Kanamycin application features for the selective screening of genetically modified *Populus × berolinensis*

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**Abstract.** The study describes the features of the kanamycin application for the effective selection of transgenic poplar expressing *nptII* gene. It has been shown that kanamycin concentration of 50 mg L\(^{-1}\) in the nutrient medium is the most suitable concentration for the effective selection of transgenic Berlin poplar plants for both regeneration and rooting stages. At the same time, 50 mg L\(^{-1}\) of kanamycin in the nutrient medium may result in the loss of the less resistant transgenic plants, so lower concentrations may be also applied. Kanamycin concentrations of 25 and 35 mg L\(^{-1}\) can give a false positive signal of transgenesis. Transgenic poplars expressing *nptII* gene can be effectively regenerated on nutrient media with kanamycin at concentrations up to 100 mg L\(^{-1}\).

1. Introduction

Genetic constructions usually used for plant transformation, in addition to the target gene, also contain a selective gene of resistance to certain toxicants or antibiotics. The most commonly used selective genes are *neo* (*nptII*), *hpt* (*hph*), *pat*, *bar*, *igRA*, *epsps* (*aroA*), *thiO*, and others.

*NptII* (*neo*) gene encodes the enzyme neomycin phosphotransferase II, which confers resistance to various aminoglycoside antibiotics, including kanamycin. *Hpt* (*hph*) gene encodes hygromycin phosphotransferase (HPH or HPT) conferring resistance to the antibiotic hygromycin B. Resistance to phosphinothricin in plants is achieved by transgenic expression of bialaphos resistance (*bar*) or phosphinothricin acetyltransferase (*pat*) genes. Such genes as *igRA*, *epsps* (*aroA*), *thiO* encoding aldo-keto reductase (AKR1), 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS), glycine oxidase respectively define the resistance to glyphosate – typical herbicide in crops genetic engineering.

Gene encoding enzyme neomycin phosphotransferase II (*nptII*) is widely used in the selective screening of transgenic plants for both fundamental and applied studies. Different plants may resist the diverse concentrations of kanamycin in the nutrient medium during *in vitro* cultivation. The kanamycin concentration in the nutrient medium both for the initial regeneration of plants that received the transgene and for the subsequent rooting of the modified plant is an important parameter for genetic transformation. The latter parameter doesn’t have to be equal to the concentration used in the initial regeneration after transgenesis and even may be lower. Moreover, the use of unnecessarily high concentrations of antibiotic for transgenic plants rooting after regeneration can lead to the loss of lines with a less pronounced effect of transgenesis.

Woody plants are an important object of biotechnology and require additional experiments to set up the methodology of their genetic engineering. Representatives of the genus *Populus*, in fact, are model
objects of genetic transformation among woody plants. The kanamycin concentration for the selective selection of transgenic poplars is species-specific and varies from 10 to 150 mg L\(^{-1}\) according to different studies (table 1). Therefore, to develop a technique for the genetic transformation of a new poplar species, additional research is required to find an effective concentration of a selective antibiotic.

Table 1. Kanamycin concentrations used for the selective regeneration of transgenic Populus.

| Species | Kanamycin, mg L\(^{-1}\) | Reference |
|---------|--------------------------|-----------|
| P. koreana × P. nigra | 150 | Noh et al., 2004 [1] |
| P. alba | 100 | Confalonieri et al., 2000 [2] |
| P. nigra var. betulifolia × P. trichocarpa | 60 | Li and Huang, 1995 [3] |
| P. nigra var. italica | 50 | Nishiguchi et al., 2006 [4] |
| P. tremula | 50 | Fladung et al., 1997 [5] |
| P. deltoides | 50 | Saraswat et al., 2016 [6] |
| P. alba × P. grandidentata | 40 | Kim et al., 1997 [7] |
| P. trichocarpa | 30 | Li et al., 2017 [8] |
| P. alba × P. grandidentata | 10 | Chun et al., 1988 [9] |

The purpose of this study was to determine the features of the use of kanamycin for the selective selection of transgenic Berlin poplar plants.

2. Materials and methods

2.1. The plant material

Berlin poplar (Populus × berolinensis C. Koch) is one of the convenient objects for woody plant micropropagation and genetic transformation. It has a high percentage of rooting (up to 100%) after the cutting during in vitro cultivation, takes up little space in test tubes due to narrow leaves (in vitro only), and also easily regenerates from roots and internodal segments. Relatively fast growth (25-30 days from rooting to cutting) makes Berlin poplar a very useful object for genetic transformation.

To study the effective concentration of kanamycin for selective screening, control (non-transgenic) in vitro plants and a comparison group were used. As the latter, the transgenic plants of Populus × berolinensis expressing uidA (GUS-reporting system) and nptII (further referred as ‘G7’ line) obtained as described in previous studies [10, 11] were used.

2.2. Nutrient media preparation and experiment design

Nutrient media for both regeneration and rooting were prepared on the base of ½ MS5524 [12] manufactured by Sigma-Aldrich with the addition of iron chelate and microelements up to full content from MS5524. The base medium was supplemented by thiamine (1 mg L\(^{-1}\)), pyridoxine (0.5 mg L\(^{-1}\)), nicotinic acid (0.5 mg L\(^{-1}\)), and meso-inositol (50 mg L\(^{-1}\)). Sucrose (2%) was used as a source of carbohydrates. To obtain a solid medium, agar (A7002, Sigma-Aldrich) was used at a concentration of 7 g L\(^{-1}\). The acidity of the medium was adjusted to pH 5.7 [11]. Medium for regeneration was supplemented with BAP (0.2 mg L\(^{-1}\)), TDZ (0.02 mg L\(^{-1}\)) and NAA (0.01 mg L\(^{-1}\)). The rooting medium contained IBA in the final concentration of 0.15 mg L\(^{-1}\). The freshly prepared medium was autoclaved (15 min at 121 °C) and dispensed by 50 ml into sterile polycarbonate GA-7-3 culture vessels (V8380, Sigma-Aldrich) and by 10 ml into glass tissue-culture tubes (Z681784, Sigma-Aldrich). Filter sterilized kanamycin sulfate (A1493, AppliChem) was added to the cooled autoclaved medium (55°C) to prevent thermal degradation of antibiotic.

Poplar stem explants (5-7 mm length) were placed on the surface of solid medium precooled to room temperature (24°C). The explants were incubated at 24 °C in an air-conditioned room with 16/8 hours of the day/night photoperiod. All vessels and test tubes with plants were exposed under T5 fluorescent lamps at 5 000 Lux [13]. Test tubes with rooting plants were exposed for 25 days. Vessels with regenerating explants were exposed 3 times per 25 days until the first regenerates were detected. Every 25 days the nutrient medium was replaced with the same one.
3. Results and discussion
In our previous studies, it was shown that the effective concentration of kanamycin for rooting of transgenic Berlin poplars expressing nptII is 25 mg L\(^{-1}\) [10, 11]. During the following experiments, it was found that this concentration might give false positive results since some of the control plants were able to take roots. In most cases, a 25 mg L\(^{-1}\) of kanamycin in the nutrient medium effectively suppressed root formation in control plants (figure 1 (A), figure 2 (1-5)).

![Figure 1. Populus × berolinensis control (non-transgenic) plants on the rooting medium with kanamycin concentration of 25 mg L\(^{-1}\) (A (1-5) – no roots, B (6-10) – weak rooting).](image)

However, in some cases, we observed weak rooting of berlin poplar in the presence of kanamycin in the nutrient medium (figure 1 (B), figure 2 (6-10)). Thereby, usage of 25 mg L\(^{-1}\) of kanamycin for the selective screening of transgenes may lead to false positive results.

![Figure 2. Zoomed picture of Populus × berolinensis control (non-transgenic) plants rooting on the medium with kanamycin concentration of 25 mg L\(^{-1}\) (1-5 – no roots, 6-10 – weak rooting).](image)

We carried out additional experiments to study this fact. For this purpose, 100 non-transgenic specimens of Berlin poplar were cut and transferred to a rooting medium with 25 mg L\(^{-1}\) of kanamycin. As a result, 75% of the plants did not take root, and 25% gave very small roots of 3-5 mm, the same as in figure 2 (6-10). With an increase in the concentration of kanamycin up to 35 mg L\(^{-1}\) (figure 3), the percentage of rooted plants decreased to 10% but 100% of rooting inhibition was still not achieved.
Figure 3. Rooting of *Populus × berolinensis* control (non-transgenic) plants on the medium with kanamycin concentration of 35 mg L\(^{-1}\) (A – no roots, B – weak rooting, C – zoomed B).

The subsequent increase of kanamycin concentrations in the nutrient medium up to 50, 70, and 100 mg L\(^{-1}\) showed that the minimum concentration completely (100%) suppressing the root formation in control plants is 50 mg L\(^{-1}\) (figure 4).

Figure 4. *Populus × berolinensis* control (non-transgenic) plants on the medium with different kanamycin concentrations (25, 50, 75, and 100 mg L\(^{-1}\)).

On the contrary, the transgenic poplar line G7 expressing *uidA* and *nptII* genes were able to take roots on the medium with kanamycin concentration of 50 mg L\(^{-1}\) and that concentration may effectively be used for selective screening of transgenic plants expressing *nptII* (figure 5).
Figure 5. Rooting of *Populus × berolinensis* transgenic G7 line on the medium with kanamycin concentration of 50 mg L\(^{-1}\) in comparison with control (non-transgenic) plant.

Additionally, we studied the optimal concentration of kanamycin in the nutrient medium suppressing regeneration of Berlin poplar from the stem explants. Stem sections of control and transgenic G7 plants were placed on a nutrient medium containing various concentrations of kanamycin (figure 6).

Figure 6. Regeneration of *Populus × berolinensis* control (non-transgenic) plants and transgenic G7 line on the medium with different kanamycin concentrations (25, 50, 75, and 100 mg L\(^{-1}\)).

As a result, it was shown that the concentration of kanamycin 25 mg L\(^{-1}\) did not completely suppress the regeneration in the control, and the appearance of false positive transformants was possible. Media with kanamycin in concentrations of 50, 75, and 100 mg L\(^{-1}\) completely suppressed regeneration in the
control. At the same time, regeneration of transgenic explants took place even at 100 mg L\(^{-1}\) of kanamycin in the nutrient medium.

4. Conclusion
It has been shown that 50 mg L\(^{-1}\) of kanamycin in the nutrient medium is the most suitable concentration for the effective selection of transgenic Berlin poplar plants for both regeneration and rooting stages. Despite the fact that individual lines of transgenic poplars are able to effectively root on a medium with 50 mg L\(^{-1}\) of kanamycin, it is necessary to conduct primary screening at lower concentrations (25 mg L\(^{-1}\)) in order to avoid the loss of less resistant transgenic plant lines. Kanamycin concentrations of 25 and 35 mg L\(^{-1}\) can give a false positive signal of transgenesis. Thus, the accidents of control plants rooting at those concentrations were observed although in less efficiency (the formed roots were always short, the yellowing and necrosis of the leaves were observed, and plant growth was inhibited in whole). Transgenic poplars expressing \(nptII\) gene can be regenerated on nutrient media with a kanamycin at concentrations up to 100 mg L\(^{-1}\). This fact may be used for the following studies requiring double transformation by genetic constructions with \(nptII\) gene.

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