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Callus Induction and Haploid Plant Regeneration from Anther Culture of Two Poplar Species

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Summary

Anthers from 5 different clones of *Populus nigra* and *Populus deltoides* were isolated from dormant flower buds before catkin elongation and exposition, in order to establish haploid and doubled haploid poplar plants in vitro. Isolated anthers were cold treated and placed on MS medium supplemented with 2,4-D (0.5 mg/l to 2.0 mg/l) and Kinetin (0.1 mg/l to 1.0 mg/l). The optimal hormone concentration for callus induction was 1.0 mg/l of 2,4-D and 0.1 mg/l of Kinetin. The callus initiation response of the genotypes ranged from 24% to 75%. The genotypes with the best callus initiation response were *P. nigra* N-90 (59%) and *P. deltoides* D-29 (75%). Calli were subcultured on MS or WPM medium supplemented with BA (0 mg/l to 2.5 mg/l) and NAA (0 mg/l to 0.2 mg/l) for plant regeneration. The highest shoot regeneration frequency (79%) was obtained after 2 subcultures on MS medium supplemented with BA (1.0 mg/l) and NAA (0.2 mg/l), and on WPM medium supplemented with BA (2.5 mg/l). The rate of shoot regeneration and number of shoots/calli ranged from 4% to 79% and 1 to 9, respectively.

Key words: Androgenesis, *Populus*.

FDC: 165.442; 176.1 *Populus nigra;* 176.1 *Populus deltoides.*

Abbreviations

MS: Murashige and Skoog; WPM: Woody Plant Medium; 2,4-D: 2,4-dichlorophenoxyacetic acid; BA: benzyladenine; NAA: naphthaleneacetic acid.

Introduction

In most tree species recurrent inbreeding to increase homozygousity is not successful due to long generation cycles, high initial levels of heterozygosity and inbreeding depression

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caused by the expression of recessive deleterious/lethal genes (STOEHR and ZSUFFA, 1990). Poplar (*Populus*) species are dioecious; staminate and pistillate flowers are born on different plants. Since they are dioecious, homozygous lines can only be obtained through Sib-mating. However, the long juvenile phase and many generations required for inbreeding via Sib-mating makes the production of homozygous lines impractical (HO and RAJ, 1985). One way of overcoming this difficulty and obtaining homozygous plants is to culture haploid plants, and perhaps the easiest way of obtaining these is by pollen and anther culture (UDDIN et al., 1988). Anther culture in *Populus* was first reported by WANG et al. (1975) who obtained haploid plants via organogenesis from pollen callus. Since then, successful plant regeneration from anther culture of various *Populus* species and interspecific hybrids has been reported (HO and RAJ, 1985; UDDIN et al., 1988; ZHU et al., 1980; STOEHR and ZSUFFA, 1990; BALDURSSON et al., 1993), but the rates of haploid plant production were low due to the very low frequency of haploid callus initiation (30%) from the microspores. The rate of shoot regeneration from haploid callus was reported to be high enough (up to 80%) in most poplar species. Consequently, the relatively low rate of callus initiation seemed to be the critical point for the in vitro androgenesis of poplar species.

Therefore our objective was to improve the rate of haploid plant regeneration through increasing the rate of callus initiation and sustaining shoot regeneration frequency at least at a level comparable to recent reports (80%, STOEHR and ZSUFFA, 1990; BALDURSSON et al., 1993).

Materials and Methods

Plant material and pretreatment

Branches with swelling buds were collected from 5 different genotypes of 2 poplar species (*Populus nigra* L. and *Populus deltoides* MARSH.) at Sárvár Research Station, Hungarian Forest Research Institute at the end of March, 1993. The name and description of the clones are as follows: N-89, *P. nigra x P. nigra* 418-1; N-90, *P. nigra x P. nigra* 418-2; D-29, *P. deltoides* cv. Illinois 29; D-30, *P. deltoides* cv. Illinois 30; D-36, *P. deltoides* cv. Illinois 36.

During the pretreatment, branches were stored in plastic bags and kept at 4 °C ± 1.0 °C for 8 to 14 days. The developmental stage of the pollen grains was determined with a stereomicroscope after staining with 2% acetocarmine solution. When microspore mother cells were in the tetrad stage or in transition from tetrad to mononucleate microspore stage, flower buds were separated from the branches, dipped in 70% ethanol for several minutes, and placed in 3% $CaHClO_2$ and agitated for 20 min. Approximately 250 to 300 anthers were isolated and incubated in each Petri dish, and at least 10 dishes were used for each incubation per clone. The total number of anthers isolated was 26000.

Culture media and conditions

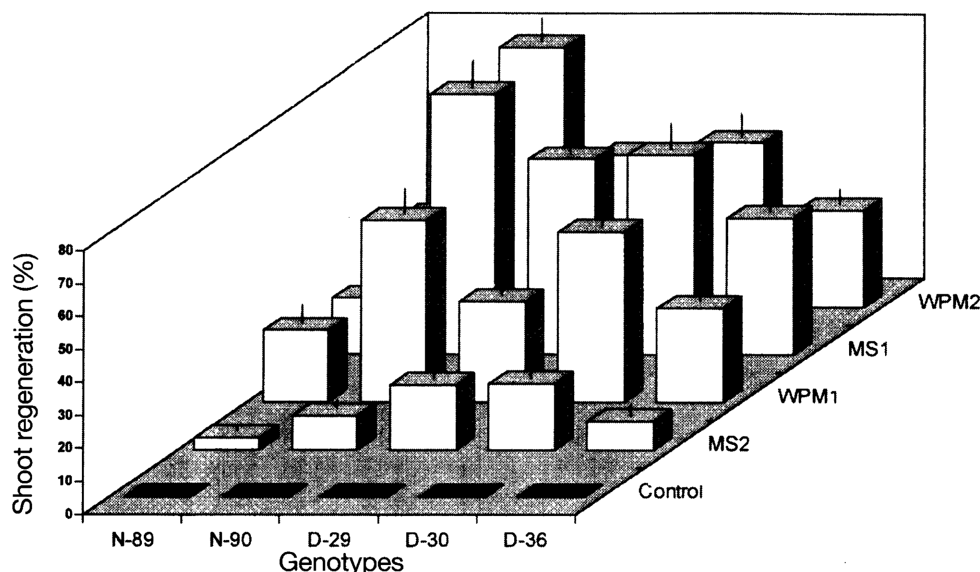
For callus induction we used MS (MURASHIGE and SKOOG, 1962) basal medium supplemented with different concentrations of 2,4-D (0.5 mg/l to 2.0 mg/l) and Kinetin (0.1 mg/l to 1.0 mg/l), with 2% sucrose. Anther cultures were incubated in the dark for 6 to 7 weeks at 25 °C. For callus proliferation, callus clumps (1 cm in diameter) were subcultured on the same medium and kept in the dark at 25 °C.

Callus clumps that developed on the anthers were transferred to either MS or WPM (LLOYD and MCCOWN, 1980) medium supplemented with various concentrations of BA (0.1 mg/l to 1.0 mg/l), and NAA (0 mg/l to 0.2 mg/l), containing 2.5% sucrose to induce organogenesis and were incubated in the light (40 µm $m^{-2}s^{-1}$) at 23 °C. Calli with or without shoots were transferred monthly onto fresh medium.

Table 1. – Effect of genotype on callus initiation in anther culture of 2 poplar species.

Species	Genotype	Treatment	Total number of isolated anthers	Anther produced callus	
			no.	no.	%
P. nigra	N-89	MS+2,4-D+Kinetin	8219	3899	47.44
		MS	1351	0	0
	N-90	MS+2,4-D+Kinetin	5384	3199	59.42
		MS	1126	0	0
P. deltoides	D-30	MS+2,4-D+Kinetin	2268	535	23.59
		MS	767	0	0
	D-36	MS+2,4-D+Kinetin	1793	931	51.92
		MS	905	0	0
	D-29	MS+2,4-D+Kinetin	3100	2316	74.71
		MS	1044	0	0
Total	P. nigra	MS+2,4-D+Kinetin	13603	7098	52.18*
		MS	2477	0	0
	P. deltoides	MS+2,4-D+Kinetin	7161	3782	52.81*
		MS	2716	0	0

*) weighted mean value



Bars represent SE

[1]) WPM1 and MS1 was supplemented with BA (1.0 mg/l) and NAA (0.2 mg/l); WPM2 and MS2 was supplemented with BA (2.5 mg/l); Control was MS without any hormones.

Figure 1. – Effect of genotype and culture media on shoot regeneration of *P. nigra* and *P. deltoides*[1]).

When shoots regenerated from calli reached 1 cm to 2 cm, they were separated from callus clumps, transferred to root induction medium and were maintained in the light. Rooting medium was hormone-free WPM supplemented with 3% sucrose. After rooting (3 to 4 weeks), plantlets were potted in a 1:1 mixture of perlite and peat, and covered with a plastic bag. They were acclimatized in a growth chamber for at least 2 to 3 weeks. After 1 week, the initial level of relative humidity (80%) was decreased gradually by punching holes in the plastic bag. At the end of the acclimatization period, plantlets were transferred to the greenhouse.

All media were solidified with 0.7% agar and pH was adjusted to 5.8 prior to autoclaving.

Cytological analysis

Chromosome number was examined in the root-tip cells and young leaves of regenerated plants. Both were pretreated in ice-water for 24 hours, fixed in a 3:1 ethanol:acetic acid fixative solution for 24 hours, and then hydrolyzed in 1N HCl at 60 °C for 12 min.

Root tips and leaves were stained by Feulgen method (DARLINGTON, 1960) before being squashed in 45% propionic acid orcein and than studied by a Leitz DMRB microscope.

Results

Callus initiation

Callus initiation from anthers was observed 2 weeks after incubation. Calli developed unorganized, parenchymatous white tissue in both species. The frequency of callus initiation was dependent on genotype, and hormone combination and concentration (*Table 1*).

The callusing response of *P. nigra* and *P. deltoides* (*Table 1*) was 52% and 53%, respectively when cultured on medium containing growth regulators (2,4-D + Kinetin), while without hormones, there was no response. The frequency of callus induction of genotypes ranged from 24% to 75%. The most responsive clones were N-90 (59%) in *P. nigra* and D-29 (75%) in *P. deltoides*.

For the most genotypes, the optimal growth regulator concentration was 1.0 mg/l 2,4-D and 0.1 mg/l Kinetin. There was no substantial difference between the 2 species regarding the mean values. In addition, callus induction decreased in each genotype with higher concentrations of 2,4-D (2.0 mg/l) and Kinetin (1.0 mg/l).

Shoot regeneration

When exposed to light, calli turned green, greenish-red, or yellow. Forty to 50 days after transplantation, shoot differentiation was observed on the calli. The frequency of regeneration varied with genotypes, media and growth regulators (*Fig. 1, Table 2*).

Without plant growth regulators, only root formation occured in both species (*Table 2*). For shoot regeneration, the combination of BA and NAA was more effective than BA alone. When used alone, the effect of BA was dependent on the basal medium. The frequency was higher in each genotype, when BA was added to WPM. The highest values (79%) were obtained after 2 subcultures on MS medium supplemented with BA (1.0 mg/l) and NAA (0.2 mg/l), and on WPM medium supplemented with BA at 2.5 mg/l (*Table 2*).

The rate of shoot regeneration and number of shoots/calli ranged from 4% to 79% and 1 to 9, respectively (*Fig. 1, 2*). With *P. nigra* clones, the average number of shoots regenerated from 1 callus clump was nearly twice as those obtained with *P. deltoides*.

Regeneration frequency on WPM basal medium was 100% higher than on MS medium in *P. nigra*, while no substantial difference between the 2 media could be detected with *P. deltoides* callus cultures. Except for D-29, the rate of shoot regeneration was higher on WPM basal medium in each genotype. The highest rate of shoot regeneration (79%) and number of shoots/callus (9 shoots) were both observed in *P. nigra* N-90 clone (*Fig. 1*).

Cytological analysis

The number of chromosomes was counted in 50 plants (10 from each genotypes). Eighty % of these had the haploid

chromosome number (n = 19) and 20% were diploid. Haploid plantlets could be identified by slower development and hard-to-root feature.

Discussion

Of the 2 steps of haploid plant regeneration from anthers (callus initiation and regeneration), the first step seems to be the limiting factor. While the frequency of shoot regeneration was as high as 80%, the rate of haploid callus initiation from anthers was found 74% in *P. deltoides* (UDDIN et al., 1988), 15% in *P. deltoides* and *P. nigra* (HO et al., 1983), 19% in *P. trichocarpa* (BALDURSSON et al., 1993), 28% in *P. maximowiczii* (STOEHR and ZSUFFA, 1990) and 5% in *P. maximowiczii x deltoides* (HO and RAJ, 1985).

In our experiments, both species showed high level of callus initiation (53% in *P. nigra;* 51% in *P. deltoides*) with a maximum frequency of 75% in N-90 (*P. nigra*) and D-30 (*P. deltoides*) clones. This achievement is in accordance with that of UDDIN et al. (1988), who described anther response of 74% in *P. deltoides,* however we used another genotype and lower hormone concentrations.

In the case of *P. nigra,* our results on N-90 represent a 5-fold increase in callus initiation (74%) compared to 15% HO et al. (1983) in N-166 anther culture. HO et al. may had a lower hormone concentration and a highly responsive genotype (N-90). 2,4-D and Kinetin were necessary for callus initiation in each genotype which confirmed previous results with *P. deltoides* and *P. nigra* (HO et al., 1983; UDDIN et al., 1988).

The efficiency of shoot regeneration varied with the genotype, medium and growth regulator. The genotype *P. nigra* N-90 showed high potential of shoot regeneration (79%), that was achieved also on several *Populus* species (BALDURSSON et al.,

Table 2. – Effect of genotype and basic medium on shoot regeneration of anther derived callus in 2 poplar species.

Species	Genotype	Medium	Total number of isolated calli	Shoot regeneration		Shoot no./calli		
				total no. calli that produced shoots	%	Min.	Mean ±SE	Max.
			no.	no.	%	no.	no.	no.
P. nigra	N-89	MS	457	52	11.38	3	3.0±0.3	4.5
		WPM	400	95	23.75	1	2.0±0.2	5.5
		Control	521	0	0	0	0.	0*
	N-90	MS	345	151	43.77	2	4.5±0.4	9
		WPM	736	482	65.49	2.5	4.5±0.4	8
		Control	432	0	0	0	0	0
	Total	MS	802	203	25.31	2.6	3.6±0.3	6.4
		WPM	1136	577	50.79	1.9	3.6±0.3	7.1
		Control	953	0	0	0	0	0
P. deltoides	D-30	MS	119	54	45.38	2	2.0±0.2	5
		WPM	114	58	50.87	2	3.5±0.3	7
		Control	221	0	0	0	0	0
	D-36	MS	134	37	27.61	0.5	1.0±0.1	2
		WPM	121	35	28.92	1	0	2
		Control	197	0	0	0	0	0
	D-29	MS	167	75	44.91	2	2.0±0.2	4.5
		WPM	118	47	39.83	2.5	2.0±0.2	4
		Control	137	0	0	0	0	0
	Total**	MS	420	166	39.50	1.5	1.7±0.2	3.8
		WPM	353	140	39.66	1.8	1.8±0.2	4.2
		Control	555	0	0	0	0	0

*) root formation only
**) weighted mean values

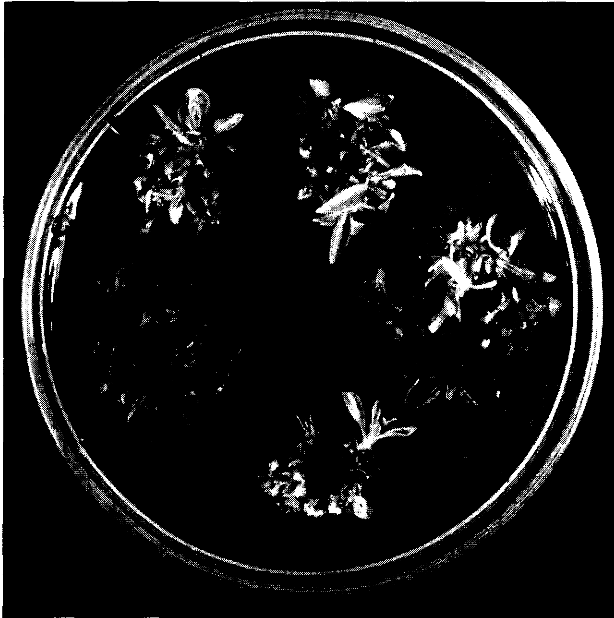


Figure 2. – Shoot regeneration in callus culture of *P. nigra* on WPM supplemented with 2.5 mg/l BA.

1993; UDDIN et al., 1988). Except for one case, WPM was more effective than MS with each genotype, which agrees well with other observations (UDDIN et al., 1988). When added together, BA and NAA showed greater effect than BA alone, while only root formation could be observed without growth regulator supplementation.

According to various isolation stages in microsporogenesis for haploid callus induction in poplar species, we isolated anthers from dormant buds, when the microspores were in transition from the tetrad to the uninucleate stage. Contrary to UDDIN et al. (1988) who observed low callus production on the anthers isolated in earlier stages, we obtained efficient callus induction in our experiments.

To conclude, a high callus initiation frequency of 53% and 51% was achieved in *P. nigra* and *P. deltoides* anther culture, respectively. Compared to previously reported results, the best genotypes (N-90 and D-30) showed at least equal or higher callus induction rate, while maintaining the best shoot regeneration capacity. These two clones seem to be suitable genetic material for both haploid and heterosis breeding work in poplar.

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