JOURNAL OF FOREST SCIENCE, *52*, 2006 (3): 101–107

Aspen micropropagation: use for phytoremediation of soils

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ABSTRACT: Toxic pollution of soils is a major environmental problem in the Czech Republic. Most contemporary remediation approaches do not provide acceptable solutions. For environmental clean-up, the use of specially selected and engineered plants capable of effective restoration and stabilization of contaminated sites is an emerging technology called phytoremediation. Aspen (*Populus* spp.) trees represent optimal plants for absorption, accumulation, storage, and degradation of environmental pollutants such as heavy metals, pesticide residues, and other waste products. The first aim of this study was to verify possibilities of micropropagating selected elite European aspen (*Populus tremula*) and hybrid aspen (*Populus tremula* × *Populus tremuloides*) mature trees growing in polluted areas. The primary cultures were established from 24 years old trees from provenance plots. Dormant buds were sampled from selected trees in spring. After sterilization of dormant buds, the shoot tips were extirpated and put on nutrient media. MS medium with a higher concentration of BAP (1.0 mg/l) and IBA (0.1 mg/l) showed to be suitable for induction of organogenesis. MS medium with lower concentration of BAP (0.2 mg/l) and higher concentration of glutamine (100 mg/l) in agar medium was used for multiplication. A high number of adventitious shoots (20–30) was produced per multi-apex culture. The losses during rooting and acclimatization were minimal, around 2%. Currently, the plantlets grow on the outside bed of the experimental nursery. Twenty-five clones were established in the gene bank. Secondly, standardization of the efficient transformation of hybrid aspen was investigated with the aim to facilitate production of transformed hybrid aspen with valuable genes for increased capability of phytoremediation in the near future. Four gene constructs of *Agrobacterium tumefaciens* comprising different promoters (bearing two genes, *NPTII* coding resistance to kanamycin, and *GUS* coding glucuronidase synthesis allowing histochemical and fluorometrical identification) were used for transformations of aspen hybrid stem segments. A promoter suitable for transformations was selected on the basis of *GUS* activity assay. The highest activity was estimated in transformants with construct 148 controlled by promoter CAMV 35S.

Keywords: aspen; micropropagation; *Agrobacterium tumefaciens*; phytoremediation

Phytoremediation is a cost-effective, environmental-friendly technology (Vaněk, Schwitzguébel 2003). Recent studies indicate that some plants have the great genetic potential to remove many toxic metals from the soil. Despite this potential, phytoremediation is not a commercially available technology yet. The genus *Populus* is an ideal candidate for absorption or degradation of environmental pollutants, heavy metals and other waste products (Soudek et al. 2004).

The aspen (*Populus tremula*) is a very modest tree species about climatic conditions and soil quality. Especially cultivated aspen clones and synthetic populations are very promising both for planting on agricultural soils (lignicultures) and as the pioneering woody species for clearings caused by air pollution (Mottl, Štěrba 1988; Joachim 1991). Aspen is amenable to tissue culture manipulation, genetic engineering, and genetic mapping. *Populus* could further be selected or engineered with remediatory function, or used to support the growth of remediatory microorganisms. Reproduction of elite mature individuals can be realized by using root cuttings but micropropagation seems to be a more

Supported by the Grant Agency of the Czech Republic, Project No. 526/04/0135, and by the Ministry of Agriculture of the Czech Republic, Project No. MZe 0002070202.

effective method. Some advantages of this method are that the donor tree is not damaged, dormant buds are used for micropropagation without affecting the root system and the donor individual can be identified with absolute accuracy. Suitability of micropropagation for clone regeneration of selected genotypes was proved by many authors, for instance by Libbby and Ahuja (1993). In the Scandinavian countries and in Germany micropropagation has been used for clone regeneration of cultivated trees (Barocka et al. 1985) for a long time. For its regeneration ability the aspen is a very perspective woody species for genetic engineering. Especially its rapid reproduction of transgenic clones highly resistant to heavy metals, salting and drought is of great importance for the arid areas (Wang et al. 1999). Genetic engineering of trees helps to compensate for conventional breeding disadvantages by incorporating known genes into a specific genetic background. Methods available for plant transformation are divided into three main groups: 1. those using biological vectors (virus or *Agrobacterium* mediated transformation), 2. direct DNA transfer technique and 3. non-biological vector systems (microprojectiles, microinjection). *Agrobacterium tumefaciens* mediated binary plasmid system is the most widely used method for transformation (Leplé et al. 1992). Our research was aimed at verifying the method of rapid *in vitro* reproduction of mature aspens growing in experimental outside plantings that proved to be suitable for extreme sites and for production of quality timber and at standardization of the efficiency of transformation procedure *via Agrobacterium tumefaciens* for hybrid aspen.

MATERIAL AND METHODS

Reproduction of mature aspen

Localities and plant material

Buds for the establishment of primary cultures were taken from 24 years old trees of hybrid aspen from provenance plots (Špičák, Přísečnice, Školní, U trianglu) in the Krušné hory Mts. Positive selection of phenotypically valuable individuals was used. Sampling of winter buds in the Krušné hory Mts. was realized in early May. The buds were collected from 40 trees. From each individual 30–50 buds were isolated and sterilized (in a solution of 0.01% $HgCl_2$ and in 1% SAVO) and washed three times with sterile water. Shoot tips from sterile dormant buds were extirpated and put on suitable nutrient media.

Induction of organogenesis

A modified medium of Murashige and Skoog (1962), MS1, was used (Table 1) for induction of organogenesis. Cultures were cultivated under air conditioning, 24°C, white fluorescent light (30 μmol/m^2/s) and 16h photoperiod.

Multiplication

During the first three months the growing cultures were transferred onto a multiplication medium, MS2 (Table 1), after 14–21 days. Transfer intervals after this period were prolonged to 4–6 weeks. Cultures were cultivated under air conditioning, 24°C, white fluorescent light (30 μmol/m^2/s) and 16h photoperiod.

Rooting

Microcuttings from multi-apex cultures were used for rooting. Rooting was done on the three times diluted basic medium MS without cytokinin into which 0.6 mg/l of IBA was added. At least 1,000 microcuttings within 20 clones rooted during the experiment.

Transformation

Plant material

Stem segments of *in vitro* multi-apex culture of hybrid aspen (*Populus tremula* × *P. tremuloides*) No. 5 from the Gene Bank of FGMRI were used for the transformation procedure.

Agrobacterium tumefaciens

Four constructs of *Agrobacterium tumefaciens*, (LBA 4404), which contain a binary vector, Ti-plasmid PAL 121 of nopaline type with virulent virus zone and different plasmids with incorporated chimeric genes: the gene for the synthesis of neomycin phototransferase II (*NPTII*), *GUS* gene and CaMv 35S, rbcS, NOS and MAN promoters were used for transformation.

Description of constructs:

148–pBI 121, comprising the reporter gene *GUS* controlled by the promoter 35S

149–pJPP1, comprising the reporter gene *GUS* controlled by the promoter NOS (nopalinesynthase)

150–pJPP4, comprising the reporter gene *GUS* controlled by the promoter MAN (mannopinsynthase)

151–pBI 131, comprising the reporter gene *GUS* controlled by the promoter *rbcS* (ribulosebiphosphate carboxylase).

These constructs were obtained from Dr. S. Rakouský, Institute of Molecular Biology of Plants,

Table 1. Media for induction of organogenesis and multiplication

Medium	BAP (mg/l)	IBA (mg/l)	Glutamine (mg/l)	Sucrose (g/l)
MS1	1.0	0.1	100	30
MS2	0.2	0.1	10	30

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Cultivation

Agrobacterium suspension grown over night in a modified liquid Yeast Mannitol Broth (YMB) medium (Tzfira et al. 1996) containing 50 μm/ml kanamycin, under air conditioning at 24°C, 250 rpm, was resuspended to a density of 10^8/ml in an MS medium. Stem segments 15–20 mm in length were immersed into the suspension of *Agrobacterium tumefaciens* for 30 min. The segments were blotted on sterile filter paper and transferred to a semisolid MS medium with concentrations of BAP 1 mg/l and IBA 0.1 mg/l. After 2 days of co-cultivation the segments were washed twice in a liquid MS medium and transferred to a fresh liquid medium supplemented with 3.5 g/l timentin for 30 min. The segments were transferred into an agar medium for adventitious bud induction, MS medium with the concentration of BAP 1 mg/l, IBA 0.1 mg/l and augmentin 500 mg/l and timentin 1,000 mg/l. Stem segments were cultivated at 25°C in the white fluorescent light (30 μmol/m^2/s). Every 7 days the explants were transferred onto a fresh medium. Simultaneously, the concentration of antibiotics (timentin 1,000 mg/l–0 mg/l, augmentin 1,000 mg/l–0 mg/l) was reduced and the concentration of selection antibiotic (kanamycin) was increased from 0 mg/l to 100 mg/l. 35–40 stem segments from aseptic cultures of the hybrid aspen and another 10 segments served as controls for each construct.

The induction of adventitive shoots started after 3–4 passages. Adventitious shoots, induced after 2–4 weeks of culture, were excised continuously and transferred onto the same multiplication medium for propagation. Routinely multiplied shoots were rooted on the agar medium.

Table 2. The influence of different constructs on regeneration of adventitive shoots from stem segments

Construct *A. t.*	Regeneration of stem segments of hybrid aspen (%)
148	68
149	26
150	45
151	50
Control	0

Evaluation of transformants

Evaluation of transformants was performed by the histochemical assay of *GUS* activity in adventitive shoots of stem segments according to Jefferson (1987) in modification of Vitha et al. (1999). 57 shoots from transformed stem segments and 25 shoots from control segments were analyzed.

RESULTS AND DISCUSSION

Reproduction of mature aspen

Primary cultures were established from isolated winter buds from 24 years old trees. The research aim was to verify a possibility of multiplication by an *in vitro* method of mature stands with reduced reproduction ability by explant cultures. Our investigation was focused on the extension of explant bank with explants taken from elite individuals, on research of effectiveness of this multiplication (i.e. number of multiplied individuals per culture) and on probable differences between clones during multiplication. MS1 medium with higher concentrations of BAP (1.0 mg/l) and IBA (0.1 mg/l) seems to be suitable for induction of organogenesis just as for induction of organogenesis from leaf discs used for the establishment of cultures from juvenile material (Malá, Michalová 1993). When compared with induction of organogenesis on leaf discs, the cultures from aspen mature trees needed higher concentrations of glutamine and casein hydrolyzate in a concentration of 100 mg/l of nutrient media. Similarly, the medium with lower concentration of BAP (0.2 mg/l) and higher concentration of glutamine (100 mg/l) in agar medium appears to be suitable for multiplication. The number of induced shoots in the multiplication stage was high (20–30 shoots per culture) during one period, i.e. 4–6 weeks (Fig. 1). Out of the total number of sampled clones 80% of trees were prepared in the spring primary cultures. The losses during the establishment of primary cultures could be caused, among other things, by low vitality of primary explants. The experiment will be repeated in order to investigate whether the regeneration ability of the clones, the stabilization of which failed in the explant culture, is not lower. During two years 39 clones were stabilized in the explant bank and 1,000 plantlets were raised. During rooting and ac-

Fig. 1. Multiplication of aspen explants

Fig. 2. Acclimatization of aspen plantlets

climatization the losses were minimal – around 2% (Fig. 2). The plantlets grow on the outside bed of the experimental nursery (Fig. 3).

We believe that this method could be used for reproduction of cultivated and selected individuals. Unlike the cuttings the identical material for the establishment of testing plots can be obtained in a relatively short time. The multiplied material is supposed to be used in practice in the form of synthetic varieties with participation of minimally 25 clones.

Transformation

The *Agrobacterium* mediated gene transfer methods are widely used for transformation of woody plants (Chun 1994; Leplé et al. 1992). The genetic transformation, avoiding the sexual process, offers opportunities for creating new varieties with important agronomic traits otherwise unavailable, such as insect pest resistance, herbicide tolerance, improvement in growth, metabolism and wood quality, and the reduction in the expression of endogenous genes that encode undesirable traits. For example, transgenic trees offer good prospects for bioremediation of contaminated soils, while the use of sterile transgenic trees reduces the genetic impact of these populations on natural forests (Confalonieri et al. 2003).

The research aims of this work were to test the possibility of genetic transformation of hybrid aspen (*Populus tremula* × *P. tremuloides*) by the bacterial vector *Agrobacterium tumefaciens*.

For its regeneration ability aspen is a very promising woody species for genetic engineering. Hybrid aspen No. 5 from the Gene Bank of For-

Fig. 3. Plantlets of aspen growing on nursery beds

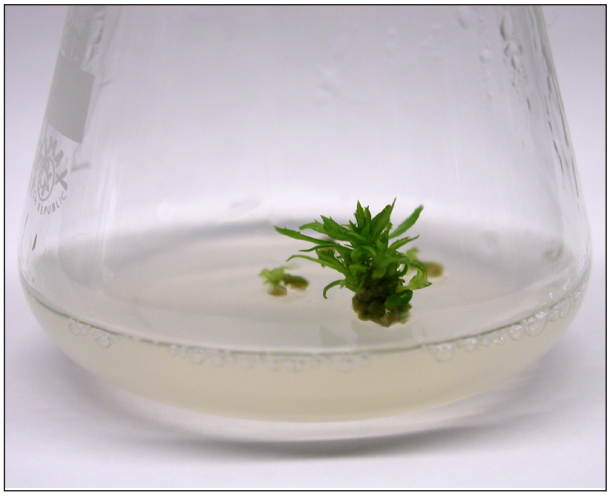

Fig. 4. Regeneration of adventitious shoots from stem segments on a kanamycin selection medium

estry and Game Management Research Institute was transformed with *Agrobacterium tumefaciens* LBA4404 using different gene constructs with four types of promoters. These constructs showed resistance to kanamycin. Several factors should be considered to improve transformation efficiency such as *Agrobacterium* strain, selectable marker system, use of a suitable antibiotic for suppression of *Agrobacterium tumefaciens* in genetic transformation. Our results confirm that the combination of timentin and augmentin is the best for elimination of this bacterium from infected aspen stem segments. Similar results were obtained by Tang et al. (2000). We used stem segments for transformation experiments.

The shoots regenerated 3–4 weeks after inoculation, mostly on the cut surface of the explants (Fig. 4). We conclude on the basis of regeneration processes on the selection medium that construct 148 (35S) and construct 151 (*rbcS*) were the most successful constructs for the transfer of genetic information in a plant model system (Table 2).

The formation of adventitious shoots of hybrid aspen on kanamycin-containing media showed the possible resistance of regenerated shoots to kanamycin. Evaluations of transformants were performed by the histochemical assay of *GUS*. The highest expression of gene *GUS* was observed in the transformed shoots of hybrid aspen by construct 148 (35S). Over 57% of the shoots showed strong positive *GUS* expression. Several transformants were selected for further analysis, and all of them showed kanamycin resistance as reflected by the high ability of rooting in the presence of kanamycin. Similar results were reported by Tzfira et al. (1997). The transgenic origin of the plants obtained during this study will be confirmed by PCR analyses.

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Received for publication September 20, 2005
Accepted after corrections November 21, 2005

Mikropropagace topolu osiky: využití pro fytoremediace půd

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ABSTRAKT: Kontaminace půd toxickými látkami je vážným celosvětovým problémem. Jednou z možností jejich regenerací je využití fytoremediací. Z hlediska využití dřevin je velmi perspektivní topol osika. Prvním cílem práce bylo vybrat a namnožit elitní stromy topolu osiky a hybridní osiky rostoucí na provenienčních plochách v Krušných horách metodou *in vitro*. Z vybraných 24 let starých stromů osiky byly odebrány větve s dormantními pupeny. Po sterilizaci byly extirpovány vzrostné vrcholy a přeneseny do nutričního média. Pro indukci organogeneze se osvědčilo MS médium s vyšší koncentrací BAP (1,0 mg/l) a IBA (0,1 mg/l). Pro multiplikaci se osvědčilo agarové médium s nižší koncentrací BAP (0,2 mg/l) a s vyšší koncentrací glutaminu (100 mg/l). Na jednu vícevrcholovou kulturu připadl vysoký počet adventivních výhonků (20–30). V průběhu zakořeňování a aklimatizace byly ztráty minimální (okolo 2 %). V současné době rostou výpěstky na venkovních plochách v pokusné školce. V genetické bance explantátů je inventarizováno 25 klonů. Druhým cílem práce bylo standardizovat efektivní postup transformace hybridní osiky k posouzení možností zavedení genů zvyšujících fytoremediační schopnosti. Pro zvýšení efektivnosti transformace stonkových segmentů osiky byly testovány čtyři konstrukty A*grobacteria tumefaciens* s různými promotory. Všechny konstrukty nesly modelové geny pro rezistenci na kanamycin a markerový gen *GUS*. Nejvhodnější je konstrukt 148 s promotorem CAMV 35S, který byl vybrán na základě aktivity markerového genu *GUS*.

Klíčová slova: topol osika; mikropropagace; *Agrobacterium tumefaciens*; fytoremediace

Kontaminace půd toxickými spady představují v České republice závažný environmentální problém. Většina používaných remediačních metod neposkytuje přijatelná řešení. Pro likvidaci environmentálního znečištění se začíná vyvíjet nový přístup, označovaný jako fytoremediace. Využívají se speciálně selektované nebo geneticky upravené rostliny, které mají zvýšenou schopnost efektivně restaurovat a stabilizovat kontaminované lokality. Optimálními rostlinami pro účely fytoremediace jsou druhy topolů osik (*Populus* spp.), které účinně absorbují, hromadí, uchovávají a degradují látky znečišťující prostředí, jako jsou těžké kovy, rezidua pesticidů a jiné kontaminující látky.

Hlavním cílem studie bylo ověřit, zda je možné mikropropagovat vybrané elitní dospělé stromy topolu osiky (*Populus tremula*) a mezidruhového hybridu topolu osiky a kanadského topolu osikovitého (*P. tremula* × *P. tremuloides*), které rostou v kontaminovaných oblastech. Primární kultury byly založeny z 24 let starých jedinců z provenienčních testovacích ploch. Dormantní pupeny byly sbírány na jaře. Po sterilizaci byl z dormantních pupenů extirpován vzrostný vrchol a umístěn na vhodná živná média (modifikace média Murashige-Skoog 1962). Pro indukci organogeneze se osvědčilo MS1 médium s vyšší koncentrací BAP (1,0 mg/l) a IBA (0,1 mg/l). Pro multiplikaci se osvědčilo agarové MS2 médium s nižší koncentrací BAP (0,2 mg/l) a vyšší koncentrací glutaminu (100 mg/l) (tab. 1). Zakládání primárních kultur je kritickou fází mikropropagace, kdy dochází k největším ztrátám především v důsledku neúspěšné sterilizace dormantního pupenu. Přesto se podařilo reprodukovat 80 % donorových jedinců. V průběhu dvou až pěti měsíců docházelo k indukci adventivních pupenů na primárním explantátu a k vytvoření vícevrcholové kultury, která představovala průměrně 20–30 adventivních výhonů (obr. 1). Tyto výhony pak byly využity k další multiplikaci nebo k dopěstování kompletních rostlin. Zakořeňování a aklimatizace probíhala při dodržení standardního postupu bez významných ztrát (do 2 %) (obr. 2). V průběhu dvou let bylo v explantátové bance stabilizováno 25 klonů a dopěstováno 1 000 výpěstků *in vitro*. Rostliny rostou na venkovním záhonu i na pokusných plochách (obr. 3). Tyto výsledky nás vedou k závěru, že zvolená metodika mikropropagace je vhodná a velmi efektivní pro namnožení dospělých stromů osiky.

Druhým cílem práce byl výzkum možností standardizace a účinné transformace hybridu topolu osiky a kanadského topolu osikovitého, aby mohly být

v blízké budoucnosti produkovány transformované hybridy, nesoucí geny determinující jejich zvýšenou schopnost fytoremediace. Pro tyto transformace stonkových segmentů hybridu byly použity čtyři genové konstrukty *Agrobacterium tumefaciens*, obsahující různé promotory (nesené gen *NPTII* kódující rezistenci vůči kanamycinu a gen *GUS* odpovědný za syntézu glukuronidasy umožňující fluorometrickou identifikaci). Promotory vhodné pro transformace byly vybrány na základě zkoušky aktivity *GUS* genu. Nejvyšší aktivitu vykazovaly transformanty obsahující konstrukt 148 kontrolovaný promotorem CAMV 35S (tab. 2, obr. 4).

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