Kanamycin-induced growth suppression of fast-growing transgenic poplar carrying the *nptII* gene

Vasiliy Pavlichenko[1*], and *Marina* Protopopova[1]

[1]Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch of the Russian Academy of Sciences, 132, Lermontov Street, Irkutsk, 664033, Russia

Abstract. In our study, a genetically modified poplar overexpressing gene encoding gibberellin-20-oxidase (*AtGA20ox1)* was analyzed for the adaptation of a protocol to maintain *in vitro* plants due to the overfilling of test tubes during fast-growing transgenic plants cultivation. It was demonstrated that these transgenic poplars were able to survive in media containing kanamycin, in contrast to the control plants, because neomycin phosphotransferase II was additionally expressed, facilitating the breakdown of kanamycin. The potential effects of kanamycin in nutrient media on the growth rate of these transgenic poplars were also investigated, despite their overall survivability. It was found that kanamycin at varying concentrations (25, 50, 75, and 100 mg L^{-1}) effectively suppressed the growth of these genetically modified poplars without affecting their mortality. Therefore, it was proposed that kanamycin be used as an effective tool for growth suppression in genetically modified plants utilizing the widely used selective *nptII* gene during micropropagation.

1 Introduction

One of the main features of *in vitro* culture of various plants is the necessity for their periodic replanting into fresh nutrient media due to the nutrient depletion. For long-term *in vitro* collections, the timing of replanting and medium replacing is crucial; longer intervals between replanting facilitate easier maintenance of cultures under artificial *in vitro* conditions. Researchers focused on developing or refining micropropagation protocols are continuously seek methods to extend the duration between replanting plants into fresh nutrient media.

The most common method for reducing the growth rate of *in vitro* plants is to lower the temperature in a climate-controlled room or to place the vessels containing plants in a refrigerator or a climatic chamber set to a low temperature, typically around to +4°C [1-3]. However, this approach has disadvantage of requiring additional infrastructure such as a dedicated cold room or a set of refrigerators to accommodate the plants after their have rooted under normal maintenance conditions. Cold storage of the *in vitro* collection ensures

* Corresponding author: vpavlichenko@gmail.com

the availability of plants for fundamental and applied research, commercial distribution, and contributes to the conservation of rare and endangered plant species.

Another effective strategy involves utilizing substances that inhibit plant growth *in vitro*. To achieve long-term suppression of plant growth during *in vitro* culture, the growth retardant chlorometachloride (CCC) is frequently employed. This substance effectively blocks the synthesis gibberellins, a type of phytohormones, in plants, thereby, significantly slowing their growth [4-7].

Highly productive or rapidly growing species present particular challenges for micropropagation and *in vitro* culture due to their metabolism, which allows for quik biomass accumulation and rapid depletion of nutrient media. Moreover, accelerated growth resulted in rapid overfilling of test tubes. Additionally, transgenic woody plant species pose a unique challenge for preservation *in vitro*, as seed material cannot be obtained in short period of time. Consequently, all obtained transgenic lines must be regularly transplanted and maintained in sufficient quantities for both conservation and experimental purposes.

During the genetic transformation of plants, *nptII* is one of the most commonly used selective genes. This gene encodes neomycin phosphotransferase II, which confers resistance to the kanamycin in the selected transgenic plants. Kanamycin is added to nutrient media during regeneration, micropropagation, and selective screening of plants following genetic transformation. It suppresses both rooting and growth in propagated plants as well as regeneration of plants from untransformed cells [8]. Kanamycin interferes with protein synthesis in the chloroplasts and mitochondria of plant cells, leading to yellowing of the green organs [8]. This process ultimately leads to the death of plant cells and entire plants. In contrast, transgenic plant lines expressing the *nptII* gene exhibit resistance to kanamycin, unlike control plants that lack this gene. Specifically, these transgenic lines can grow and reproduce in media containing kanamycin [9]. However, it is reasonable to assume that high concentrations of kanamycin may still impact the growth of transgenic plants, as they will continue to expend some resources on deactivating kanamycin.

In this paper, we evaluated the potential of using high concentrations of kanamycin in a nutrient medium to slow down the growth rate of rapidly growing transgenic poplars that additionally expressing neomycin phosphotransferase II.

2 Materials and methods

2.1 The plant material

Berlin poplar (*Populus × berolinensis* K. Koch) is a convenient model for the micropropagation and genetic transformation of woody plants. It exhibits a remarkably high rooting success rate (up to 100%) after the cutting during *in vitro* cultivation. Additionally, its narrow leaves (*in vitro* only) allow it to occupy minimal space in test tubes. The plants also demonstrate a strong ability to regenerate from roots and internodal segments. Furthermore, its relatively rapid growth taking only 25 to 30 days from rooting to cutting makes Berlin poplar a highly valuable species for genetic transformation efforts.

For the study, we used *Populus × berolinensis* (line #15) transformed by gene *AtGA20ox1* encoding gibberellin 20-oxidase from *Arabidopsis thaliana* (L.) Heynh., obtained from our previous study [9]. Gibberellin 20-oxidase is a key enzyme involved in the active production of gibberellins in plants, making it a good target for genetic manipulation aimed at enhancing growth rate. The primary phenotypic effect following the transformation included a significant elongation of the stem due to increased internode size, a slight thinning of the stem, and both lengthening and narrowing of the leaves.

Additionally, the gene encoding the enzyme neomycin phosphotransferase II (*nptII*) was incorporated for selective screening of transgenic plants. Hence, the transgenic poplars used in this study also expressed the *nptII* gene, which conferred their resistance to kanamycin.

Control (non-transformed) plants of Berlin poplar typically grow *in vitro* for 3 to 4 months until they reach the closure cap of the 15 cm test tube. Consequently, we usually cut the shoot tips of the control plants and root them in fresh nutrient medium after 2 or 3 months, depending on the experiment. In contrast, fast-growing genetically modified poplars significantly faster growth than control plants, reaching the closure cap of 20 cm tube in just 25 to 30 days (Figure 1).

Fig. 1. Comparison of two-month-old fast-growing transgenic Berlin poplar in 20 cm test tube (B) with a control plant of the same age in 15 cm test tube (A).

This rapid growth complicates the *in vitro* storage of transgenic plants, as they require regular transfer to new tubes supplemented with fresh nutrient medium every 20 days. Even using longer test tubes has proven ineffective in equalizing the relocation periods for control and transgenic plants in *in vitro* culture.

Prolonged storage (more than 2 month) of the fast-growing transgenic poplars in the 20 cm tubes resulted in stem twisting. This compaction of the plants within the tubes ultimately led to deformation of both the stem and leaves (Figure 2). After cutting the apical part of the shoots for rooting, we observed that irregular shape of the plants complicated their translocation into new tubes. Moreover, the percentage of rooted plants decreased due to lignification processes and mechanical deformation of the apical buds.

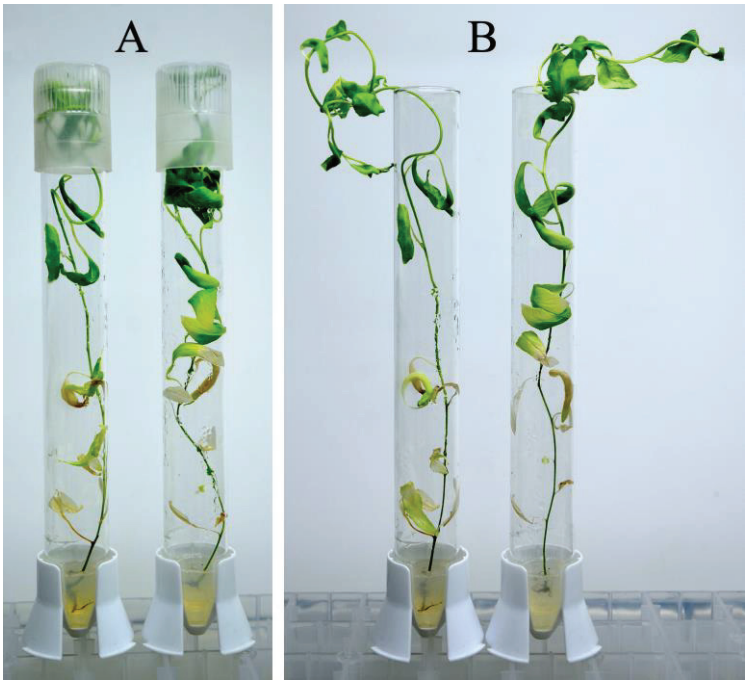

Fig. 2. Three-month-old fast-growing transgenic poplars in 20 cm test tubes with closed (A) and opened (B) closure caps.

2.1 Nutrient media preparation, *in vitro* conditions and experiment design

Nutrient medium for rooting was prepared according to the composition of Murashige and Skoog [10] basal salt mixture manufactured by Sigma-Aldrich (MS5524). The full 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 solidification, agar (A7002, Sigma-Aldrich) was added at a concentration of 7 g L-1. The medium pH was adjusted to 5.7, and rooting media contained indole-3-butyric acid (IBA) at a final concentrations of 0.15 mg L^{-1}. The freshly prepared medium was autoclaved for 15 min. at 121 °C and 220 kPa, then dispensed in 10 ml aliquots into pre-autoclaved and dried 25 × 150-mm borosilicate glass tissue culture test tubes (Z681784, Sigma-Aldrich) and/or 25 × 200-mm test tubes with semi-transparent closure caps (118158C, Globe Scientific Inc.). Filter-sterilized kanamycin sulphate (A1493, AppliChem) was added to the cooled autoclaved medium (55°C) to prevent its thermal degradation. The following concentrations of kanamycin were tested: 25, 50, 75, and 100 mg L^{-1}.

Initially, control plants of non-transformed Berlin poplar were evaluated for their rooting ability in the presence of kanamycin in the nutrient medium. Subsequently, all tested concentrations of kanamycin were applied to fast growing transgenic poplars expressing both *nptII* and *AtGA20ox1* genes.

Throughout the experiment, all *in vitro* plants were cultivated at 24 °C in an air-conditioned room without humidity control, under a photoperiod of 16 hours light and 8 hours dark, with illumination provided by fluorescent TL5 HE 28W/865 lamps (BMC, China) delivering an intensity of 5000 lux.

3 Results and discussion

Our results showed, that all tested concentrations of kanamycin in the nutrient medium (25, 50, 75, and 100 mg L^{-1}) suppressed the rooting of control (non-transformed) plants and finally led to the death of the experimental specimen (Figure 3). We observed no concentration-depended relations in the experiment with the control plants.

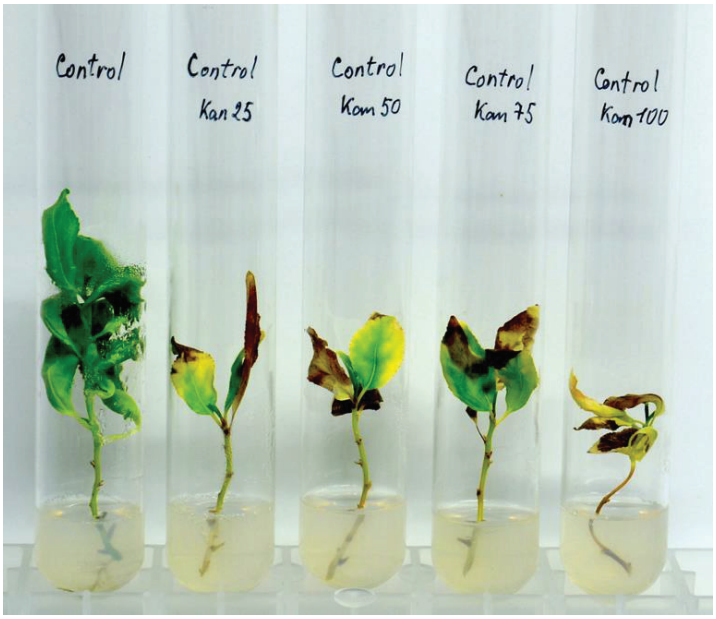

Fig. 3. Rooting absence of control Berlin poplar plants in the presence of kanamycin in different concentrations (25, 50, 75, and 100 mg L^{-1}) in the nutrient medium in comparison with control without antibiotic.

The same concentrations of kanamycin (25, 50, 75, and 100 mg L^{-1}) were applied to the fast-growing transgenic poplars expressing both gibberellin-20-oxidase from *A. thaliana* (*AtGA20ox1*) and neomycin phosphotransferase II (*nptII*), which confers resistance to kanamycin. Notably, kanamycin did not increase mortality among the transgenic poplars at any of the concentrations tested, in contrast to the control plants. However, we did observe an effect of kanamycin on the growth rate of transgenic plants. The maximum growth suppression induced by kanamycin was observed at a concentration of 100 mg L^{-1}. Specifically, transgenic plants rooted in the presence of kanamycin at this concentration exhibited heights comparable to those of control plants rooted without kanamycin after 25 days of experimentation (Figure 4). General decreasing of the plant height was 50% in comparison with fast-grooving poplar rooted without antibiotic. Kanamycin concentrations of 25 and 50 mg L^{-1} produced nearly identical effects, reducing the growth rate of the fast-growing transgenic poplars by approximately 25% compared to transgenic plants rooted in the nutrient medium without kanamycin (Figure 4). An approximate 50% reduction in growth was noted for transgenic plants rooted in medium supplemented with kanamycin at a concentration of 75 mg L^{-1} (Figure 4).

Fig. 4. Influence of different kanamycin concentrations on the growth rate of fast-growing transgenic polar expressing neomycin phosphotransferase II. Test tubes length – 20 cm. A – control poplar in the nutrient medium without kanamycin; B – F – transgenic poplars (line #15): B – without kanamycin, C – 25 mg L^{-1}, D – 50 mg L^{-1}, E – 75 mg L^{-1}, F – 100 mg L^{-1}. Age of the plants – 25 days.

Overall, the results of the experiment demonstrated a classical dose-response relations, with minimal influence observed at lower dose and maximum effect at higher doses.

These findings suggest that kanamycin can be an effective tool for micropropagation and enhancing the efficiency of *in vitro* culture for fast-growing transgenic poplars. By varying kanamycin concentrations in the nutrient medium, it is possible to control the height of transgenic poplars in the test tubes and plan schedules for replanting of *in vitro* plants.

In particular, using kanamycin at the highest studied concentration of 100 mg L^{-1} enables the production of transgenic poplars with growth rates comparable to those of control plants. This allows for the use of standard 15 cm test tubes for the cultivation of fast-growing transgenic Berlin poplar.

4 Conclusion

It has been demonstrated that kanamycin, at various concentrations (25, 50, 75 and 100 mg L^{-1}) in the nutrient medium, can effectively suppress the growth of fast-growing genetically modified poplars expressing neomycin phosphotransferase II as a product of selective gene expression, without effecting their mortality. Consequently, kanamycin may serve as a growth suppressor and can be utilized as an effective tool in the micropropagation and *in vitro* culture of the genetically modified fast-growing Berlin poplar. The application of kanamycin to inhibit the growth of these transgenic poplar plants allows for an extended duration between plant relocations to fresh nutrient media, thereby enhancing the efficiency (in terms of time and cost) of maintaining the *in vitro* culture of this transgenic line. Thus,

kanamycin can be used as an effective tool for growth suppression of other genetically modified plants that also carry the widely used selective *nptII* gene during their micropropagation.

Acknowledgements

The research was carried out within the state assignment of Ministry of Science and Higher Education of the Russian Federation for Siberian Institute of Plant Physiology and Biochemistry of the Siberian Branch of Russian Academy of Sciences (Project State Registration No – 122041100049-0). The research was done using the equipment of The Core Facilities Center "Bioanalitika" and collection of The Core Facilities Center "Bioresource Center" at Siberian Institute of Plant Physiology and Biochemistry of the Siberian Branch of Russian Academy of Sciences (Irkutsk, Russia).

References

1. B. M. Reeoi, Fruit Varieties Journal **46**, 98 (1992)
2. I. Kovalchuk, Y. Lyudvikova, M. V. Volgina, and B. M. Reed, Plant Cell, Tissue and Organ Culture **96**, 127 (2009)
3. H. Alzubi, L. M. Yepes, and M. Fuchs, In Vitro Cellular & Developmental Biology - Plant **55**, 334 (2019)
4. W. Rademacher, Annual Review of Plant Physiology and Plant Molecular Biology **51**, 501 (2000)
5. S. Altintas, African journal of biothechnology 10 (2011)
6. S. D. Koutroubas and C. A. Damalas, Bioscience Journal **32**, 1493 (2016)
7. P.-W. Jing, X.-F. Li, Q.-F. Shi, H.-N. Liu, M.-S. Pei, T.-L. Wei, D.-L. Guo, and Y.-H. Yu, Scientia Horticulturae **313**, 111891 (2023)
8. C. Chen, X. Fu, R. Peng, Y. Tian, and Q. Yao, Biotechnology & Biotechnological Equipment **34**, 673 (2020)
9. V.V. Pavlichenko and M.V. Protopopova, Russian Journal of Plant Physiology **71**, (2024)
10. T. Murashige and F. Skoog, Physiologia Plantarum **15**, 473 (1962)