'Hamdi *et al.*, 2003; Kashani *et al.*, 2012). These differences may be due to multiple factors associated with the transformation conditions or the type of explant used (Lagunes, 2009). Nevertheless, the main cause may be that regeneration and transformation assays are genotype-dependent on the type of crop used in the experiments (Orbegozo *et al.*, 2013). In future transformation works of cv. Spunta using hygromycin as a selection agent, this parameter should be adjusted and higher concentrations should be used.

This protocol is a first approximation to the genetic transformation (of a potato cultivar of broad economic and agronomic interest in Argentina, as is the cultivar Spunta) mediated by *Agrobacterium tumefaciens* using the antibiotic hygromycin as a selection agent.

Conclusions

Transgenic potato plants cv. Spunta resistant to the antibiotic hygromycin were obtained. It was possible to establish a reproducible transformation protocol that can be applied, as a first approach, to other cultivars of economic and agronomic interest in Argentina for which no transformation protocols have been reported. For each case, the response of each cultivar to the method developed for Spunta will need to be studied.

Finally, after carrying out the dose-response curve with the selective agent hygromycin on potato plantlets cv. Spunta, it was concluded that the variables analyzed were adequate for determining sensitivity to the antibiotic, and that the concentration of 8 $mg \cdot L^{-1}$ hygromycin, although it made it possible to distinguish transgenic potato plants cv. Spunta from WT plants was not entirely sufficient, and it is necessary to increase its dose in the culture media. The variables evaluated, “appearance of new leaves”, “root emergence”, and “absolute growth”, are the most suitable to take into account when selecting transgenic plants that use the antibiotic hygromycin as a selection agent.

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