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REGULAR ARTICLE

ADVANCING PROTOCOLS FOR POPLARS *in vitro* PROPAGATION, REGENERATION AND SELECTION OF TRANSFORMANTS

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ABSTRACT

Poplars (genus *Populus*) have emerged as a model organism for forest biotechnology, and genetic modification is more advanced for this genus than for any other tree. So far several protocols for microclonal propagation and regeneration for *Populus* species have been developed. However it is well known that these protocols differ for various species and need to be adapted even for different clones of the same species. This work was focused on developing of protocols for propagation, regeneration and putative transformant´s selection of aspen *Populus tremula* L. and other two fast-growing *Populus* species (*P. nigra* L., *P. x canadensis* Moench). The regeneration ability for black poplar explants was demonstrated to be three times higher compared to those for aspen and hybrid poplar. It was found that concentration 1 mg/L of phosphinothricin and 25 mg/L of kanamycin is toxic for non-transgenic plant tissues of *P. x canadensis* and can be applied in transformation experiments when genes of resistance to the corresponding selective agents into the plant genome are introduced.

Keywords: *Populus* sp., *in vitro* culture, microclonal propagation, regeneration, kanamycin and phosphinothricin selection.

INTRODUCTION

Poplar (genus *Populus*) is fast growing tree often cultivated for inexpensive hardwood timber. In addition it is widely used for the manufacture of paper; production of packing materials, furniture, matches, particle board, viscose etc. There is also interest in using poplar as an energy crop for biomass and bio-fuel and finally, poplar trees are often used for planting in forest shelter belts and as ornamental plants. Currently poplar has emerged as a model organism for forest biotechnology, and genetic modification is more advanced for this genus than for any other tree **(Tuskan *et al.* 2006)**.Through this technology it is possible to increase plant growth and the resistance of plants to environmental stresses and herbicides; modify quality of the wood (decreasing or modifying the lignin content); utilize the plants for phytoremediation; and make the changes in the plant morphology **(Confalonieri *et al.* 2003, Taylor, 2002, Giri *et al*. 2004, Herschbach and Kopriva, 2002, Rishi *et al.* 2001, Lin *et al.* 2006, Kutsokon, 2011)**. So far several protocols for microclonal propagation and regeneration for *Populus* species have been developed **(Confalonieri *et al.* 2003, Tzfira *et al.* 1997, Han *et al.* 2000, Meilan, Ma, 2006, Yevtushenko and Misra, 2010)**. However it is well known that these protocols differ for various species and need to be adapted even for different clones of the same species.

Since we plan to introduce polar transformation protocols in our laboratory in the first step we focused on developing of protocols for propagation, regeneration and putative transformant´s selection of aspen *Populus tremula* L. and other two fast-growing *Populus* species (*P. nigra* L., *P. x canadensis* Moench).

MATERIAL AND METHODS

Plant material

Three *Populus* species – aspen *P. tremula* L., black poplar *P. nigra* L. and hybrid poplar *P. x canadensis* Moench were introduced into *in vitro* culture. *P. nigra* and *P. x canadensis* Moench as fast-growing clones were provided by the Institute of Forestry and Forest Melioration (Kharkiv, Ukraine). Plants were grown aseptically on propagation medium (1.230 g/L WPM salt mixture (DUCHEFA), 1 ml/L MS vitamins (DUCHEFA), 20 g/L sucrose, 7 g/L plant agar, pH 5.8) in glass jars under 16 hrs day period at 26 °C.

Shoot regeneration and rooting

As explants for plant regeneration – the leaves, petioles and stamen segments were used. Callus-inducing medium (CIM) with 2.15 g/L MS salt mixture (DUCHEFA), 1 ml/L MS vitamins, 20 g/L sucrose, 0.27 g/L MES, 0.02 μM TDZ, 7 g/L plant agar, pH 5.8 was used to induce direct regeneration from tree explants **(Tzfira *et al.* 1997)**. Explants were cultivated on Petri dishes with CIM medium within 3 weeks. Then they were transferred on the shoot inducing medium (SIM), that contained 2.15 g/L MS salt mixture, 1 ml/L MS vitamins, 20 g/L sucrose, 0.27 g/L MES, NAA 0.5 μM, zeatin 1 μM, 7 g/L plant agar, pH 5.8 **(Yevtushenko, Misra, 2010)**. Plants were grown on SIM medium until they reached the length of 1-2 cm then were transferred into the jars with propagation medium.

Evaluation of selective concentration of kanamycin and phosphinotricin

In order to determine concentration of selective agent that is toxic for non-transgenic poplar plants the explants of leaves and petioles of hybrid poplar *P. x canadensis* Moench were applied on above mentioned media supplemented with 0.5; 1; 2; 3; 4; 5 mg/l of phosphinothricin (ppt) and 25; 50; 100; 150; 200 mg/L of kanamycin (Km). Twenty four explants were tested for each selective agent concentration. The evaluation of selective pressure was carried out after 10 days and 1 month; while the presence of green explants as well as explants with regenerants was monitored.

Acclimatization

When shoots reach a length of 3-5 cm, they were carefully taken from jars, the roots were washed with tap water and the plants were transferred to pots with regular potting mixture. Plants were placed inside plastic bags in a growth room. After about 2 weeks plastic bags were removed.

RESULTS AND DISCUSSION

In vitro propagation and regeneration of poplars

Leaves, stems and petioles were taken from the plants of three poplar clones, growing on propagation medium (Fig. 1), were cultivated 3 weeks on CIM; and then transferred on SIM (Fig. 2). As it is shown in Figures 2 and 3, regeneration ability of black poplar explants was 3 times higher compared to aspen and hybrid poplar.

P. nigra L., clone Gradizhska | *P. x canadensis* Marsh., clone Guliver | *P. tremula* L.

Figure 1 Poplars in *in vitro* culture

a) **b)** **c)**

Figure 2 Regeneration of poplar plants from different explants:
a) leaves regenerants of *P. nigra,* b) leaves regenerants of *P. x canadensis*,
c) stem and petiols regenerants of *P. tremula*

Determination of selective concentration of kanamycin and phosphinotricin

As our research is planned to be focused on genetic transformations, firstly we decided to determine the concentrations of selective agents that may be used for selection of transformed poplars following the transformation with the constructs harboring selective genes – *bar* (selection by ppt) or *nptII* (selection by Km). Phosphinotricin was found to be more effective selective agent than kanamycin, as the plants of hybrid poplar became brown and died very quickly when they were grown on media with concentration 1 mg/L of ppt (Fig. 4). Only the explants growing on media supplemented with ppt 0.5 g/L were able to survive after 10 days as well as 1 month.

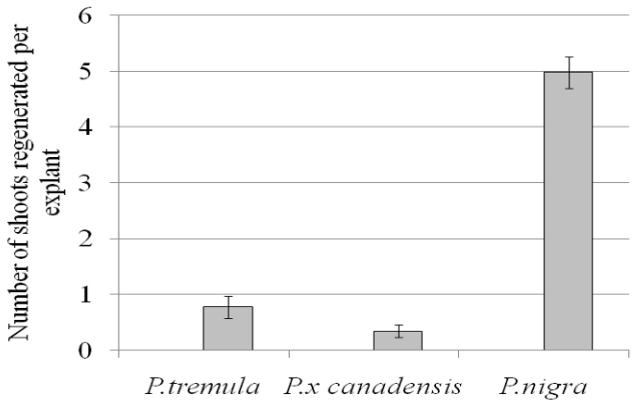
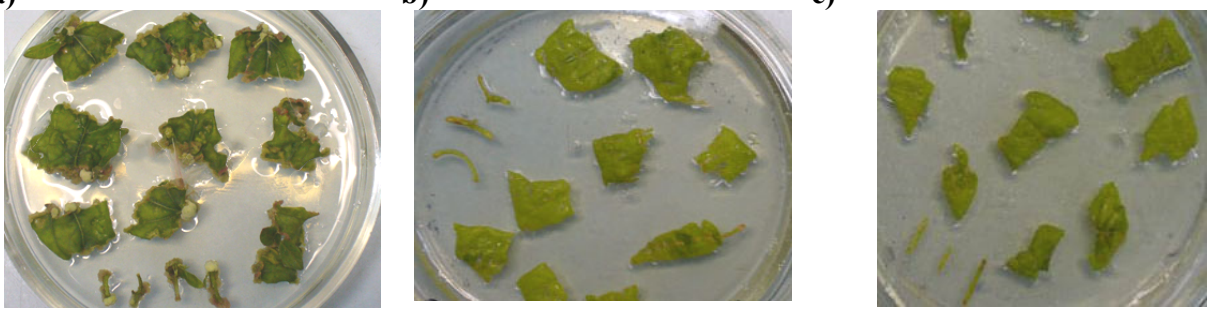


Figure 3 The regeneration activity of poplars leave explants

a) **b)** **c)**



d) **e)** **f)**

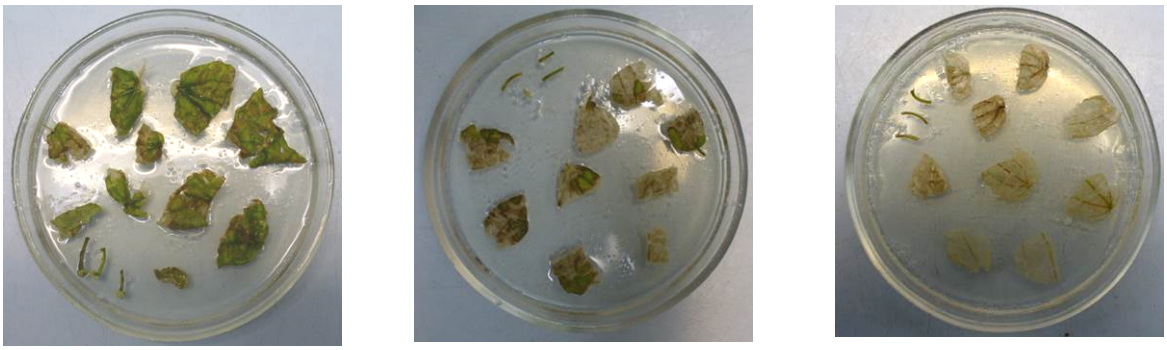


Figure 4 Effects of phosphinothricin and kanamycin on *Px canadensis* leave explants after 1 month growth on CIM
a) 0 mg/L; b) Km, 25 mg/L; c) Km, 200 mg/L; d) ppt, 0,5; e) ppt, 1 mg/L; f) ppt 2 mg/L.

Contrary the selection of *P. x canadensis* explants on media containing kanamycin as a selection agent was not clear. All explants, growing on media with all tested concentration of kanamycin left green during all the time of observations, but no regenerants were formed (Fig. 4). Regarding clone of hybrid poplar *P x canadensis* Moench, it appeared to be sensitive to both ppt and Km, at concentrations 1 mg/L and 25 mg/L, respectively. When **Confalonieri *et al*., (2000), Tzfira *et al*., (1997), DeBlock, (1990)** tested other poplar species/clones concentration of both selective agents recommended for transformation experiments was higher – up to 100 mg/L for kanamycin and– 5-10 mg/L for phosphinotricin.

CONCLUSION

In this study we improved the protocols for *in vitro* propagation, regeneration and putative transformant´s selection of selected poplar species/clones. The regeneration ability for black poplar explants was three times higher compared to those for aspen and hybrid poplar. It was found that concentration 1 mg/L of ppt and 25 mg/L of Km may be used as selective for determining transformants. Results obtained will be applied in planned genetic transformation experiments.

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