Organogenesis and Sonication-Assisted Agrobacterium-Mediated Transformation of Poplar Roots

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Abstract

Functional genomics along with genetic transformation and plant regeneration are used to identify genes of interest, comprising essential tools to obtain plants with desired characteristics. In this study, we described an organogenesis and a genetic transformation protocol for *Populus tremula x Populus alba* clone 717-1B4 using roots. In the organogenesis experiments the PGRs zeatin, BA and kinetin at different concentrations were evaluated. The effect of explant age was evaluated and at 30 days “old” roots (6 months old) showed a higher regeneration rate when compared to “young” roots (2 months old). In the genetic transformation experiments kanamycin concentration, the use of sonication (SAAT), co-cultivation period and explant age were evaluated. Sonication positively affected transformation, while co-cultivation time did not interfere. Regarding explant age, no statistical differences were observed. Quantitative real-time PCR (qPCR) data showed that among the six transgenic plants evaluated, two presented two copies and the others only one copy of the gene. In this study, efficient protocols of organogenesis and genetic transformation for poplar roots are presented with a transformation efficiency of 58%.

Introduction

Populus is the main model plant for functional genomic studies of woody plant growth, adaptation and development (Busov et al. 2010). Its compact genome (~500 Mb, n=18) was the first among forest species to be sequenced (Tuskan et al. 2006). Model plants, such as *Arabidopsis* and tobacco, are characterized by small genome, have short life cycle and must have highly efficient genetic transformation and *in vitro* regeneration protocols (Zhu 2001).

In the last decade, gene editing increased considerably the number of genes that can be modified in the target plant, making gene function studies in model plants faster and more precise *Populus* was the first forest species successfully transformed by CRISPR Cas9, using transformation via *Agrobacterium tumefaciens*. The system was considered a promising tool to precisely edit genomic sequence and effectively create knockout mutations in woody plants (Fan et al. 2015; Zhou et al. 2015). These studies are facilitated by using *Populus*, since the forestry species are normally highly recalcitrant to tissue culture techniques, being unfeasible for gene function investigation by genetic transformation.

The first step for gene function studies based on genetic transformation is the development of efficient *in vitro* regeneration protocols. Several studies of genetic transformation and shoot organogenesis using poplar have been published, and the explants commonly used are leaves and stem segments (Ma et al. 2004; Filichkin et al. 2006; Song et al. 2006; Du et al. 2012; John et al. 2014; Li et al. 2017).

Few authors describe the use of roots as explants, which shows advantages when compared to the above mentioned: less oxidation, high regenerative capacity, easy manipulation and high *Agrobacterium tumefaciens* susceptibility (Son and Hall 1990; Márton and Browse 1991; Sharma 2012). Moreover, by using the entire plant during the organogenesis and genetic transformation protocols, it is possible to

optimize space in the growing room, time and reagents, and could expand the time among the subcultures of micropropagation step.

The use of roots in organogenesis experiments was already reported by Son and Hall (1990), Vinocur et al. (2000), Chaturvedi et al. (2004), Tsvetkov et al. (2007), Yadav et al. (2009), Sherif and Khattab (2011). In these studies, the effects of plant growth regulators in different concentrations were evaluated using different *Populus* species. Regarding genetic transformation, to our knowledge, there are no studies using this type of explant. When stem segments and leaves were used, transformation efficiencies ranged from 10 to 88% (Leplé et al. 1992; Ma et al. 2004; Filichkin et al. 2006; Song et al. 2006; Jhon et al. 2014).

Several factors affect the transformation efficiency and regeneration during genetic transformation via *Agrobacterium tumefaciens*, including the plant growth regulators and concentrations, the explant source and age and co-culture time period, among others. Alternative methods such as sonication-assisted *Agrobacterium tumefaciens* (SAAT) also demonstrated to be efficient for several plant species, although it has not been tested for *Populus* transformation so far.

Therefore, the objective of this study was to develop a shoot organogenesis and genetic transformation protocol using roots as explants for *Populus tremula x Populus alba* clone 717-1B4.

Materials And Methods

Plant material and growth conditions

Plants of *P. tremula x P. alba* clone 717-1B4 were kindly provided by Dr. Gilles Pilate, INRAE, France. For organogenesis and genetic transformation experiments 2 cm length roots segments from 2 to 6 months old plants (since the last transfer to fresh medium) were used as explants. *Populus* plants used as explants sources were micropropagated by stem internodes with two axillary buds, on MS medium (Murashige and Skoog 1962) containing 3% sucrose and 7% agar, with pH adjusted to 5.8. All cultures were maintained in growth room at 23±2ºC under a 16 h photoperiod (40 $\mu mol.m^{-2}.s^{-1}$).

Shoot organogenesis

To determine the optimal medium, three experiments were performed. In the first, different growth regulators BA, kinetin and zeatin at concentrations of 2.2, 4.4 and 8.8 µM were tested. In the second experiment only zeatin was tested at the concentrations of 7, 14 and 21 µM. In the third experiment, the effect of root age was evaluated (roots segments were isolated from plants micropropagated every 2 or 6 months). Explants from 6 months old plants were called “old” and from 2 months old plants “young”. The basal induction medium was composed of WPM (Lloyd and McCown 1981) salts and vitamins supplemented with 3% sucrose 14 µM of zeatin and 7% agar. The pH of the media was adjusted to 5.8 prior to autoclaving (121 °C, 20 min at 1 atm. pressure). The cultures were maintained in growth room at 23±2ºC under a 16 h photoperiod (40 $\mu mol.m^{-2}.s^{-1}$).

Agrobacterium strain and plasmid

The hypervirulent *Agrobacterium tumefaciens* strainAGL1 harbouring the plasmid pCAMBIA2301 (Cambia, Australia) was used for genetic transformation of *Populus tremula x P. alba* clone 717-1B4. The plasmid contains *nptII* (kanamycin resistance) and *uidA* reporter gene under the control of 35S constitutive promoter.

Root transformation protocol: explants age, SAAT treatment, co-cultivation period and kanamycin concentrations

Transformation was performed using an overnight culture of *A. tumefaciens* (from a glycerol stock culture kept at -80°C) at optical density (OD_{600}) of 0.5-1.0, grown in liquid LB medium with 50 mg L^{-1} of kanamycin. The bacterial suspension was centrifuged in microtube at 12000 xg for 10 min and suspended with 1.0 mL of half strength MS medium (Murashige and Skoog 1962). The bacterial solution was transferred to a flask containing 50 mL of half strength MS medium, where the explants (from 2 or 6 months old *in vitro* plants) were immersed, submitted to sonication for 30 s, and then remained under gentle shaking for 30 minutes.

A control without the bacteria was also tested. In this treatment, the *A. tumefaciens* were added to the MS medium with the explants after the sonication. Subsequently, the explants were dried on sterile filter paper and transferred to Petri dishes with 25 mL of co-cultivation medium (WPM medium without PGRs and antibiotics).

The cultures were kept in the dark in BOD incubator at 23º±2°C C for 24 or 48 h. After co-cultivation, the explants were transferred to WPM medium containing 14 µM of zeatin, 250 mg L^{-1} of the antibiotic amoxicillin to eliminate the *Agrobacterium* and 0, 30, 50 or 70 mg L^{-1} of kanamycin, as the selective agent. Explants were subcultured to fresh medium at two-week intervals. The regenerated shoots were isolated with approximately 2 cm and micropropagated in flasks containing 50 mL of MS medium (with 3% sucrose and 0,7% agar and supplemented with 250 mg L^{-1} of amoxicillin and 50 mg L^{-1} of kanamycin). Shoots were transferred to fresh medium at 30 days intervals. Shoot elongation and rooting were carried out in the same medium.

Acclimatization of in vitro regenerated plants

Plantlets about 7 cm high and the caps of the flasks were kept half opened for two days. After that, roots were washed in running tap water to remove medium culture residues and were transferred into 1.7 L pots filled with the substrate Agrinobre TNMIX previously autoclaved. Plants were then placed in fitotron at 23±2°C, 80% of humidity under a 16 h photoperiod and watered at two days intervals. From the organogenesis experiment with different zeatin concentrations 10 plants were used (another 3 plants were used as control) with 2 replicates. From the transformation experiments 3 events confirmed as transgenic and one control were acclimatized, each with 5 replicates.

Molecular analysis

DNA extraction and transgene integration analysis by PCR

The insertion of *gus* and *uidA* genes detected by PCR using genomic DNA from leaves. Plant tissues (150 mg) from control not transformed plants and regenerated putative transformants were transferred to microtubes along with tungsten beads for tissue mechanical disruption with TissueLyser® (Qiagen) system. DNA extraction was performed using CTAB 2% protocol (Ferreira and Grattapaglia 1998). Plasmid DNA was used as a positive control. The samples were submitted to PCR for amplification of *uidA* and *nptII* genes, using the following program: 1 cycle at 94^{o}C for 4 min, 30 cycles at 94°C for 1min, 55^{o}C for 1 min, 72^{o}C for 1 min and a final extension at 72^{o}C for 7 min for *uidA* and 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, 1 cycle at 72 °C for 7 min for *nptII.* The PCR reactions were setup in a total volume of 25 µL containing: 0,2 µg/µL genomic DNA; 2,5 µL PCR buffer (50 mM KCl; 0,1% Triton X-100; 10 mM Tris-HCl pH 8,8); 1 µL $MgCl_2$ (50mM); 2,5 µL de dNTPs (10 mM); 1 µL of each *primer forward* and *reverse* (10 µM each) and 1 µL *Taq* DNA polymerase. The primers used were: uidA F: 5´-CAGCGCGAAGTCTTTATACCG-3´; R: 5´-ATGCGTCACCACGGTGATATCG3´ and nptII F: 5´-TCGGCTATGACTGGGCACAACAGA-3´; R: 5´- AAGAAGGCGATAGAAGGCGATGCG-3´. The amplified DNA was visualized under UV light after electrophoresis on a 1% (w/v) agarose gel with ethidium bromide staining.

Copy number quantification by qPCR and Comparative Ct ($2^{-\Delta\Delta Ct}$)method

All qPCR analysis were carried out with a LightCycler® 480 Real-Time PCR System (Roche Molecular Systems Inc., Indianapolis, USA) in a 25 µL volume, containing 4 µL of DNA sample and 21 µL of PCR master-mixture by LightCycler® 480 SYBR Green I Master kit (Roche Molecular Biochemicals, Germany) following the manufacturer's instructions. The gene-specific primers were: *uidA* F: 5' - CAGCGCGAAGTCTTTATACCG - 3' and R: 5' – ATGCGTCACCACGGTGATATCG - 3' and the constitutive gene *LEAFY/FLORICAULA* (*PTLF*) F: 5' - GGTTTCTCTGAGGAGCCAGTACAG - 3' and R: 5' – GCCTCCCATGTCCCTCTTC - 3' (Li et al., 2008); 1 µM/µL of primer concentration in the reactions. qPCR conditions were 95ºC for 5 min to pre-incubation, then 45 cycles of 95ºC for 30s, 56ºC for 30s, and 72ºC for 10s. Each DNA sample was analyzed in three technical replicates and the average was used for calculation of copy number based on the comparative Ct (cycle threshold) method (Bubner and Baldwin 2004).

Experimental design and statistical analysis

Shoot organogenesis experiments were evaluated after 30 and 60 days while genetic transformation after 30, 60 and 90 days. Experiments were performed twice, and treatments had four replicates, each composed of one Petri dish with 10 explants. All experiments were carried out using the Completely Randomized Design. The experiment of different cytokinins was carried out using factorial design (3x3) and the analyzed factors were type and concentration of cytokinins. The variable analyzed was the percentage of responsive explants (capable of regenerate shoots). The homogeneity of variances was

tested using Bartlett's test and data were analyzed by analysis of variance (ANOVA). When the ANOVA indicated statistical significance, a Tukey's comparison test was carried out at the 5% probability level. All analyses were performed using the program ASSISTAT version 7.7.

The genetic transformation efficiency was calculated as follows:

(Number of PCR confirmed plants / total number of regenerated plants) x 100.

Results And Discussion

Shoot organogenesis using roots

Effect of different cytokinins

The effect of different concentrations of zeatin, kinetin and BA on shoot organogenesis from roots of clone 717-1B4 at 30 days was evaluated (Table 1). Data from 60 days showed the same response pattern (data not shown). The higher shoot induction rate was observed when the lowest concentration of BA was used, at 30 days (Table 1). In general, on zeatin at all concentrations, BA at 2.2 and 4.4 µM and kinetin at 2.2 µM higher shoot induction rates were observed. By increasing the BA concentration, a reduction on responsive explants was observed.

When kinetin was used, at all concentrations the shoot induction rates was below 50%. Similar results were observed by Yadav et al. (2009) for two clones of *P. deltoides*. The authors observed 43-45% of roots regenerating shoots in liquid medium supplemented with 1.16µM of kinetin. However, in works involving *Populus* shoot organogenesis from roots, the percentages of responsive explants were normally not reported.

The morphogenic response of *Populus* is mostly affected by the species, genotype and can be manipulated and controlled *in vitro* by the ratio auxin/cytokinin, the media composition and other environmental factors (Confalonieri 2003). Coleman and Ernst (1989), evaluating the influence of cytokinins on shoot regeneration from internodal segments of three genotypes of *Populus deltoides,* tested different concentrations of BA, zeatin and 2iP and founded better responses for all genotypes with zeatin. They also reported a phytotoxic response that caused tissue death when BA was used in concentrations above 2.22 µM.

Although BA and zeatin led to similar results, on media supplemented with BA shoots showed abnormal morphology and did not develop, remaining stunted (Figure 1), while on media supplemented with zeatin the explants showed a better development. Similar results were found for internodes and nodes of *Populus deltoides* Bartram ex Marsh. using WPM supplemented with 4,56µM of zeatin, which induced more and healthier shoots than the media supplemented with BA alone or combined with zeatin and NAA (Cavusoglu et al. 2011).

Effect of zeatin concentration

There was no statistical difference the different concentrations of zeatin, and the shoot induction ranged from 80 to 87.5% (C.V. 18.49%) after 30 days (Fig. 1). After 60 days he same response was observed (data not shown).

The survival rate of *Populus* plants acclimatized was evaluated. The rates ranged from 90% for plants cultivated on medium supplemented with 7 µM of zeatin to 95% at 14 and 21 µM. These results were similar to other works were *Populus* roots were used as explants (Son and Hall 1990; Chaturvedi et al. 2004; Yadav et al. 2009). The plants showed normal morphology and development.

Influence of explant age

The effect of root age was evaluated on basal induction medium. After 30 days a higher percentage of responsive explants was observed when “old” roots were used. However, after 60 days, the results between “young” and “old” did not differ statistically, showing that “young” roots may have a regeneration delayed in the first 30 days of development (Table 2).

Plant cells seem to show a regeneration capacity negatively affected the longer they are cultured, and also an altered morphogenetic potential. Regarding callus induction, young tissues still passing through cell division usually form callus faster than explants from older parts. Also, the callus formed is more likely to regenerate shoots (Gahan and George 2008)

Better results using young tissues were found for *Eucalyptus camaldulensis* hypocotyls (Ahad et al. 2018) and *Rhizophora apiculata* leaves (Fadhilasari et al. 2018). Otherwise, in Son and Hal (1990) study with *Populus grandidentata* roots from plants with 15, 30, 45, 60, 75 and 90 days the best results were found when 60 days plants were used.

Evaluating the effect of plant maturity on shoot regeneration rate and number of shoots per explants for the triploid (*Populus alba* × *P. glandulosa*) × *P. tomentosa* leaves, Zeng et al. (2019) founded that explants from the intermediate node (node 3) showed better results for the two parameters than young leaves (node 1) and older (node 5). In a similar study, Sharma et al. (2020) verified that the organogenic capacity in very young (3^{rd} from the top) and old (7^{th} from the top) leaves of *Populus deltoides* were inferior when compared to the 5^{th} leaf from the top.

In some studies, the age of the explant does not lead to a different regeneration response as found for *Vaccinium corymbosum* (Cao and Hammerschlag 2000). In this latter work, this experiment was performed in order to develop the best strategy for plant multiplication using internodes.

The fact that young and old roots did not show statistical difference in the second evaluation (at 60 days) facilitates the genetic transformation experiments because the material can be kept on in vitro culture without subcultures without losing its regeneration potential. Although the final evaluation was performed at 60 days, the explants maintained the ability of regeneration for at least 90 days (period that the experiment was sustained).

It is possible that the period of organogenic capacity of the roots can be even higher, allowing the obtention of an elevated number of shoots. When *Citrus aurantifolia* roots were cultivated in vitro a retention of the regenerative capacity for three years, after several passages, was observed by Bhat et al. (1992). In all organogenesis experiments described the regeneration occurred without the visible formation of callus, which was also found by Son and Hall (1990), Tsvetkov et al. (2007) and Sheriff and Khattab (2011) with *Populus* roots.

Genetic transformation using roots

Kanamycin concentration

Among the different kanamycin concentrations tested in this study, the appropriate was 50 mg L^{-1} (Table 3). In the control (0 mg L^{-1}) a high number of escapes were observed. The regeneration rate reached 93%, while the percentage of transformants was 19% (Table 4). In the concentration of 30 mg L^{-1} the selection was still not efficient, and when 50 mg L^{-1} was used the best results were observed, around 49% of regeneration and the transformation rate reached 58%. The higher concentration was toxic and inhibited the organogenesis (Table 3).

The use of 50 mg $L^{-1}$of kanamycin was also reported in other studies with *Populus* for transformants selection using leaves and stem segments as explants, sometimes increasing the concentration to 100 mg L^{-1} after a period (Han et al. 2000; Ma et al. 2004; Song et al. 2006; Movahedi et al. 2014). The correct dose of the selective agent varies with the type of explant used, the size and developmental state of the cell or tissue, and also depends on the species (Hinchee et al. 1994). Integration of *gus* and *nptII* genes of 6 randomly selected putative transgenic *Populus* plants was confirmed by PCR (Fig. 2).

Presence of Agrobacterium tumefaciens during sonication

The presence of *A. tumefaciens* during sonication positively influenced the transformation efficiency. When sonication was carried after addition of bacterial suspension in the medium, up to 40% of the explants were transformed, while only 4% transformation efficiency was observed if sonication occurred previously to the addition (C.V. 58.75%).

Sonication technique commonly increases genetic transformation due to the formation of micro-wounds (caused by cavitation) on the tissue that facilitates A. tumefaciens passage (Trick and Finner 1997). Also, these wounds may increase the bacteria infection enhancing the production of phenolic compounds that induce vir genes, involved in the transfer of the T-DNA (Trick and Finner 1997; Santarém et al. 1998).

Positive results using sonication in genetic transformation experiments were described by several authors for different species sometimes combined with vacuum infiltration: *Eucalyptus grandis × E. urophylla* (González et al. 2002), *Phaseolus vulgaris* L. (Liu et al. 2005), *Pisum sativum* L. (Švábová et al. 2005), *Lens culinaris* Medik. (Chopra et al. 2012); *Eucalyptus globulus* (de La Torre et al. 2014), *Withania*

somnifera (Sivanandhan et al. 2015), *Catharanthus roseus* (Alam et al. 2017), *Dierama erectum* Hilliard (Koetle et al. 2017).

Trick and Finner (1997) related that the presence or absence of *Agrobacterium* during sonication did not alter the transient expression in their studies. On the contrary, when González et al. (2002) compared ultrasound applied prior to *Agrobacterium* inoculation (0, 30, 60, 90 and 120 s) with ultrasound exposition during inoculation (0, 15, 30), and 120 s exposure before the addition of the bacteria showed the best results. In the present work, it is possible that the wounds formed may close before the addition of *A. tumefaciens*, explaining the low transformation efficiency in the treatment without it. However, if the bacteria presence or absence during sonication will improve, cause damage to *Agrobacterium* decreasing the transformation efficiency or even not affect it at all, it is a subject poorly discussed in literature and probably will vary with the species, treatments, type of explant, sonication period, among other factors.

Influence of co-cultivation period

Many factors can interfere in the genetic transformation efficiency and the co-cultivation time may vary among species and explants of the same species. The effect of co-cultivation period (24 or 48h) on genetic transformation and shoot regeneration of *Populus* roots was evaluated. No statistical differences between the two treatments for shoot regeneration or for transformation rates were observed (40% for 24 h and 48% for 48h, respectively (C.V. 26.06%)).

For *Populus* genetic transformation, the period of 48 h of co-cultivation is the most commonly used and described in literature: *Populus trichocarpa* internodal segments and leaves (Ma et al. 2004); leaf and internodal segments from *P. tremula x P. alba* and *P. tremula x P. tremuloides* (Filichkin et al. 2006); *Populus trichocarpa* leaf, stem and midrib explants (Song et al. 2006); leaf explants from the two hybrids *P. alba x P. berolinensis* and *P. davidiana* x *P. bolleana* (Wang et al. 2011) and *Populus tomentosa* leaf explants (Du et al. 2012). However, in some studies the transformation efficiency was improved when different co-cultivation times were evaluated. For *P. deltoides* × *P. euramericana* leaves, 72 h of co-cultivation provided the best results when compared to 24 and 48 h (Movahedi et al. 2014).

In another study, with *Populus angustifolia* and *Populus balsamifera* stem and axillary buds, when the authors tested 24, 48 and 72 h of co-cultivation the highest transient expression (100%) was observed with 72 h. However, this long explant exposition to *A. tumefaciens* resulted in bacterial overgrowth that led to explant necrosis. The use of 24 h provided a transformation frequency of 58-71% and 81-89% using 48 h (Maheshwari and Kovalchuk 2016). John et al. (2014) reported that for *Populus deltoides* nodes, internodes and leaves, 24 h of co-cultivation is sufficient to the success of the genetic transformation. With 48 h they observed problems with the bacterial overgrowth. In the present work the overgrowth of the *A. tumefaciens* was not observed and 24 h of co-cultivation appears to be enough for the genetic transformation.

Influence of explant age

The transformation efficiency was not affected by explant age, varying from 26.7 to 31.7 (C.V. 40.81%). However, “old” roots were more efficient in regenerating shoots 90 days after transformation experiment, when compared to “young” roots. While 63.3% of “old” roots regenerated new shoots, on young leaves 50% regeneration was observed (C.V. 15.11%).

Information about the influence of explant age on genetic transformation are scarce and usually higher efficiency rates are obtained with younger tissues. Based on scanning electron microscopy (SEM), Han et al. (2016) observed that young leaves of the hybrid *Populus davidiana* Dode *× P. bollena* Lauche showed higher infection by *A. tumefaciens* when compared to older explants (60 and 90 days old). Authors attributed this result to changes on cell wall composition at different physiological and developmental stages which, as leaf age increases, can cause losses of binding sites to bacteria. Similar results were observed by Verma et al. (2008) for sorghum leaves. On the contrary, in the present work, on older roots higher shoot regeneration was observed. This may occur due to the higher resistance of older roots to stresses like ultrasound. However, other analysis such as TEM and histology must be performed to confirm this theory.

Conclusion

In this study, efficient protocols of organogenesis and genetic transformation for *Populus tremula x Populus alba* clone 717-1B4 using roots as explants are presented. To the best of our knowledge, this is the first report of transgenic *Populus* plants obtained by *Agrobacterium tumefaciens* with this kind of explant. Roots showed great regenerative potential and easy genetic transformation comprising a remarkable source of explant. Using “old” roots as explants it is possible to space subculture for micropropagated plants, lowering the costs with inputs and labor. BAP and Zeatin were the most suitable growth regulator for this clone and explant among the PGRs tested. For genetic transformation, the presence of *A. tumefaciens* during sonication was essential improved the transformation efficiency.

Declarations

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Conflicts of interest/Competing interests

Not applicable

Availability of data and material

Not applicable

Code availability

Not applicable

Authors' contributions

Not applicable

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Tables

Table 1 Effect of different cytokinins (zeatin, cinetin and BA) at concentrations 2.2, 4.4 of 8.8 µM on the shoot induction explants of *Populus tremula x Populus alba* clone 717-1B4 at 30 days.

Concentration (μM)			
PGRs	2.2	4.4	8.8
Zeatin	67.50aA	78.75aA	82.50aA
Kinetin	46.25aA	23.75bA	40.00bA
Ba	83.75aA	53.75abAB	16.25bB
C.V. (%)		25.44	
Means followed by the same lowercase letter in the column and uppercase letter in the row do not differ from each other according to Tukey's test at 5% probability.CV: coefficient of variation.			

Table 2 Effect of explant age on the percentage of responsive explants and number of shoots of *Populus tremula x Populus alba* clone 717-1B4at 30 and 60 days.

Age	Responsive explants (30 d)	Responsive explants (60 d)
“Young”	37.5b	95[a]
“Old”	82.5[a]	100[a]
C.V. (%)	23.15	20.32
Means followed by the same lowercase letter in the column and uppercase letter in the row do not differ from each other according to Tukey's test at 5% probability. CV: coefficient of variation.		

Table 3 Effect of different concentrations of kanamycin on shoot regeneration and transformation efficiency of *Populus tremula* x *Populus alba* clone 717-1B4 at 90 days.

Kanamycin concentration (mg L^{-1})	Regeneration rate (%)	Transformation efficiency (%)
0	93.0a	19.0b
30	54.0b	32.0b
50	49.0b	58.0a
70	0c	0c
C.V. (%)	17.14	41.53
Means followed by the same lowercase letter in the column and uppercase letter in the row do not differ from each other according to Tukey's test at 5% probability. CV: coefficient of variation.		

Table 4 is not available with this version

Figures

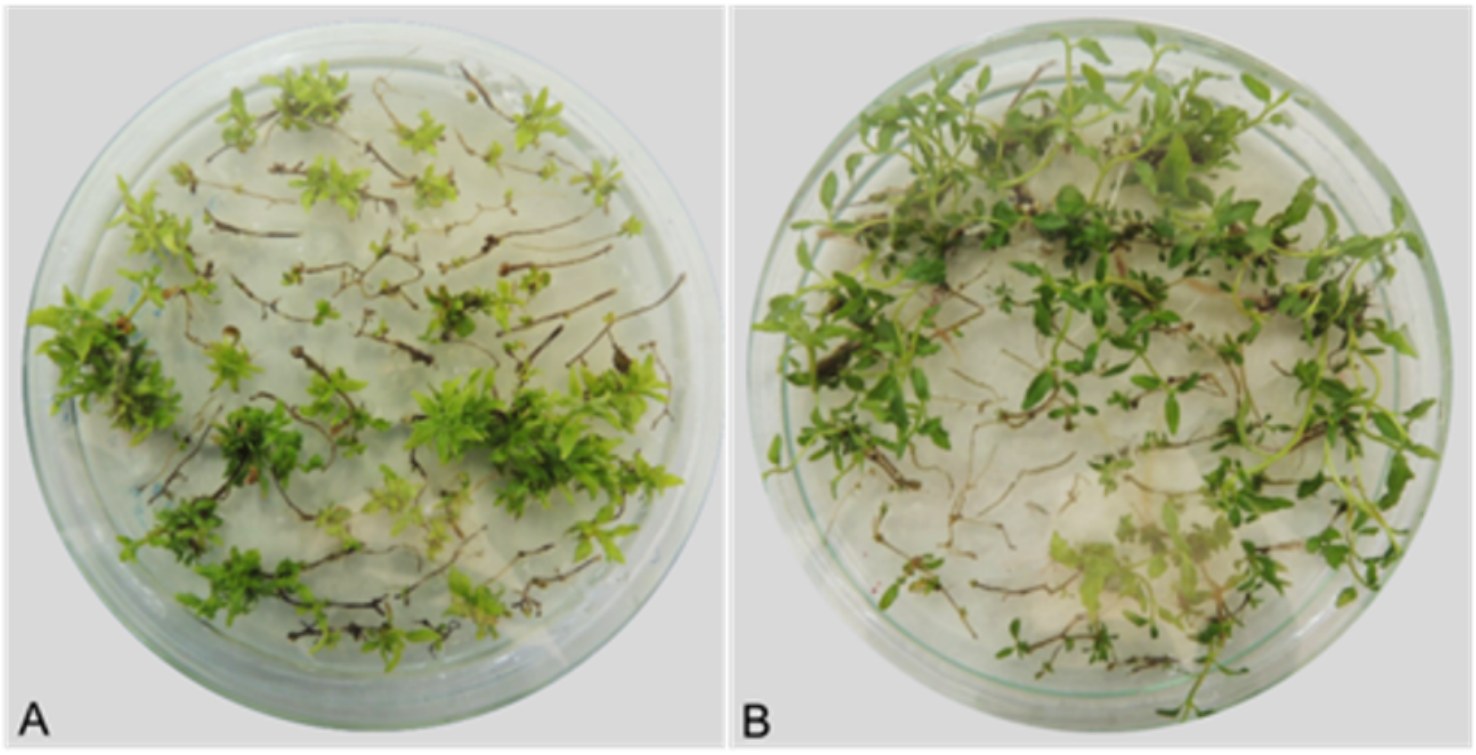

Figure 1

Shoots from roots of Populus tremula x Populus alba clone 717-1B4 from the experiment using different cytokinins at 30 days. A. 2.2 µm of BA. B. 8.8 µm of zeatin

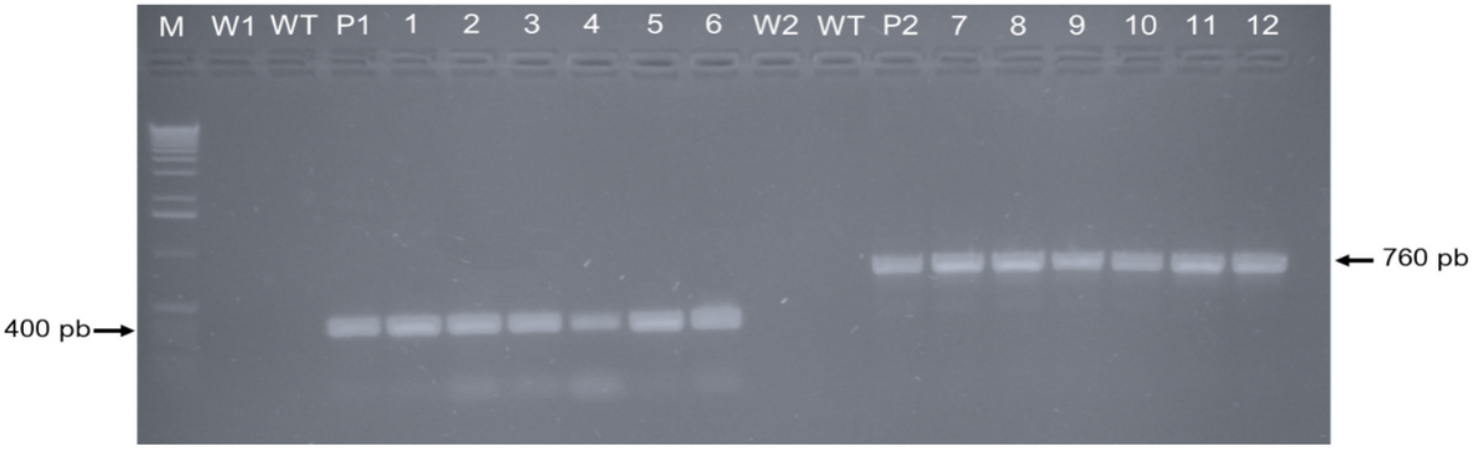

Figure 2

PCR of genomic DNA isolated from leaves of non-transformed control and putative transgenic Populus tremula x Populus alba clone 717-1B4 plants using specific primers for amplification of gus (400 bp) and nptII (760 bp) genes. M. Molecular marker (1 kb), W1. PCR product with double distilled water as template, WT. PCR product with wild type plant genomic DNA as template (negative control), P1. PCR product with pCAMBIA2301 plasmid DNA as template (positive control), 1-6. PCR products from 6 randomly Populus plants using specific gus primers, W2. PCR product with double distilled water as template, WT. PCR product with wild type plant genomic DNA as template (negative control), P2. PCR product with pCAMBIA2301 plasmid DNA as template (positive control), 7-12. PCR products from 6 randomly selected Populus plants using specific nptII primers.