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In vitro Propagation of Ornamental Hybrids of *Populus* L

Anna Alekseevna Erst[1], Viktor Timofeevich Bakulin[2], Andrey Sergeevich Erst[3], Alexander Alexandrovich Kuznetsov[4], and Eugene Zhumanovich Bayahmetov[5]

[1]Central Siberian Botanical Garden of the Siberian Branch of Russian Academy of Sciences, Zolotodolinskaya str., 101; 630090, Novosibirsk, Russia;
Altai State University, Lenina st. 61; 656049, Barnaul, Russia.
[2]Central Siberian Botanical Garden of the Siberian Branch of Russian Academy of Sciences, Zolotodolinskaya str., 101; 630090, Novosibirsk, Russia.
[3]National Research Tomsk State University, Laboratory of Biodiversity and Ecology, prospekt Lenina, 36; 634050, Tomsk, Russia;
Central Siberian Botanical Garden of the Siberian Branch of Russian Academy of Sciences, Zolotodolinskaya str., 101; 630090, Novosibirsk, Russia.
[4]National Research Tomsk State University, Laboratory of Biogeochemical and of Remote Methods of Monitoring Environmental, prospekt Lenina, 36; 634050, Tomsk, Russia.
5National Research Tomsk State University, Laboratory of Biodiversity and Ecology, prospekt Lenina, 36; 634050, Tomsk, Russia
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To propagate two hybrids of the genus Populus (Siberian silver poplar #12 and «Strojnyi» poplar #18/4) and two species (P.alba and P.nigra) the culture technique for plant tissues and organs in vitro was used. The following ways of morphogenesis in vitro were revealed: direct (adventitious shoot-forming, development of axillary buds) and indirect (bud formation). The dependence of explants response on the maternal plant genotype was shown. The best morphogenic response was obtained for Populusnigra and P.alba (72% to 100%). The key factor for the shoots developing from the callus culture is the introduction of gibberellic acid into the medium. At the stage of shoot multiplication the best media are Murashige, Skoog and Gamborg, Eveleigh supplemented by 6-benzylaminopurine 1μM and gibberellic acid 5μM. The microshoots obtained by both direct and indirect morphogenesis were successfully rooted on the double reduced Murashige, Skoog medium. To propagate and preserve the unique poplar hybrids (Siberian silver poplar #12 and «Strojnyi»poplar #18/4) we recommend the direct morphogenesis as the most efficient in its parameters and providing better genetic stability of the regenerated plants.

Key words: Populus, hybrids, in vitro propagation, morphogenesis.

The forest-forming tree species grown on an industrial scale and in the urban environment are an important biological resource playing a significant role in the economics and ecology of ground ecosystems. They also carry a tremendous aesthetic and spiritual load[1].

The genus Populus L. (poplar and aspen) comprises about 30 species growing in the moderate zone of the Northern hemisphere. Since it grows fast and easily vegetates, the poplar is widely used in many countries to green up the populated areas, for protective and industrial planted afforestation[2]. However, it is noted that the wild poplar species feature some limitations: a weak vegetative propagation (with hardwood cuttings), susceptibility to heart rot, leaf rust, etc.,

* To whom all correspondence should be addressed.

which prevents the species from meeting the production criteria. The necessary breeding of the poplar is performed by various methods of selection and vegetative propagation of economic spontaneous forms and artificial exposure of the genotype followed by the selection of prospective forms (interspecific hybridization, experimental polyploidy and mutagenesis).

In the long-term selection in different countries, the total number of the elite poplar forms and breeds significantly exceeds the number of species described in the nature. However, in Siberia the poplar breeding was first performed by BakulinV.T.[3]in the Central Siberian Botanical Garden of Siberian Branch of the USSR Academy of Sciences (CSBG). As a result of this work a significant breed reference of fast-growing poplars was prepared for Novosibirsk region. The poplar hybrids created by BakulinV.T. serve as a basis for planted growing in the southern areas of West Siberia[4].

One of the factors hindering the quick introduction of the promising forms and hybrids of the tree plants is that in order to estimate their prospects it is necessary to study the grown up trees, which are difficult to reproduce with the traditional methods of vegetative propagation. The modern biotechnologies are the alternative for the existing methods of propagation and are able to solve a number of problems, as they facilitate the process of breeding as well as accelerate the mass propagation of the prospective genotypes and creation of the bank of these plants for their further involving into the breeding process. High response, reproducibility and stability of the culture in vitro are the necessary features for the requirements of the forest biotechnology.

The genus Populus is a model system both in the field of the tree plants biotechnology and in the sphere of obtaining the trees with the improved characteristics[5]. The genus is the subject of nearly half of all the works concerning the genetic transformation of the tree breeds[6]. However, the success of these technologies depends directly on the developing of the effective protocols of in vitro propagation[7]. The species and hybrids of genus Populus propagate in the culture of plant tissues and organs from the various types of explants, i.e., buds, leaves, and roots[8-10]; from internodes[11,12]; from costa[13]; from primordia of pistillate aments[14], etc.

The present study considers the factors affecting the morphogenetic potential of the various types of the explants of two decorative hybrids (Siberian silver poplar #12 and «Strojnyi» poplar #18/4) and two species of poplar (P.alba and P.nigra) which have good prospects to be used in the urban plantations.

MATERIALS AND METHODS

Plant material

The objects of the study are two fast-growing winter-hardy decorative poplar hybrids obtained in the CSBG and two wild poplar species. Siberian silver poplar #12 was obtained in 1980 by breeding white poplar (from the Ob river bottom land) with Bolle's poplar (P.alba×P.bolleana). It is a slim decorative tree with a straight trunk and narrow cylinder canopy. It is characterized by a very fast growth, especially in the trunk diameter. The breed is close to white poplar in its seasonal development rhythm. It reaches the height of 7.4 m by the age of 6, and 20.6 m, by the age of 20. The light-demanding tree is comparatively drought and gas resistant, it is not susceptible to rust. The rooting of the winter (hardwood) stem cuttings in the open ground can reach 65%[15].

«Strojniy» poplar #18/4 is obtained in 1981 by the open pollination of the polyploid form C_0 (P.nigra×P.pyramidalis). It is a decorative tree with a straight trunk and very narrow canopy. The canopy shape and long-staying green leaves make the tree especially attractive. It is a diploid (2n=38) with a small admixture of polyploid cells. It reaches the height of 8.4 m by the age of 7, and 20 m, by the age of 20. The tree features a high field resistance to rust and leaf blight. It propagates well with the hardwood stem cuttings[16].

P.alba and P.nigra are isolated in the natural plantations in the Ob river bottom land in the outskirts of Novosibirsk. Their age is about 25 years. Both species are very winter-hardy, ecologically adaptive, and fairly resistant to diseases and insect pests. At the same time, they propagate poorly with the hardwood stem cuttings, which hinders its wider use in forestry.
The 1-2-year-old shoots were cut from the young (5-15-year-old) trees of the second vegetative generation grown on the CSBG territory; the

shoots of P.alba and P.nigra were taken from the 25-year-old trees (in November). The initial explants were the axillary buds and young leaves obtained after shoot forcing. Two variants of the surface sterilization were tested: 1) 0.1% solution of sulfohlorantin (10 minutes), then 70% ethanol (3 seconds), and 0.1% solution of mercuric chloride (20 minutes); 2) 70% ethanol (3 seconds), then 0.1% solution of $HgCl_2$ (30 minutes). The sterilization done, the plant material was rinsed three times with sterile distilled water. The buds were cleared of all top scales and part of the leaves, leaving two small leaves of the deepest location, after that the explants were placed into the medium.

Cultivation medium

The various compositions of media were tested: MS (Murashige, Skoog)[17], W (Winton)[18], N6 (Simola)[19], B5 (Gamborg, Eveleigh)[20]. The media contained 3% sucrose and 0.6% agar (Difco, USA). The media were autoclaved at 121°C for 20 minutes, pH of the media were brought to 5.8. The mineral foundation was supplemented with various growth regulators and physiologically active additives (Tabl.). The explants were cultivated under the following conditions: photoperiod, 16/8 hours of light/darkness; illuminance, 2-3 kilolux; temperature, 24±1°C. The calluses were cultivated in thermostat at the temperature of 24±2°C in the dark.

For adaptation of the regenerated plants the transparent plastic containers filled with sphagnum moss were used.

Statistic analysis

All experiments were repeated 3-5 times. The statistical processing of the results was performed by calculating with the use of Microsoft Excel packet of statistical analysis. The tables show the arithmetic means and confidence intervals. The confidentiality of the estimated parameters was taken at the level of $P<0.05$[21].

The morphogenesis of the poplar explants in vitro was observed in the Center of collective use of CSBG SB RAS (Novosibirsk) on the Stereo Discovery V 12 microscope (Carl Zeiss, Germany).

RESALTS AND DISCUSSION

Direct organogenesis

The use of the poplar as a model object in the forest biotechnology resulted in the developing of a number of protocols of propagation for the species and hybrids of the genus. Whitehead and Giles[22] used the axillary buds for introduction into the poplar culture in vitro. The explants of P.nigra, P.deltiodes×P.nigra and P.yunnanesis were cultivated on the media of various compositions, with the shoots forming observed after 6-8 weeks. The same method was used for other poplar species[23]. The regeneration of the poplar plants from the meristems was obtained by Rutledge and Douglas[24] in order to dispose of a viral infection. Welander *et al.*[25] developed a technology of micropropagation of Populus×wilsocarpa, a highly decorative hybrid, also using the culture of axillary buds.

In our study we used the culture of axillary buds as one of the most efficient and genetically stable systems of micropropagation. The buds of the studied poplar hybrids and species were introduced into the culture in vitro in autumn (November). After two weeks of cultivation almost all buds responded on the MS medium supplemented with BAP 5 µM and CH 200 mg/l. On this medium the «Strojnyi»poplar# 18/4 and P.nigra developed the adventitious shoots at the bud base, whereas the Siberian silver poplar #12 and P.alba showed the development of the axillary buds. The difference in the morphogenic response is likely to be explained by the hybrids genesis. «Strojnyi»poplar # 18/4, the hybrid of P.nigra×P.pyramidalis, featured the same morphogenic response in the culture in vitro as P.nigra. Siberian silver poplar #12, the hybrid of P.alba×P.bolleana, had the same response as P.alba. The data once again corroborate the strict dependence of the morphogenic response of the explant on the genotype of the maternal plant. The largest number of the buds was developed with P.nigra and «Strojnyi»poplar # 18/4 as they formed a great amount of the adventitious buds (23.8 and 15.4, respectively) (Fig. 1,2).

The MS medium supplemented with BAP 5 µM and CH 200 mg/l proved to be the universal one for all studied hybrids and species at the stage of introduction of the buds into the culture in vitro, however, further cultivation of the explants on this medium affected the parameters of growth and development. Our data show that to initiate the adventitious buds and stimulate the development of axillary buds at the stage of introduction into

the culture in vitro BAP concentrations have to be high. For the further growth and development of the buds it is necessary to cultivate the explants on the media of the same composition but with 5-times less content of cytokinins.

Callus culture and in vitro shoot organogenesis

In 1934, Gautheret[26] obtained the poplar callus in the culture in vitro from the cambial tissue. Mathes[27] observed a long-existing callus obtained from a stem segment of P.tremuloides. Later a large part of the species and hybrids of the poplars were tested for their ability to form callus out of various types of the explants: internode, leafstalk, leaf, and root[23,28,29]. Beside the genotype of the plant and the explant type the following factors influence the callus-formation and the further regeneration of the shoots through the callus culture: the mineral composition of the medium, concentration and combination of the growth regulators, especially auxins, and the cultivating conditions.

To obtain the calluses the young leaves were divided into several parts and placed with their adaxial side over the media. Callusogenesis in tested media was observed 20 days after the cultivation in an incubator in the dark conditions. All the studied species and hybrids of poplar were capable of forming calluses; tissue dedifferentiation was primarily in the areas of cutting. Callusogenesis frequency on MS and N6 media was 100%, and approximately 60% on the medium W. Using the MS supplemented with 2,4-D proved to be effective for the induction of callusogenesis and many other representatives of the genus Populus: *P. angustifolia, P. balsamifera, P. deltoides*[12].

Regeneration of shoots in the studied hybrids and poplar species, as in the photoperiod and in the dark on callusogenesis initiation media, did not occur. It is known that for the initiation of meristematic centers and shoot development,

Table 1. Composition of nutrient medium

Components	B5		MS											N6			W	
	1	2	1	2	3	4	5	6	7	8	9	10	11	1	2	3	1	2
Macronutrients	+	+	+	+	+	+	+	+	½	+	+	½	½	+	+	+	+	+
Micronutriens	+	+	+	+	+	+	+	+	½	+	+	½	½	+	+	+	+	+
Vitamins	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
BAP 1 µM	+		+	+	+													+
5 µM		+				+												
7 µM							+											
8 µM								+										
10 µM									+									
KN 1,25 µM											+							
2,5 µM "														+				
5 µM																	+	
ZN 25 µM															+	+		
GA_3 5 µM					+			+	+							+		
NAA 0.3 µM							+											
IBA 5 µM													+					
IAA 1.25 µM											+							
2,4-D 0.2 µM																	+	
5 µM			+	+														
10 µM														+				
CH 200 mg/l						+			+									
1000 mg/l			+	+														
AA 100 mg/l								+	+				+					
CW 5%				+						+								

BAP - 6-benzylaminopurine; KN-kinetin; ZN-zeatin; GA3- gibberellicacid; NAA- 1-Naphthylacetic acid; IBA-Indole-3-butyric acid; IAA - 3-Indoleacetic acid; 2,4-D - 2,4-dichlorophenoxyacetic acid; CH - caseinhydrolysate; AA - ascorbicacid; CW - coconutwater.

exogenous cytokinin growth regulators are usually required. A wide range of cytokinins (BAP, ZN, TDZ (thidiazuron), KN, 2-iP(2-isopentyl adenine)) induce shoot formation in representatives of g. Populus. Optimal concentrations vary for each cytokinin[28,29,30]. For example, for ZN and BAP, the greatest morphogenic response in poplars explants was observed at a concentration of 1 - 4μM, and 1 - 5 μM respectively[29]. TDZ causes morphogenic response at low concentrations (0.05 - 2μM) while kinetin and 2-iP induce weak morphogenic response in explants poplar. High levels of BAP, ZN and TDZ cause the formation of leaves (without the formation of shoots) and vitrification of tissues in representatives of g. Populus[31].

To study the effect of growth regulators on the morphogenesis in vitro, calli were transplanted to the shoot regeneration medium: MS supplemented with BAP-1, 5, 7, 8, and 10 μM, MS supplemented with equal concentrations of KN and 1.25 μM IAA, hormone-free MS supplemented with 5% CW, N6 + ZN 25 μM, N6 + ZN μM + GA3 5 μM and W + BAP 1 μM in the conditions of photoperiod. In the light, they attained green and green-red color. On media W +

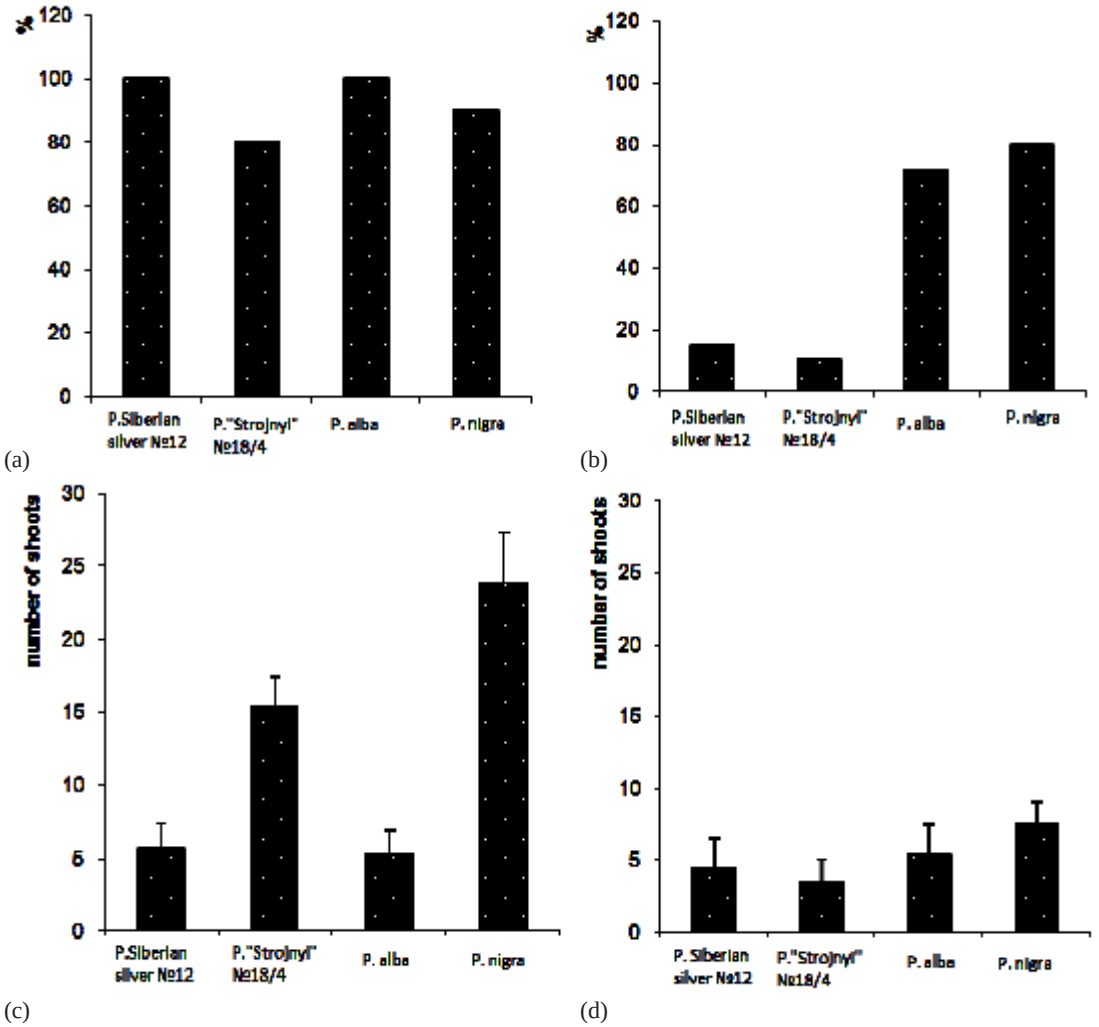

Fig. 1. Morphogenicresponseof explants of poplar´s speciesand hybrids in *in vitro* culture, n=10.A. % of regeneration by directmorphogenesis on MS+BAP 5 μM + CG 200 mg/l. B. % of regeneration by indirect morphogenesis on N6+ZN 25 μM + GA3 5 μM. C. Number of shoots by directmorphogenesis on MS+BAP 5 μM + CG 200 mg/l. D. Number of shoots by indirect morphogenesis on N6+ZN 25 μM + GA3 5 μM

BAP 1 µM, Siberian silver poplar #12 demonstrated inception of meristematic centers, but further development of shoots on this medium did not occur. In the media containing coconut water, only rhizogenesiswas observed.

A stable morphogenic callus culture of all studied hybrids and poplar species was obtained on N6 + ZN 25 µM medium. This medium allows longstanding maintenance of callus culture without loss of morphogens. We have noted that the further development and growth of shoots occurred only when added to the medium with ZN, GA_3 5 µM. Gibberellins are used mostly to pull the shortened shoots, such as *P.*×*euramericana*[11]. The role of $GA_3$is manifested in a synergistic effect with other growth regulators, including cytokinins. It is

Fig. 2. Clonal micropropagation of hybrids of Populus. A. Development ofaxillary buds of P. Siberian silver #12 on MS+BAP 5 µM+CG 200 mg/l. B. Nonmorphogeniccallus of P. Siberian silver #12 on MS+2,4-D 5 µM+BAP 1 µM+CG 1000 mg/l. C. Morphogenic callus and regeneration of shoots of P. Siberian silver #12 on N6+ZN 25 µM+GA3 5 µM. D. Development ofadventitious budsat the base ofthe explant of P. «Strojnyi» #18/4 on MS+BAP 5 µM+CG 200 mg/l. E. Nonmorphogeniccallus of P. «Strojnyi» !18/4 on MS+2,4-D 5 µM+BAP 1 µM+CG 1000 mg/l. F. Morphogenic callus and regeneration of shoots of P. «Strojnyi» #18/4 on N6+ZN 25 µM+GA3 5 µM. G. Multiplication of shoots of P. Siberian silver #12 on MS+BAP 1 µM. H. Rhizogenesis in vitro of P. Siberian silver #12 on 1/2MS. I. Adapted plants of P. Siberian silver #12

known that treatment of plants with gibberellins greatly increases the mitotic activity[32]. For example, morphogenic response in Betulaschmidtiiwas obtained only when initiated to the GA_3 culturing medium[33].

The intensity of the shoot regeneration for Siberian silver poplar #12 and «Strojniy» poplar #18/4 hybrids was 15 and 10%, respectively, for the species P. alba and P. nigra, this figure was significantly higher (72 and 80%, respectively). Low frequency of the shoot regeneration of poplar hybrids may be explained as by hybridogeneous nature of these forms, and long-term cultivation in the medium with auxins. In the paper by P. Maheshwari and I. Kovalchuk[12] it was noted that only a short period of the explants cultivation (6-10 days) on the medium for callusogenesis contributes to further high rates of shoot regeneration. The number of shoots per callus was also species-specific, the largest number of shoots was developed in P. nigra (see Fig.1,2).

Selecting a method of cultivation of plants in vitro is fundamental for the development of breeding protocols for forms, varieties, hybrids and unique genotypes in relation to the possibility of somaclonal changes. Such factors as the source of an explant, the presence and concentration of growth regulators can lead to somaclonal variations in the plants. So, variations in leaves, roots sprout structure, branching and growth rate were described for the representatives of g. Populus[34]. Somaclonal variations are often associated with the induction of callusogenesis, which is not obligatory for the micropropagation of the studied hybrids and poplar species and, thus, the methods of cultivation (meristems axillary cultures) reduces the risk of genetic variations.

Multiplication and rhizogenesis of shoots

For micropropagation of shoots obtained by direct and indirect ways, MS and B5 media were used. It was reported earlier that the use of MS medium was effective for such representatives of the genus Populus as *P.deltoides, P.×euramericana*[9,11]. Our studies show that the use of high concentrations of BAP (greater than 5 μ“) on both MS and B5 media affects negatively all investigated poplars, except for P. nigra. The media supplemented with BAP 1 μ“ GA3 and 5 μ“ (coefficient of multiplication - from 5 to 16 shoots/explant for different species and hybrids) were optimal for the development and growth of shoots. Siberian silver poplar #12 shoots were also characterized by good growth on the MS medium, supplemented with KN 1,25 μ“ and IAA 1,25 μ“, the coefficient of multiplication was 8,4 ± 1,2 shoots/explant.

Some researchers note that auxins are required to initiate rooting. So, IBA and NAA were identified as the most effective auxins for poplar microshoots rooting[28]. Typically, rooting of explants in vitro occurs in 3 weeks of culturing at low concentrations of auxin. ½ MS and ½ MS, supplemented with 5μM IBM media were used for microshoots rooting in our studies. All representatives of the genus Populus studied have shown the ability to root in these environments. Rhizogenesis was faster on the medium with IBA, but the development of callus was also observed. Therefore, hormone-free medium was selected as an optimum for rhizogenesis stage.

An analysis of the external morphology of regenerated plants, obtained by direct and indirect ways, showed no differences.

Adaptation of micropropagated plants

Sphagnum moss was used to adapt the resulting plants. Adaptation period took 3 weeks. Then, the plants were planted in a soil mixture and grown under greenhouse conditions. The yield of adapted viable plant material was 90%.

Thus, the direct (adventive shoot formation, the development of axillary buds) and indirect (gemmogenesis) ways of morphogenesis for hybrids and poplar species were identified. P. nigra and P. alba are characterized by the highest flexibility, showing its high ability for morphogenesis in culture in vitro. For development following the direct way, a bud is to be cultivated on MS medium or B5 + BAP 5 μM + CH 200 mg/L, and after initiation of shoots, it is to be cultured in media with a low content of cytokinins. Optimum environment for indirect hemmogenesis from the leaf explants was N6 + ZN 25 μM + GA_3 5 μM medium, where GA_3 plays a crucial role in the development of the initiated buds. Microshoots obtained directly and indirectly were successfully rooted on MS medium with a twice as reduced content of mineral elements.

To reduce the risk of somaclonal variations, when culturing hybrid poplar in vitro, we recommend using direct way of hemmogenesis

followed by the rooting of the obtained microshoots. This method of reproduction is also the most optimal in terms of efficiency of micropropagation, since it provides a high percentage of these hybrids morphogenesis (80 to 100%).

CONCLUSIONS

Thus,direct(adventive shoot formation, the development of axillary buds) andindirect (gemmogenesis) pathmorphogenesis of poplar´s hybridsandspecieswere observed. P. nigraandP. albawere characterized bythe highestductility,has shown itsability tomorphogenesisinin vitro culture.For development ofthe directpath buds hadto be cultivatedon a mediumMS +5µMBAP +CH200mg/ l, and after emplacement shoots cultured inmedia witha low content ofcytokinins. Optimum environment for indirect gemmogeneza of leaf explantswasN6 +ZN25µM+$GA_3$50 µM, with a crucialrole in the developmentof the budsplays GA_3. Obtained directly and indirectly by microshoots successfully were rootedon MS mediumwith a reducedcontent ofmineral elementstwiceandthenadaptedin sphagnummoss. Although not a comprehensive remedy for the difficulties associated with working with trees for biotechnological research, shoot cultures are a major and often essential tool. Shoot cultures provide tissues to begin manipulations and offer an effective avenue for moving plant materials from culture to testing or production. For a program beginning work on a selection of poplar, establishing the genotype in shoot culture will sensitize researchers to its idiosyncrasies in the microculture environment.

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