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THE POSITIVE EFFECT OF ANTIBIOTIC PAROMOMYCIN COMPARED WITH KANAMYCIN FOR SELECTION OF TRANSGENIC PLANTS WITH *NPT*II GENE ON THE EXAMPLE OF *NICOTIANA TABACUM*

Plant genetic transformation is an important tool for crop improvement and research in genetics [1]. Transformation processes require multiple steps and numerous media. For example, in *Agrobacterium*-mediated transformation, the process starts with the isolation of explants. Then the explants are inoculated with *Agrobacterium* in an inoculation media. Following the inoculation step, the *Agrobacterium* and plant explants are co-cultured together for a period of several hours to several days under conditions suitable for growth and T-DNA transfer. After co-culture, the presence of *Agrobacterium* is deleterious to plant tissue culture via typically moving of the explants to fresh medium containing antibiotics in order to inhibit the growth of bacteria. Then explants are placed on the selection media to selection of transgenic events and regeneration media for regeneration to plantlets that can be moved to soil afterwards. Selection step is optional but makes it much easier and faster to obtain transformed plants. A selective gene that provides plant resistance to antibiotics or herbicides is inserted in vectors for genetic transformation along with the gene of interest. One of such selective genes frequently used in vector design for plant transformation is *npt*II gene which encodes neomycin phosphotransferase II enzyme that inactivates aminoglycoside antibiotics so as kanamycin, neomycin, geneticin and others. Typically, for selection of plants transformed with *npt*II gene antibiotic kanamycin is effectively used as inexpensive and stable. However, the negative effect of kanamycin on regenerative ability of explants was observed for some species [2–8]. This fact significantly reduces the transformation frequency and forces researchers to change selection conditions or replace vectors containing *npt*II gene with other selective genes. Aminoglycoside antibiotic paromomycin successfully used for selection of transgenic plants with *npt*II gene has been reported in the literature [9, 10]. In the present study we aimed to assess the efficiency of paromomycin for selection of transgenic plants compared to kanamycin on tobacco, the model object for plant biotechnology.

Materials and methods

Aseptically grown tobacco plants *Nicotiana tabacum* cv. Retit Havana were employed as the plant material in the experiments. Plants were cultivated on hormone free MS medium [11] under 16-hour light period illumination at 24 °C.

In the control experiment on the influence of antibiotics on the tobacco tissue, the leaf explants of the nontransformed tobacco 1–1.5 cm^2 were placed on MS regeneration media containing 1 mg/l BAP, 0,1 mg/l NAA, 100 mg/l kanamycin sulfate (Km) or 100 mg/l paromomycin (Pm).

Genetic transformation of leaf explants *N. tabacum* was carried out by conventional method [12] using *Agrobacterium tumefaciens* strain ABI harboring the vector containing *npt*II gene driven by 35S promoter of cauliflower mosaic virus. Explants were cultured for 3 days on regeneration medium without antibiotics under disseminated light at 22 °C and then transferred to a selective regeneration medium containing 500 mg/l cefotaxime (Cx) to inhibit the growth of *Agrobacterium* and 50 mg/l Km or 50 mg/l Pm for selection of transgenic plantlets. After 3 weeks the explants were transferred to a fresh regeneration medium with 500 mg/l Cx and 100 mg/l Km or 100 mg/l Pm respectively. The replacement of the medium to a fresh one was done every 3 weeks. The regenerants were placed into jars with hormone free MS medium which contained 500 mg/l Cx and 100 mg/l Km or 100 mg/l Pm according to the selection scheme. Regeneration frequency (RF) on selective media was calculated as the ratio of the number of explants that formed regenerants to the total number of explants, expressed as a percentage.

Total DNA was isolated from the leaves of antibiotic resistant plants using the CTAB [13]. Analysis of plant DNA on the presence of *npt*II gene was carried out by polymerase chain reaction (PCR). To eliminate the possibility of contamination

of plant material by *Agrobacterium,* before analysis on selective gene, the amplification was carried out on the presence of *vir*-D1 gene [14]. In the PCR for *npt*II gene the following pair of primers was used: 5›-CCTGA ATGAA CTCCA GGACG AGGCA-3 ‹and 5›-GCTCT AGATC CAGAG TCCCG CTCAG AAG-3›. The size of amplicon for *vir*-D1 gene was 432 base pairs (b.p.) and for *npt*II – 547 b.p. The reaction mixture for PCR (20 µl) contained 0.75 units DreamTaq DNA polymerase (Thermo Scientific), 2 µl 10× DreamTaq Green buffer, 2 µl 200 mM each dNTP, 0.1 mM of each forward and reverse primer and 30 ng of plant DNA. As positive controls to correct the reaction performance, total DNA of transformed plants containing gene *npt*II or *Agrobakterium* total DNA were used. As a negative control DNA of nontransformed tobacco and no template control were used. The program for PCR was the following: one cycle at 94 °C for 4 min and subsequent 34 cycles (denaturation at 94 °C for 30 s, renaturation at 58 °C for 30 s, elongation 72 °C for 27 s for gene *vir* D1 and 37 s for *npt*II). The ultimate elongation was 10 min at 72 °C followed by rapid cooling to 4 °C. The reactions were performed in Mastercycler personal (Eppendorf). Amplification products were separated in 1.2 % agarose gel with ethidium bromide (0.5 mg/ml) in 0.5 × TBE buffer (45 mM Tris-Cl, 45 mM H_3BO_3, 1 mM EDTA, pH 8.0) at 6 V/cm. The frequency transformation by PCR (FT) was calculated as the ratio of the number of samples containing gene *npt*II to the total number of samples, expressed as a percentage.

Results and discussion

In the beginning, we conducted a comparative description of antibiotic action (kanamycin and paromomycin) for leaf explants of the nontransformed tobacco plants. The negative effect of paromomycin on the explant regeneration ability was milder compared to kanamycin (6 % explants formed plantlets). Whereas the explants remained on medium with kanamycin became totally bleach (fig. 1). We can assume that number of nontransformed plants selected as transformants on paromomycin is higher than with the use of kanamycin as a selective agent.

The next stage of the study was the *Agrobacterium*-mediated genetic transformation of tobacco with a vector containing the *npt*II gene, followed by parallel selection of transgenic plants on media with kanamycin or paromomycin. Three weeks after transformation multiple regeneration (3 to 10 regenerants for a single explant) was observed on selective regeneration medium for explants both on kanamycin as well as paromomycin containing media. After 8 weeks after the transformation the regeneration frequency (RF) was evaluated for all explants on selective media. Among 127 explants, which were kept on medium with kanamycin 27 bleached. For explants on the medium with paromomycin only 9 of 120 were sensitive (table). So, paromomycin had less negative impact on the regeneration of plants compared to kanamycin. Among 328 regenerants that were planted on kanamycin containing medium, 97 proved to be sensitive, representing 29.57 %. The portion of sensitive regenerants obtained on the medium with paromomycin was 44.24 % (table). Finally, the number of kanamycin resistant lines of plants was 231, and the paromomycin resistant ones was 155. Despite the fact that the frequency of regeneration on

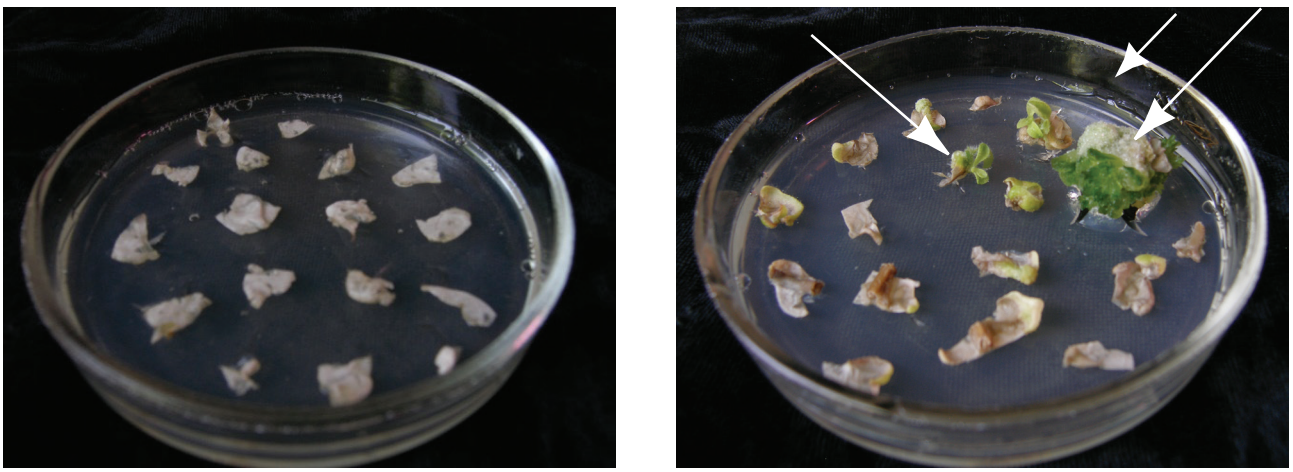


Fig. 1. The effect of kanamycin (A) and paromomycin (B) on leaf explants of the nontransformed tobacco plants. The arrows indicate on regenerated explants

Table

Comparative description of antibiotics as selective agents in *Agrobacterium*-mediated transformation

Antibiotics	RF, %	Planted regenerants				PCR-analysis of regenerants		
		total, pcs.	resistant, pcs.	sensitive		total, pcs.	*npt*II «+», pcs.	TF, %
				pcs.	%			
Kanamycin	78.5	329	231	97	29.6	20	13	65.0
Paromomycin	92.5	284	155	123	44.2	13	12	92.3

N o t e s: TF, transformation frequency; RF, regeneration frequency.

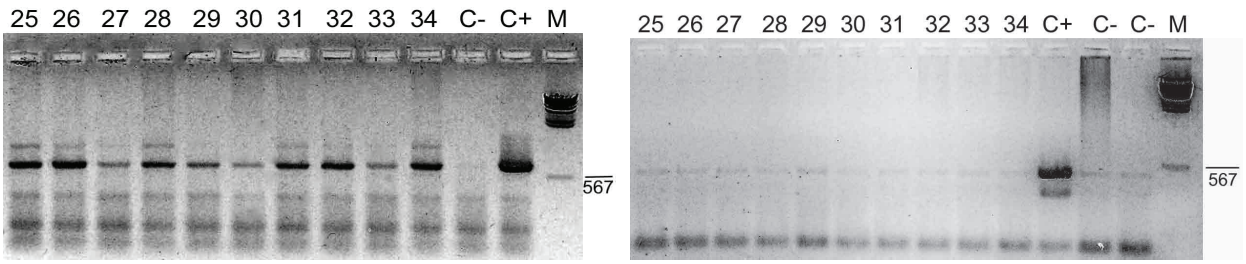
the medium with paromomycin was higher compared to that containing kanamycin as the selective agent, but the total number of antibiotic-resistant plants was lower because of the high percentage of sensitive to antibiotics plants observed during cultivation. Thus, the research confirmed the assumptions made by us as a result of the cultivation of leaf explants from nontransformed plants on media with antibiotics that the number of nontransformed plants selected as transformants on media with paromomycin is higher than with kanamycin.

Antibiotic-resistant plants were studied by the PCR for the presence of *npt*II gene. Total DNA from 33 lines of regenerants generated on both antibiotics was prepared. The presence of *npt*II sequence (fig. 2 A) in plants regenerated on both selective media was confirmed. In addition, the PCR showed the absence of bacterial contamination of plant material (fig. 2 B). The transformation frequency estimated by PCR was 65.0 % for plants selected on kanamycin and 92.3 % for regenerants selected using paromomycin.

Therefore, paromomycin inhibits the regeneration potential of tobacco explants much less compared to kanamycin. Its use for the selection in *Agrobacterium*-mediated tobacco transformation allows obtaining transgenic plants in sufficient number with high frequency. The use of paromomycin for selection of transformants with *npt*II gene may be promising for those species, for which kanamycin has a strong negative impact, particularly species *Brassica* [3, 4] and cereals [7, 8]. For example, the efficiency of paromomycin as a selective agent to produce transgenic plants of corn has been shown [9, 10].

Conclusions

It was shown that paromomycin has less negative impact on the regeneration potential of plant explants compared to kanamycin. Use of paromomycin at the stage of selection after *Agrobacterium*-mediated transformation of tobacco allows us to obtain transgenic plants containing *npt*II gene, with high frequency. Thanks to the mild effects on plant explants paromomycin can be used for transgenic plants production of the other species for which kanamycin being inefficient.



A B

Fig. 2. Electrophoretic analysis of the PCR products from tobacco regenerant lines for the presence of *npt*II (A) and *vir* D1 (B) genes. Lanes 25–29, DNA of tobacco regenerants obtained on kanamycin; lanes 30–34, DNA of tobacco pants regenerated at paromomycin; C-, negative control; C+, positive control; M, molecular weight marker λ DNA/ HindIII

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Aims. To investigate the efficiency of antibiotic paromomycin as a selective agent for the production of transgenic plants with *npt*II gene compared with kanamycin sulfate on the example of *Nicotiana tabacum*. ***Methods.*** A vector containing the *npt*II gene under the control of 35S promoter of cauliflower mosaic virus was used in *Agrobacterium*-mediated genetic transformation of leaf explants from *Nicotiana tabacum*. Following parallel explant selection on media with paromomycin or kanamycin plant regeneration was observed. Total DNA of the regenerated plants was tested for the presence of *npt*II gene by PCR. ***Results.*** It has been shown that the use of antibiotic paromomycin is effective for obtaining transgenic tobacco plants. Paromomycin revealed a softer effect on plant explants compared with kanamycin sulfate without causing severe depression on regenerative ability of plant tissues, leading to a higher frequency of regeneration on selective medium and transformation. ***Conclusions.*** Transgenic tobacco plants were received on selective media containing paromomycin with high frequency. Due to the mild effects on plant explants, paromomycin is an attractive agent for selection of transgenic plants with *npt*II gene, especially for those species in which the kanamycin sulfate has a strong negative impact on the regenerative potential.

Keywords: Paromomycin, kanamycin, *npt*II, *Nicotiana tabacum*, selection of transgenic plants.