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In vitro culture initiation and regeneration of two highly productive clones of poplar

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Abstract

In vitro shoot regeneration is a fast and reliable method for propagation of valuable tree genotypes and important step in genetic manipulations. This study aimed to establish an optimal *in vitro* culture initiation and regeneration protocol for highly productive poplar clones 'Novoberlinska-3' (*Populus pyramidalis* × *P. laurifolia*) and 'Volosystoplidna' (*P. trichocarpa*). *In vitro* culture was initiated either on MS (IM-MS) or WPM (IM-WPM) medium both containing BAP (0.2 mg.L^{-1}) and IBA (0.1 mg.L^{-1}). Results demonstrated that the best initiation medium for the clone 'Volosystoplidna' was IM-WPM on which 93.3 % of explants survived, while explants from 'Novoberlinska-3' better survived on IM-MS. After establishing both genotypes into aseptic culture, regeneration experiments were started using two types of explants, leaf and petioles, planted on callus induction medium containing 2iP (1.02 mg.L^{-1}) and NAA (1.86 mg.L^{-1}). Furthermore, microshoots were placed on shoot induction medium supplemented with 0.04 mg.L^{-1} thidiazuron, and then transferred on shoot elongation medium with 0.2 mg.L^{-1} BAP. Regeneration protocol showed better performance of poplar 'Novoberlinska-3', but it was also efficient for 'Volosystoplidna'. Plant regeneration from leaf explants in the clone 'Novoberlinska-3' showed the highest regeneration percentage (92.3 %) and the number of shoots per explant (3.1), which significantly exceeded other explants. Our results showed a significant difference between the survivability of two clones during culture initiation. The differences in regeneration rates between the clones as well as leaf and petiole explants were also determined. Obtained aseptic cultures of highly productive poplar hybrids will be used in our further studies, especially for genetic transformation.

Introduction

Poplars *(Populus spp.)* belong to the plants of the *Salicaceae* family, which are ecologically and economically important tree species. These fast-growing trees are widely used for biofuel production, wood, pulp, and paper industries, environmental protection, and restoration of degraded soils (Gordon 2001; Pilipović *et al.* 2019; Galović *et al.* 2021). Poplars are also trees of great scientific importance due to ease of vegetative propagation, short rotation cycle, small genome size, and the ability to regenerate *in vitro*. These important features, combined with the complete sequencing of the genome of *Populus trichocarpa*, made poplar a model plant offering molecular tools for basic research in genetics, physiology, and

biochemistry of trees (Han *et al.* 2000; Tuskan *et al.* 2006; Kutsokon *et al.* 2020).

In recent decades, short-rotation plantations of fast-growing trees for biomass production have been increasingly established worldwide (Wullschleger *et al.* 2002; Aylott *et al.* 2008; Kutsokon *et al.* 2017; Moravčíková *et al.* 2017). For the effective management of these plantations, it is important to properly select highly productive and easily propagated intensively growing clones. It is known that some valuable poplar hybrids do not reproduce vegetatively or reproduce poorly.

The method of micropropagation can overcome this problem by providing genetically homogeneous planting material identical to the original form. Application of *in vitro* culture methods makes it possible to produce high-quality plants at any time, quickly and in large quantities. In addition, micropropagation preserves the genotype of the original plant, allows to control the growth environment, monitor the physiological and biochemical parameters of the plants, as well as to work regardless of weather conditions and season (Giri *et al.* 2004).

Micropropagation of trees is much more difficult than the micropropagation of herbaceous plants. It requires the development of specific cultivation techniques for different species. In addition to selection of the nutrient medium composition for macro- and micronutrient content, it is also necessary to add various vitamins, phytohormones or growth regulators. It may be also necessary to add absorbents and antioxidants, as woody plant species can release phenolic compounds into the growth environment. These compounds can be rapidly oxidized and may lead to plant death (Shahzad *et al.* 2017; Abiri *et al.* 2020).

In vitro shoot regeneration is not only a rapid and reliable method for valuable genotypes propagation but also the indispensable prerequisite for some biotechnological techniques, such as genetic transformation and gene editing. Genetic manipulations are able to increase the plant growth and resistance to biotic and abiotic stresses, change the quality of wood (e.g., reduce or change lignin content), and improve phytoremediation characteristics (Confalonieri *et al.* 2003; Kutsokon 2011; Song *et al.* 2019). However, not all tree species can be easily *in vitro* regenerated. Their ability to morphogenesis depends on the genotype, tissue types or explants, cultivation environment, media nutritional components, and growth regulators (Giri *et al.* 2004; Musienko and Panyuta 2005; Ferreira *et al.* 2009). The ratio of auxins to cytokinin plays a crucial role in the success of plant regeneration (Hill and Schaller 2013; Bidabadi and Jain 2020). Several authors have shown that the effective plant regeneration methods should be optimized for each clone individually (Noël *et al.* 2002; Thakur *et al.* 2012; Kutsokon *et al.* 2013; Kwon *et al.* 2015; Pokorná *et al.* 2017). Additionally, although many studies have been reported using the *Populus* explants for tissue culture propagation, those studies are limited to a small number of reports on shoots regeneration from leaf and petiole explants for different species that is an important step in genetic manipulations.

In our previous work, poplar clones 'Novoberlinska-3' (*Populus pyramidalis* × *Populus laurifolia*) and 'Volosystoplidna' (*Populus trichocarpa* Torr. Et Gray) were found to be between the most productive and energy-valuable clones in the short-rotation trials (Kutsokon *et al.* 2017). These clones can easily be multiplied by woody cuttings; however, for regeneration, gene transformation, and other *in vitro* manipulations, a homogeneous quality of the plant material is needed, thus the use of micropropagation is strongly recommended (Müller *et al.* 2013).

One of the clones studied, *P. trichocarpa,* as described for genotype 'Nisqually-1', is known as recalcitrant in *in vitro* conditions, though few protocols are known for regeneration (Kang *et al.* 2009) and transformation (Li *et al.* 2015) of 'Nisqually-1' clone. A limited number of papers about *in vitro* cultivation and genetic transformation of hybrids *P. laurifolia x nigra 'Italica'* (*P. x berolinensis)* were published, but they described only partial steps of the protocol (Pavlichenko *et al.* 2016; Zolotovskaya *et al.* 2018). Nevertheless, the clones 'Novoberlinska-3' and 'Volosystoplidna' have not been used in *in vitro* culture in the studies before. Therefore, in this paper we reported the optimal conditions for their *in vitro* culture initiation and regeneration which are the key steps for further genetic transformation.

Experimental

Plant material and surface sterilization

Buds and young softwood shoots of poplar clones 'Novoberlinska-3' *(P. pyramidalis × P. laurifolia)* and 'Volosystoplidna' (*P. trichocarpa* Torr. Et Gray) were collected at the beginning of the growing season. The plants in the juvenile stage of development, maintained in a pot stock plant collection, kept at the Institute of Cell Biology and Genetic Engineering NAS of Ukraine, were used as softwood shoot sources.

Explants were surface sterilized using our previously established protocol (Khudolieieva *et al.* 2017), by treating them with the different solutions in three subsequent steps: 1) concentrated soap solution (2 min); 2) commercial bleach solution of sodium hypochlorite diluted with distilled water to 30 % (10 min); 3) 70 % ethanol solution (1 min). After each step, the decontaminated explants were rinsed three times with sterile water**.**

Culture initiation, shoot regeneration, media, and growth conditions

In order to evaluate the effect of basal salt medium on plant growth during culture initiation, surface-sterilized stem explants of poplar clones 'Novoberlinska-3' and 'Volosystoplidna' were placed on two different types of initiation media, either on MS (IM-MS) or woody plant (IM-WPM) medium. Both nutrient media were modified with 0.2 mg.L^{-1} 6-benzylaminopurine (BAP) and 0.1 mg.L^{-1} indole-3-butyric acid (IBA). Detailed compositions of all nutrient media used in this research are summarized in Table 1.

Table 1. Composition of the nutrient media.

IM-MS	2.18 g.L^{-1} MS, 2 ml.L^{-1} WPM vitamins, 0.1 g.L^{-1} myo-inositol, 30 g.L^{-1} sucrose, 0.26 g.L^{-1} MES, 0.2 g.L^{-1} L-glutamine, 7.5 g.L^{-1} agar, 0.2 mg. L^{-1} BAP, 0.1 mg. L^{-1}IBA, pH 5.8
IM-WPM	1.15 g.L^{-1} WPM, 2 ml.L^{-1} WPM vitamins, 20 g.L^{-1} sucrose, 7.5 g.L^{-1} agar, 0.2 mg. L^{-1} BAP, 0.1 mg. L^{-1} IBA, pH 5.8
CIM	2.18 g.L^{-1} MS, 2 ml.L^{-1} WPM vitamins, 0.1 g.L^{-1} myo-inositol, 30 g.L^{-1} sucrose, 0.26 g.L^{-1} MES, 0.2 g.L^{-1} L-glutamine, 7.5 g.L^{-1} agar, 1.86 mg. L^{-1} NAA, 1.02 mg. L^{-1} 2-IP, pH 5.8
SIM	2.18 g.L^{-1} MS, 2 ml.L^{-1} WPM vitamins, 0.1 g.L^{-1} myo-inositol, 30 g.L^{-1} sucrose, 0.26 g.L^{-1} MES, 0.2 g.L^{-1} L-glutamine, 7.5 g.L^{-1} agar, 0.04 mg. L^{-1} TDZ, pH 5.8
SEM	2.18 g.L^{-1} MS, 2 ml.L^{-1} WPM vitamins, 0.1 g.L^{-1} myo-inositol, 30 g.L^{-1} sucrose, 0.26 g.L^{-1} MES, 0.2 g.L^{-1} L-glutamine, 7.5 g.L^{-1} agar, 0.2 mg. L^{-1} BAP, pH 5.8
PM-MS	2.18 g.L^{-1} MS, 2 ml.L^{-1} WPM vitamins, 0.1 g.L^{-1} myo-inositol, 30 g.L^{-1} sucrose, 0.26 g.L^{-1} MES, 0.2 g.L^{-1} L-glutamine, 7.5 g.L^{-1} agar, pH 5.8
PM-WPM	1.15 g.L^{-1} WPM, 2 ml.L^{-1} WPM vitamins, 20 g.L^{-1} sucrose, 7.5 g.L^{-1} agar, pH 5.8

IM-MS – initiation media contained MS (Murashige and Skoog) salts; IM-WPM – initiation media contained WPM (McCown Woody Plant Medium) salts; CIM – callus induction medium; SIM – shoot induction medium; SEM – shoot elongation medium; PM-MS – propagation media contained MS (Murashige and Skoog) salts, PM-WPM – propagation media contained WPM (McCown Woody Plant Medium) salts; BAP – 6-benzylaminopurine; IBA – indole-3-butyric acid; MES – 2-(N-morpholino) ethanesulfonic acid; NAA – α-naphthaleneacetic acid; TDZ – thidiazuron.

All chemicals were purchased from Duchefa (The Netherlands) unless otherwise stated. Fifteen explants per variant were planted, each in a separated glass tube. The explants were maintained in a tissue culture growth room at 24 °C under a 16-h photoperiod. The percentage of surviving stem explants was determined after 30 days of cultivation on induction media.

Successfully initiated into *in vitro* culture poplars 'Novoberlinska-3' and 'Volosystoplidna' were micropropagated on PM-MS and PM-WPM respectively (Table 1) and maintained by sub-culturing at 4-week intervals. Well-developed one-month plants were used for indirect shoot regeneration according to the protocol of Meilan and Ma (2006). This protocol was successfully performed in our previous studies for the regeneration of several poplar clones (Khudolieieva *et al.* 2014; 2017). Therefore, it was tested in the current study. Leaf and petiole explants were cultivated on Petri dishes with callus-inducing medium (CIM) supplemented with growth regulators 1.02 mg.L^{-1} N6-(2-isopentenyl) adenine (2-IP) and 1.86 mg.L^{-1} α-naphthaleneacetic acid

(NAA) (Table 1) at 21 °C in the dark for 3 weeks. The explants produced calli were transferred on Petri dishes with shoot induction medium (SIM) supplemented with 0.04 mg.L^{-1} thidiazuron (TDZ) (Table 1). Plants were cultured in the tissue culture growth room at 24 °C under a 16-h photoperiod for 2 weeks. Then the explants with microshoots (1 – 1.5 cm) were transferred to shoot elongation medium (SEM) containing 0.2 mg.L^{-1} BAP (see Table 1).
At least 15 leaf and 15 petiole explants were collected per clone at the initial stage of regeneration. The efficiency of regeneration was evaluated after six weeks since beginning the experiment by the percentage of explants producing at least one shoot to the total number of explants used as well as by the number of shoots per regenerated explant.

Statistical analysis

Statistical processing of the results was performed according to standard methods. Data are expressed as % ± s_p or as mean ± standard error of the mean. Data normality and variance homogeneity were checked using Shapiro-Wilk and Levene's tests, respectively. Because the data were not normally distributed, nonparametric tests were applied. Significant differences in the survivability of explants and percentages of explants producing the shoots were tested with the chi-square test. The differences in the number of shoots per regenerated explant were tested using the Mann-Whitney U test. All tests were declared significant at $P \leq 0.05$. All statistical analyses and tests were performed using OriginPro9.0 software (OriginLab Corporation).

Results and Discussion

Survivability of stem explants initiated into in vitro culture

In this study, two poplar clones 'Novoberlinska-3' and 'Volosystoplidna' were established in aseptic cultures (Fig. 1), and the optimal composition of the IM for the first stage of cultivation was selected. As shown in Fig. 2, the best initiation nutrient medium for the poplar clone 'Volosystoplidna' was the IM-WPM on which the survival rate was 93.3 %. For poplar clone 'Novoberlinska-3', a higher survival rate (73.3 %) was observed on IM-MS compared to IM-WPM (46.7 %), but in this case, the difference between the rates was not significant.

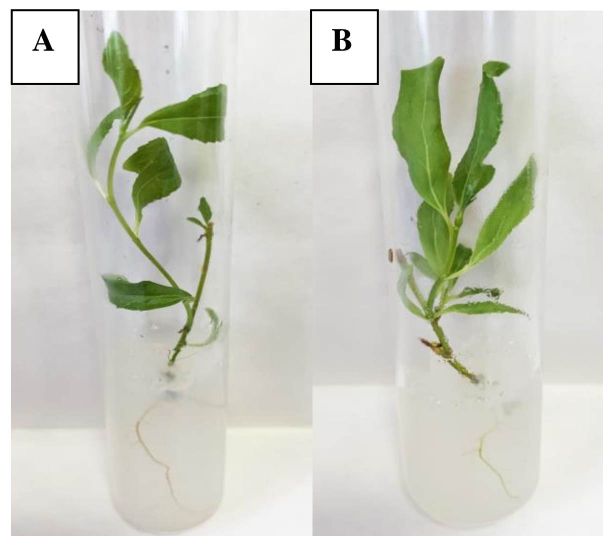

Fig. 1. Establishment of aseptic cultures of poplars from stem explants: clone 'Novoberlinska-3' on the IM-MS (**A**) and clone 'Volosystoplidna' on the IM-WPM (**B**).

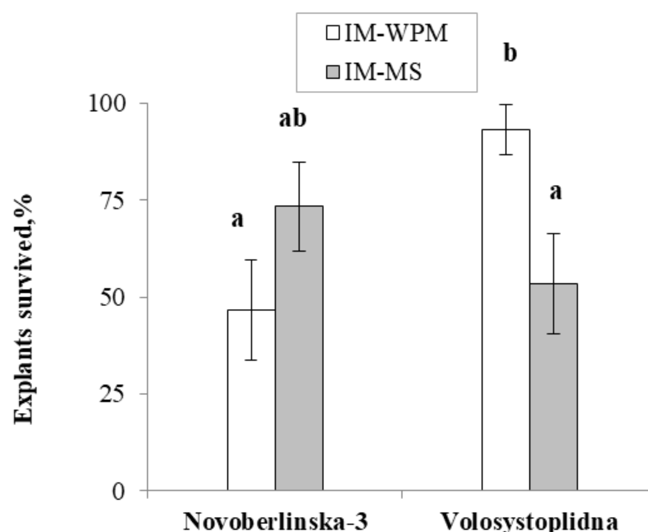

Fig. 2. Survivability of stem explants of the poplar clones 'Novoberlinska-3' and 'Volosystoplidna' while initiated into *in vitro* culture. IM-WPM and IM-MS were both supplemented with 0.2 mg.L^{-1} BAP and 0.1 mg.L^{-1} IBA. Different letters above the bars indicate significant differences in survivability between the samples (% ± s_p) after the chi-square test ($P \leq 0.05$), n = 15 replicates for each genotype.

The survival rates of both poplar clones on the IM demonstrated that initiation into *in vitro* culture requires an individual approach for each clone separately. In general, the use of growth regulators has different effects on plant regeneration and rooting efficiency in *Populus* species (Bannoud and Bellini 2021). In particular, the study by Pardhi *et al.* (2019) showed that the addition of 0.5 mg.L^{-1} BAP promoted the initiation of *P. nigra* explants into *in vitro* culture on the 21-st day of the experiment. An application of 0.1 µM IBA showed high efficiency in rooting of Himalayan poplar shoots (*P. ciliata* Wallich ex Royle) (Aggarwal *et al*. 2015).

Shoot indirect regeneration from leaf and petiole explants

After establishing both poplar genotypes, and their micropropagation in aseptic cultures, regeneration experiments were started. Both types of explants, the leaf and petioles, formed callus within three weeks on CIM with the growth regulators 2-IP and NAA (Fig. 3A).

The explants of both poplar clones, transferred on SIM with TDZ (0.04 mg.L^{-1}), formed the microshoots after two weeks of cultivation, more actively in poplar 'Novoberlinska-3' compared to poplar 'Volosystoplidna'(Fig. 3B and 3C). Later, these microshoots were transferred on SEM with the addition of BAP (0.2 mg.L^{-1}) (Fig. 3D) and within two weeks regenerants from leaf and petiole explants in both poplar clones were obtained (Fig. 3E and 3F) with different regeneration efficiencies (Table 2).

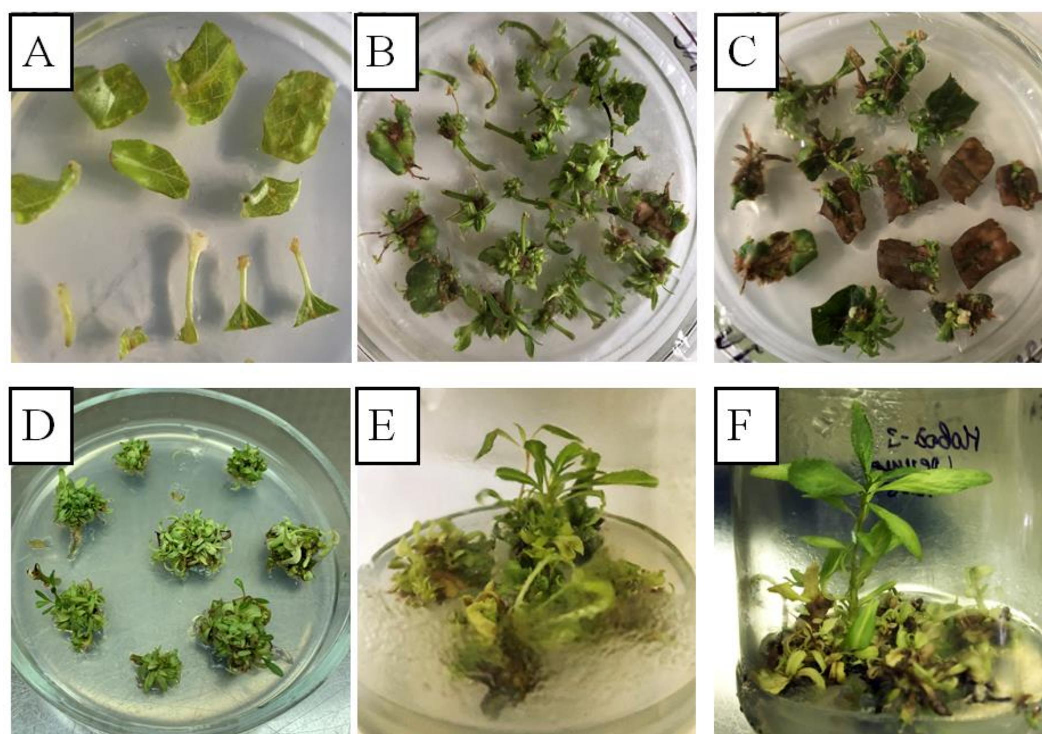

Fig. 3. Regeneration of poplar clones 'Volosystoplidna' (**A**, **C**, **E**) and 'Novoberlinska-3' (**B**, **D**, **F**) from leaf and petiole explants. **A** – callogenesis on CIM after 10 days of cultivation; **B**, **C** – formation of microshoots after two weeks of cultivation on SIM; **D** – microshoots transferred on SEM; **E**, **F** – shoots growing on SEM during two weeks.

Table 2. Shoot regeneration from leaf and petiole explants of poplar clones 'Novoberlinska-3' and 'Volosystoplidna'.

Clone	Type of explant	The number of explants planted	The number of explants with shoots	The number of shoots per explant
'Novoberlinska-3'	Leaves	39	36	112
	Petioles	30	27	43
'Volosystoplidna'	Leaves	38	19	30
	Petioles	31	18	33

The poplar clone 'Novoberlinska-3' showed higher regeneration efficiency. In particular, the percentage of regeneration of this clone from leaf (92.3 %) and petiole (90 %) explants was significantly higher than in the poplar clone 'Volosystoplidna', where the percentage of regeneration was lower, at 50 % from both leaf and petiole explants (Fig. 4).

As shown in the Table 2, the number of shoots obtained from leaf explants in the clone of poplar 'Novoberlinska-3' was 112, and from petiole explants – 43, while in the clone 'Volosystoplidna' the number of shoots obtained from leaf and petiole explants was 30 and 33, respectively. Therefore, the number of shoots per regenerated explant in the clone of poplar 'Novoberlinska-3' from leaf explants was 3.1 (Fig. 4B) that significantly exceeded all other variants by 1.5 – 2 times. Thus, plant regeneration from leaf explants in the clone 'Novoberlinska-3' showed the highest efficiency, which significantly differed from all other explants.

As our experiments have shown, the composition of the nutrient medium plays an important but different role in the regeneration of the shoots of individual poplar clones. Many studies (Khatab 2011; Cai *et al.* 2015; Kwon *et al.* 2015; Pardhi *et al.* 2019) have demonstrated that the optimal composition of the nutrient medium and concentration of phytohormones should be selected separately for each species and even for each cultivar and the type of explants. In particular, studies (Tsvetkov *et al.* 2007) have shown that the addition of thidiazuron to the main nutrient medium at both high and low concentrations has different effects on shoot regeneration in white poplar *(P. alba).* The greatest regeneration efficiency was observed when lower concentrations were added (0.11 – 0.56 µM) compared to higher concentrations (14 µM). Similarly, thidiazuron applied to stem, leaf, and root explants of *P. berolinensis* in higher concentrations (0.1 – 0.5 $mg.L^{-1}$), than we did (0.04 $mg.L^{-1}$), was inefficient for regeneration. In those experiments only very weak efficiency was determined for petiole explants under the lowest concentration of TDZ (0.1 $mg.L^{-1}$) (Pavlichenko *et al.* 2016). Addition of 0.5 µM NAA induced callus formation in *P. euphratica* Oliv. (Cai *et al.* 2015). Studies by Garcia-Angulo *et al.* (2018) showed that the addition of 0.5 µM NAA leaded to the highest frequency of shoot regeneration in two hybrid clones of *Populus (P.×euramericana)* and *(P.×interamericana)*, but the shoots formed roots better in medium without NAA. All these studies suggest that the appropriate composition of the nutrient medium must be selected at each stage of organogenesis.

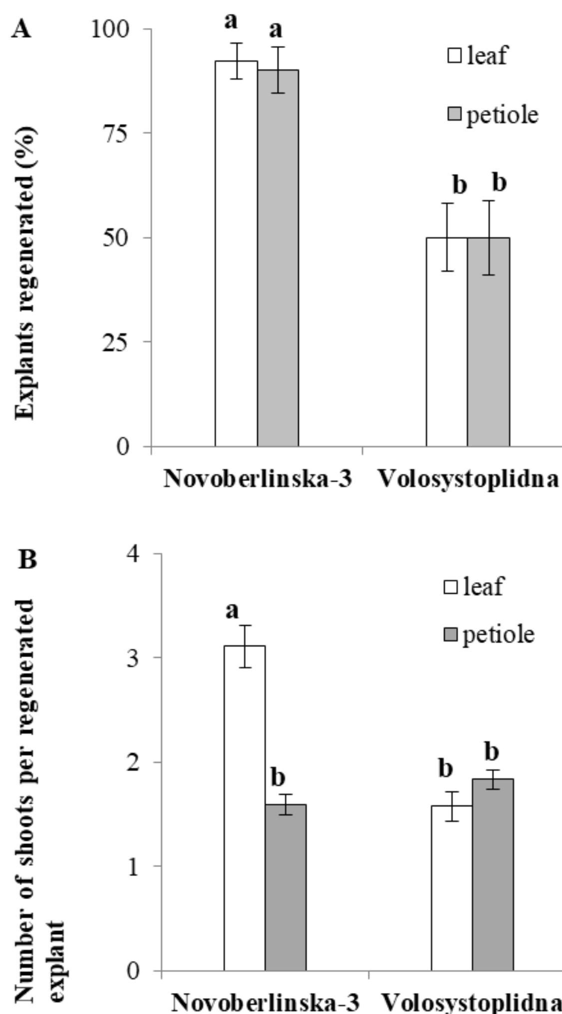

Fig. 4. The efficiency of regeneration from leaf and petiole explants of poplar clones 'Novoberlinska-3' and 'Volosystoplidna' *in vitro*. The percentage of regenerated explants (% ± s_p) (**A**) and the number of shoots per regenerated explant (mean ± standard error of the mean) (**B**) are presented. Different letters above the bars indicate significant differences between the samples in the percentage of regenerated explants and the number of shoots per regenerated explant after the chi-square and the Mann-Whitney U test, respectively ($P \leq 0.05$), n = 15 replicates.

Our results showed a significant difference between the survival rates of two clones during the culture

initiation. The differences in regeneration rates were also determined between the clones as well as leaf and petiole explants at the same composition of medium and growth regulators. Thus, we can assume that the regeneration of shoots in poplar clones depends on both the genotype of the plants and on the tissues to be regenerated, and therefore requires the selection of optimal conditions for each clone.

Conclusion

In this study, two clones of poplar 'Novoberlinska-3' and 'Volosystoplidna' were introduced into *in vitro* culture. An optimal initiation nutrient medium for poplar 'Volosystoplidna' was IM-WPM supplemented with BAP (0.2 mg.L^{-1}) and IBA (0.1 mg.L^{-1}), on which the survival rate was 93.3 %. It was significantly higher than the survival rate of explants cultivated on IM-MS with the same hormone composition. For poplar clone 'Novoberlinska-3', although better survival rate (73.3 %) was observed on IM-MS than on IM-WPM (46.7 %) but without a significant difference. The protocol chosen for indirect *in vitro* regeneration showed better performance of poplar clone 'Novoberlinska-3', but was also efficient for the clone 'Volosystoplidna'. The percentage of regeneration in the clone 'Novoberlinska-3' from leaf (92.3 %) and petiole (90 %) explants was significantly higher than in the poplar clone 'Volosystoplidna', where the percentage of regeneration in each type of explants equalled 50 %. The regeneration rate of leaf explants of clone 'Novoberlinska-3' (3.1) was also significantly higher than that of petiole explants and exceeded the rates of explants of clone 'Volosystoplidna'. Obtained aseptic cultures of two highly productive poplar hybrids will be used in our further studies, and optimal protocols for regeneration will be applied for their genetic transformation.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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